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Assessment of the Role
of Plant Mitochondrial Respiratory Complexes
in Cytoplasmic Male Sterility
and
Structural Analysis of Plant Cytochrome c Oxidase

presented by

Wendy Eileen Peiffer

has been accepted towards fulfillment
of the requirements for

Ph.D. **degree in** Biochemistry

S. Ferguson-Miller
Major professor

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ASSESSMENT OF THE ROLE OF PLANT MITOCHONDRIAL RESPIRATORY COMPLEXES IN CYTOPLASMIC MALE STERILITY AND STRUCTURAL ANALYSIS OF PLANT CYTOCHROME *C* OXIDASE

Volume I

By

Wendy Eileen Peiffer

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1992

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ABSTRACT

ASSESSMENT OF THE ROLE OF PLANT MITOCHONDRIAL RESPIRATORY COMPLEXES IN CYTOPLASMIC MALE STERILITY AND STRUCTURAL ANALYSIS OF PLANT CYTOCHROME *C* OXIDASE

By

Wendy Eileen Peiffer

The mechanism of action of the fungal toxin causing Southern corn leaf blight in plants with the mitochondrially-encoded trait cytoplasmic male sterility (CMS) is not completely understood. Some evidence suggests that respiratory complexes are involved in mediating both toxin action and CMS. Studies reported here provide evidence that the ATP synthase is involved in toxin action and CMS through interaction with a 13 kDa CMS-specific peptide. The ATPase inhibitors dicyclohexylcarbodiimide and quercetin inhibit toxin-induced mitochondrial swelling. Furthermore, toxin inhibits ATPase activity in mitochondria from CMS but not fertile plants. Finally, maximal ATPase activity in mitochondria from CMS plants is about 20% lower than in fertile plants. These findings suggest that the 13 kDa peptide may be physically associated with the ATPase. To test this hypothesis, we analyzed fractions from anion-exchange fast protein liquid chromatography (FPLC) of solubilized maize mitochondria for reactivity with antibody against the 13 kDa peptide. All protein-

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containing fractions contained this peptide, indicating non-specific association, but this does not rule out a specific effect on the ATPase. A similar experiment with male sterile petunia was inconclusive.

Cytochrome oxidase has also been postulated to be involved in CMS, making it of interest to purify and characterize. Using commerical wheat germ to overcome the difficulty in isolating large amounts of plant mitochondria, we purified highly active cytochrome oxidase by anion-exchange FPLC in lauryl maltoside. SDS-gels show a subunit composition in which subunits I–IV resemble the yeast oxidase in size and antigenicity while three to four smaller peptides are dissimilar to other eukaryotic oxidases. A 9 kDa peptide appears in the wheat germ but not seedling oxidase, suggesting a developmental or tissue-specific form of the oxidase.

Amino acid sequence analysis of subunit II verified one site of RNA editing, suggesting that a conserved cysteine ligand to Cu_A is restored. Electron paramagnetic resonance and visible absorption spectra confirm that the environment of Cu_A is not altered in wheat oxidase. However, resonance Raman and visible spectroscopy extends previous observations in plant mitochondria indicating that the environment of heme *a* is different from other oxidases.

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Good luck to all

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Kathy, Diane, C
Joe L., and Carol

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Thanks also to the past and present grad students and postdocs of my laboratory: Bob Ingle, Linda Gregory, Krishnakumar Rajarathnam, Jianli Cao, Taha Taha, Bridgette Barry, Jon Hosler, Eileen Allison, Claudia Lerma, and John Fetter. I have been fortunate that the many undergrads and high school students who have worked in the lab have provided me with friendship, idealistic inspiration, and in many cases, technical assistance: Becky Dutch, Donna Maki, Jason Takeuchi, Barbara Ellis, Thomas Lee, Grace Ho, Terrie Rife, and Amara Siva. Special thanks are in order for Terrie, in particular for the wonderful immunoblot in chapter 7. Good luck to all of you in the future!

I must thank Dr. Robert Bandurski for his insightful suggestion that we try to make mitochondria from wheat germ. I would also like to thank collaborators Julio dePaula in Dr. Babcock's lab, Ken Korth in Dr. Levings lab, and Marie Connett in Dr. Maureen Hanson's lab, for some good work, good fun, and a fresh perspective. Thanks and best wishes also to Sherry, Kathy, Diane, Carrie, Warren and Pam, Jamie, Gerry and Lorri, Joe G., Joe L., and Carol.

Most
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Thanks Jef
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Most of all, I thank my family: Mom, Dad, Jeff, and George. Your love and support helped me through some seemingly hopeless times. Thanks Jeff, for not falling asleep during my corn story. The world is safe for Frito's! Thank you George, for your MEGA help and endless love.

Lastly, I want to thank Carl Sandburg, for writing this simple but wonderful poem which has served as an inspiration to me since I was in the third grade:

Circles

The white man drew a small circle in the sand
and told the red man
"This is what the Indian knows."
And drawing a big circle around the small one,
"This is what the white man knows."

The Indian took the stick
And swept an immense ring around both circles:
"This is where the white man and the red man
know nothing."

– Carl Sandburg

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SDS
SDS-PAGE
SHAM
TES

TFA
TMPD

List of Abbreviations

BSA	bovine serum albumin
CAPS	3-cyclohexylamino-1-propane sulfonic acid
CCCP	carbonyl cyanide *m*-chlorophenylhydrazone
CMS	cytoplasmic male sterility
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ENDOR	electron-nuclear double resonance
EPR	electron paramagnetic resonance
EXAFS	extended X-ray absorption fine structure
FPLC	fast protein liquid chromatography
HPLC	high performance liquid chromatography
MCD	magnetic circular dichroism
PMSF	phenylmethylsulfonyl fluoride
PVP	polyvinylpyrrolidone
RCR	respiratory control ratio
RR	resonance Raman spectroscopy
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SHAM	salicylhydroxamic acid
TES	N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid
TFA	trifluoroacetic acid
TMPD	N,N,N',N'-tetramethyl *p*-phenylene diamine

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Chapter 1 – Plant mitochondria and respiration

1.1 Overview

In all living organisms energy is extracted from the environment and conserved in the form of a high energy chemical bond, often the triphosphate bond of ATP, in order to do cellular work. For animals and many bacteria, the source of energy is the bonds between atoms of organic molecules which are sequestered from the environment. Carbohydrates, fats, and proteins in foodstuffs are broken down into simpler molecules such as CO_2. The energy is channeled into ATP through oxidative phosphorylation in the mitochondria.

In contrast, the energy source of plants and some bacteria is sunlight. In the process of photosynthesis, absorbed photons of light stimulate a cascade of reactions in the chloroplasts which allows ATP to be produced, thus storing the energy gained. Since sunlight is not available all the time, plants store large amounts of energy in the chemical bonds of starch. In the dark, the starch is broken down to CO_2 and ATP is produced in the mitochondria, by a process analogous to animal respiration.

Because of the different energy-securing strategy of plants, plant mitochondria have assumed somewhat different metabolic roles than their animal counterparts. In the light, for instance, mitochondrial respiration is usually suppressed, but plant mitochondria participate in the cycle of photorespiration, decarboxylating glycine produced in the peroxisomes and returning the NADH via the malate shuttle. The unique roles of plant mitochondria have produced some interesting structural and functional

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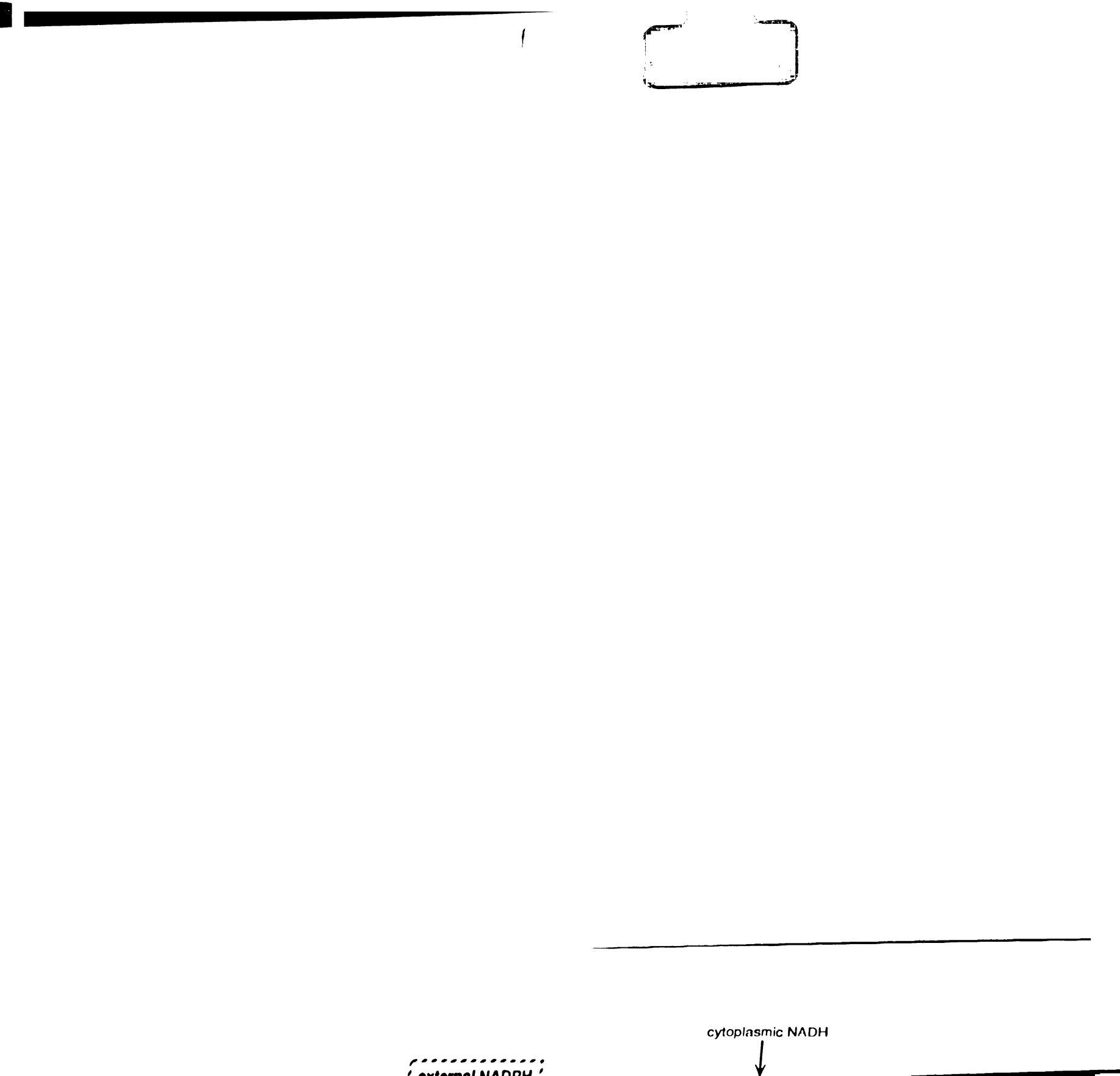
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differences between the metabolic pathways and their components of plant and animal mitochondria.

1.2 Respiratory pathway

The main pathway of energy conservation is remarkably similar between all species, from animals to plants to bacteria. Electrons extracted from the breakdown of organic molecules in glycolysis and the tricarboxylic acid cycle are stored temporarily as NADH and $FADH_2$. These carriers donate their electrons to the respiratory chain in the mitochondria, a series of multisubunit complexes which channel the energy of electron transfer into the creation of an electrochemical gradient across the inner mitochondrial membrane, in part by the vectorial movement of protons across the membrane. The controlled release of the proton gradient by the ATP synthase drives the synthesis of ATP from ADP. A representation of the respiratory chain is shown in Figure 1.1

The NADH dehydrogenase (complex I) transfers the electrons donated by NADH through the FMN and Fe-S centers of the protein to a pool of lipid-soluble coenzyme Q (Q). Alternatively, electrons can feed into the chain through the succinate dehydrogenase (complex II). In contrast to the other complexes, transfer of electrons from $FADH_2$ to the Q pool by complex II is not coupled to ATP synthesis; the enzyme does not pump protons. Electrons from the Q pool then pass through the cytochrome *c* reductase (complex III, also called bc_1 complex because of its *b* and c_1 cytochromes) to cytochrome *c* in the intermembrane space. Four electrons are donated to cytochrome *c* oxidase (complex IV) sequentially from cytochrome *c* and are transferred through heme A and copper centers to


cytoplasmic NADH
external NADPH

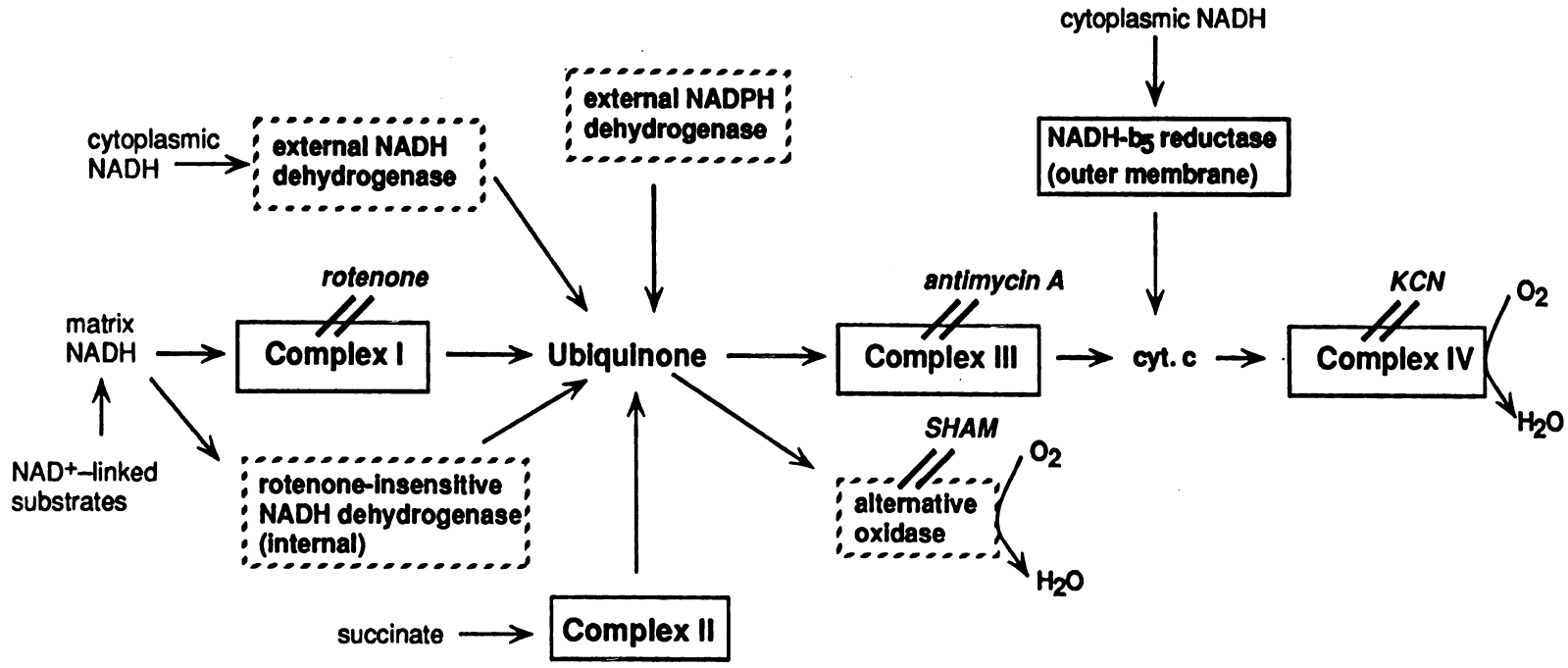


Figure 1.1: Diagram of the respiratory chain of mitochondria.

all mitochondria

plant mitochondria only

indicates inhibitor site

O_2, which is reduced to water. Protons built up in the intermembrane space from proton pumping are transported through the membrane-bound F_0 region of the ATP synthase, catalyzing the release of newly synthesized ATP by the F_1 region protruding into the matrix.

1.3 Structure and function

The mitochondrion has a smooth outer membrane and a highly invaginated inner membrane enclosing the matrix. A major component of the outer membrane is porin, which acts as a gate to regulate traffic of small metabolites in and out of the mitochondrion as well as a binding site for a variety of enzymes, such as the hexokinase. The protein-dense inner mitochondrial membrane houses the electron transfer chain as well as many auxiliary proteins and transport systems necessary for energy metabolism, including the ADP/ATP translocase and P_i transporter. The matrix contains the enzymes of many important metabolic pathways, including the tricarboxylic acid cycle, amino acid metabolism, heme synthesis, mitochondrial DNA and protein synthesis, and in most eukaryotes, fatty acid oxidation (Tzagoloff, 1982). In plants, however, most steps of fatty acid oxidation occurs in a separate subcellular compartment called the glyoxysome.

1.3.1 Genome

Mitochondria contain their own DNA and protein synthesis machinery. Mitochondrial DNA (mtDNA) in mammals is a compact 16 kb circle (Anderson, 1981; Attardi *et al.*, 1981). Genes coding for several of the larger subunits of the respiratory enzymes such as cytochrome oxidase



subunits I, II, and III alternate between tRNA genes. Yeast mtDNA is somewhat more complicated with an 80 kb circle encoding a similar set of proteins but with additional intervening sequences and introns (Borst and Grivell, 1978). Plant mtDNA is even more complex, with genome sizes ranging from 200 kb in *Oenothera* (Brennicke, 1980) and *Brassica* (Lebacq *et al.*, 1981) to 2400 kb in muskmelon (Ward *et al.*, 1981).

The purpose of the larger genome size of plant mitochondria is unclear. A high proportion of the DNA is transcribed, between 30% (Makaroff and Palmer, 1987) and 100% (Finnegan and Brown, 1990), suggesting that a greater number of proteins are encoded by the mitochondrial DNA. However, a set of proteins similar to other eukaryotes are found to be encoded (Levings and Brown, 1989) with the addition of the ATP synthase α subunit, which is nuclear-encoded in both mammals and yeast (Boutry *et al.*, 1983; Hack and Leaver, 1983; Isaac *et al.*, 1985). Many of the genes contain large introns as in yeast (Fox and Leaver, 1981; Wissinger *et al.*, 1988), unlike animal mtDNA, but since the protein-coding regions which contain the introns make up only a small part of the genome, this cannot account for the larger size of plant mtDNA. The DNA is only moderately repetitive (Ward *et al.*, 1981), suggesting that the "extra" DNA may be of external origin. Indeed, sequences have been found which are highly homologous to chloroplast DNA (Stern *et al.*, 1982; Lonsdale *et al.*, 1983; Stern and Palmer, 1984, 1986). Transfer of chloroplast sequences may have taken place via RNA using a reverse transcriptase coded for in the mitochondrial genome (Schuster and Brennicke, 1987). Possibly, the integration of reverse transcribed foreign RNA sequences contributes to the large genome size.

Direct repeats scattered throughout the "master circle" cause homologous recombination, resulting in many smaller circle sizes (Palmer and Shields, 1984; Lonsdale *et al.*, 1984). Recombination could have resulted in pseudogenes which have diverged and been maintained, increasing the genome size. However, the mitochondrial DNA from one *Brassica* species contains no repeats and is always found as a single circular molecule (Palmer and Herbon, 1987) of 208 kb, of similar size to other plants. Possibly the extra DNA could facilitate mRNA processing and modification; however, this has not been demonstrated, and the purpose and source of the large mitochondrial genome in plants remains largely unknown.

1.3.2 Origins

The similarity of the mitochondrial and bacterial electron transfer pathway and protein synthesis machinery led to the proposal that mitochondria arose from an endosymbiotic event of a eubacteria with a prokaryote (Margulis, 1970), perhaps similar to the nonsulfur purple bacteria such as *Paracoccus* or *Rhodobacter* (Whatley, 1981). Comparison of protein and nucleic acid sequences of various species allows evolutionary trees to be constructed showing the relatedness of species. From these, Dayhoff and Schwartz (1981) conclude that mitochondria of animals, fungi, and plants originated from one endosymbiotic event preceding the divergence of the kingdoms. Recent comparisons have pinpointed the origins of mitochondria to the α division of the purple bacteria, some of whose members are known to exist in intimate intracellular associations with eukaryotic cells (Yang *et al.*, 1985), for example *Agrobacterium tumefaciens*, which is commonly used to transform plants. However,



small and large subunit rRNA sequences from plant mitochondria compare more favorably to sequences from bacteria than to non-plant mitochondria, suggesting that plant mitochondria may have arisen from a second later endosymbiotic event (Gray *et al.*, 1984; Gray *et al.*, 1989).

1.3.3 Defects of the respiratory chain

Since the protein complexes of the respiratory chain have components coded in both the nucleus and mitochondria (Sebald *et al.*, 1972; Mason and Schatz, 1973), any deficiency in respiration may be the result of a large number of defects in either genome. Respiratory-deficient yeast are called petite (ρ-) mutants because they are characterized by small size and slow growth (Borst and Grivell, 1978). They have been relatively well-studied because of the organism's ability to survive by metabolizing fermentable carbon sources. Defects in respiration are less commonly observed in animals because there is no alternative source of energy, thus serious defects are often fatal; however, an increasing number of "mitochondrial myopathies" have been characterized in humans over the past five years. Respiratory mutants in plants have also been identified, resulting in the abnormal conditions of cytoplasmic male sterility and non-chromosomal stripe (discussed below). A common element between these types of respiratory defects is mitochondrial gene rearrangements which sometimes lead to large deletions of the DNA, possibly caused by homologous recombination between repetitive sequences.

1.3.3.1 Human Mitochondrial Myopathies

These disorders (reviewed in Capaldi, 1988) most often affect tissues with high aerobic metabolic demands, specifically brain, skeletal muscle, and heart. Sometimes only one tissue type is affected in an individual,



possibly due to differential segregation of defective mitochondria during development or because defects have occurred in tissue-specific subunits. Some myopathies are maternally inherited while others are not, indicating that both mitochondrial and nuclear genes may be involved. Defects have been localized to several respiratory complexes which in some cases appear to be missing one or more subunits.

The mtDNA of patients with Kearns-Sayre syndrome and progressive opthalmoplegia shows large deletions. One very common deletion is found to be flanked by a 13-base direct repeat in normal mtDNA, suggesting that homologous recombination is responsibile for the deletions (Schon *et al.*, 1989; Holt *et al.*, 1989). Other deletions are also found to be flanked by direct repeats of varying length (Tanaka *et al.*, 1989; Yuzaki *et al.*, 1989). However, no evidence of a mtDNA deletion is found in patients with myoclonus epilepsy with ragged red fibers where a selective defect in cytochrome oxidase subunit II is indicated by Western blots of mitochondrial protein (Schon *et al.*, 1988). Clearly, the mechanisms of mitochondrial myopathies can vary greatly.

1.3.3.2 Plant conditions associated with respiratory defects

Cytoplasmic male sterility. Cytoplasmic male sterility (CMS) is a maternally-inherited trait in which pollen production is aborted. In most species examined, CMS is correlated with mitochondrial gene rearrangements which presumably occur by homologous recombination as a result of the large number of repeats in the plant mitochondrial genome, leading to production of novel or altered proteins. Several genes for respiratory chain complexes are involved; however, no one gene seems to be common to these alterations (see chapter 2). CMS can be produced artificially by setting up an incompatibility between the nuclear and mitochondrial genomes by



protoplast fusion or recurrent backcrossing. Recurrent backcrossing (crossing the female offspring of each generation with the original male parent) produces offspring which contain the mitochondria and chloroplasts of one original parent (female) and the nuclear DNA of the other original parent (male). Thus any evolved "matching" of the nuclear and mitochondrial genomes is lost. The mechanism of disruption of mitochondrial function in the anthers has yet to be determined. It is assumed that the novel proteins are involved, but it not understood why their presence does not affect the normal functioning of the plant. Cytoplasmic male sterility is discussed in detail in chapter 2.

Non-chromosomal stripe. Non-chromasomal stripe (NCS) is a maternally-inherited trait in maize characterized by poor growth, decreased yields, and variable leaf striping (Schumway and Bauman, 1967; Coe, 1983) . Plants survive because each contains a mixture of normal and mutant mitochondria. NCS originated spontaneously in the Texas CMS variety, but does not seem to correlate with the CMS condition. Several varieties have been identified, each containing a deletion in an essential mitochondrial gene (for instance cytochrome oxidase subunit II) which appears to have been generated by recombination between short repeats from 6 to 31 bp (Newton *et al.*, 1990; Lauer *et al.*, 1990; Hunt and Newton, 1990).

1.4 Unique aspects of plant mitochondria

The many unique aspects of plant mitochondria may be the result of their unique respiratory function in the plant or their possibly separate origin from animal mitochondria. Several differences occur in the

components of the respiratory chain and transport systems. Moreover, in addition to their larger genome size, plant mitochondria edit their mRNA and, unlike animal mitochondria, translate their proteins using the universal code.

1.4.1 Additional NADH Dehydrogenases

Mammalian mitochondria do not oxidize exogenously supplied NADH because the rotenone-sensitive complex I binds NADH on the matrix (internal) side of the inner mitochondrial membrane (Lehninger, 1955) and no transport system exists to allow NADH to cross the inner membrane (LaNoue and Schoolwerth, 1979). However, like fungi (vonJagow and Klingenberg, 1970; Weiss *et al.*, 1970), plant mitochondria rapidly metabolize NADH and NADPH in a non–rotenone-sensitive Ca^{+2}-dependent manner (Palmer and Passam, 1971; Koeppe and Miller, 1972). This has led to the conclusion that plant mitochondria also contain a NADH dehydrogenase which can accept NADH on the outer side of the membrane, although endogenous NADH and NAD-linked substrates are preferred (Bergman and Ericson, 1983; Dry *et al.*, 1983; Day *et al.*, 1985). Differences in pH optima and responses to chelators and inhibitors suggested that NADH and NADPH utilize distinct dehydrogenases (Møller and Palmer, 1981; Edman *et al.*, 1985). Further studies have indicated the presence of an internal dehydrogenase which is not rotenone-senstive and has lower affinity for NADH (Møller and Palmer, 1982). The physiological significance for two internal dehydrogenases is unclear, although it has been suggested (Palmer, 1979; Møller and Lin, 1986) that the energy-conserving complex I is associated with electron flow through the cytochrome pathway while the non-rotenone sensitive dehydrogenase,

which is non-energy conserving, is associated with the alternative pathway (see below). Plant mitochondria, like animal mitochondria, also have an outer membrane NADH dehydrogenase which feeds electrons to the respiratory chain at the level of cytochrome *c*. The physiological role of this complex is unclear.

1.4.2 Alternative oxidase

The observation that plant mitochondria are incompletely inhibited by cyanide led to the proposal that the respiratory chain of plant mitochondria contains two terminal oxidases (James and Elliot, 1955; Bendall and Bonner, 1971). In addition to the CN-sensitive "cytochrome" pathway in which electrons are transferred through cytochrome *c* reductase and oxidase, electrons from the Q pool may instead go to a CN-insensitive "alternative" oxidase (AO; Storey, 1976) which is inhibited by substituted hydroxamic acids like salicylhydroxamic acid (Schonbaum *et al.*, 1971). O_2 is reduced to water as in the cytochrome pathway; however, this step is not coupled by proton pumping to ATP synthesis as are the cytochrome reductase and oxidase (Moore *et al.*, 1978; Moore and Bonner, 1982; Ishida and Palmer, 1988). Energy is not conserved and may therefore be given off as heat. The inflorescence of some aroid species has been measured at 12–14°C higher than ambient temperatures, correlating with a dramatic increase in AO activity prior to flowering (Elthon *et al.*, 1989a). The higher temperature may allow volatilization of a compound produced by the plant which attracts insects for pollination (Smith and Meeuse, 1966). Another possible function for the AO may be to accomodate "overflow" of reducing equivalents into the cytochrome pathway (Lambers, 1980); however, Dry *et al.* (1989), have demonstrated AO activity when the



cytochrome pathway is not saturated. Alternatively, shunting electrons through the AO pathway could allow the TCA cycle to produce carbon skeletons and reducing equivalents while not generating unneeded and potentially inhibitory ATP (Palmer, 1976). The nuclear-encoded AO has at least one subunit near 36 kDa (Elthon and McIntosh, 1987; Rhoads and McIntosh, 1991), but it is immunologically undetectable in many monocot species such as wheat and maize under normal conditions (Hiser and Peiffer, personal communication).

1.4.3 Transport systems

A number of unique transport systems exist in plant mitochondria. First, the inner membrane of mitochondria has been thought to be largely impermeable to oxaloacetate (but see Passarella *et al.*, 1985) and NAD^+ (vonJagow and Klingenberg, 1970); however, plant mitochondria readily transport these metabolites into the matrix (Douce and Bonner, 1972; Neuburger and Douce, 1978; Tobin *et al.*, 1980). Oxaloacetate transport has been shown to be mediated by a specific carrier separate from that responsible for malate transport based on differences in inhibitors (Day and Wiskich, 1984). Its physiological role has yet to be determined, but the results of Ebbighausen *et al.* (1987) suggest a role in the return of NADH from mitochondria to peroxisomes during glycine oxidation in green leaves of C_3 plants.

NAD^+ transport is thought to be mediated by a specific carrier because uptake exhibits Michaelis-Menton kinetics (Neuburger and Douce, 1978; Tobin *et al.*, 1980) and is inhibitable by a substrate analog (Neuburger and Douce, 1983). The carrier may allow manipulation of the matrix NAD^+ concentration and thus regulates the internal rotenone-insensitive pathway

(Neuburger *et al.*, 1985). Additionally, uptake of NAD^+ has been suggested to have a role in the transition from a dormant stage to a stage of active growth, such as the process of germination (Shibasaka and Tsuji, 1988).

Adenine nucleotides are also actively accumulated in plant mitochondria in a carboxyatractyloside-insensitive manner, particularly under conditions of high electrochemical potential (Abou-Kahlil and Hanson, 1977, 1979), possibly enabling plant mitochondria to maintain the total matrix nucleotide pool during mitochondrial proliferation (Douce and Neuburger, 1989). A further difference in metabolite transport is that in plant mitochondria, unlike animal mitochondria, glutamate and aspartate exchange does not appear to be electrogenic. This unique transport property may be explained by the presence of the externally-facing NADH dehydrogenase of the inner membrane and thus plant mitochondria do not require complex exchange systems for the transfer of reducing equivalents from the cytosol to the matrix (Douce and Neuburger, 1989).

1.4.4 RNA editing

For many years researchers thought that plant mitochondria protein translation did not follow either the universal code or the "mitochondrial" code. By comparing predicted protein sequences from plants with homologous proteins from other eukaryotes, Fox and Leaver (1981) deduced that CGG coded for tryptophan rather than arginine. An additional unexpected finding was that two well-conserved residues in cytochrome oxidase subunit II thought to be copper ligands (see chapter 5) were predicted from the DNA sequence to be different in wheat (Bonen *et al.*, 1984). When RNA was sequenced either directly or from cDNA, however, these unexpected changes disappeared, and the conserved amino acids as

well as the universal code were restored (Gualberto *et al.*, 1989; Covello and Gray, 1989; Hiesel *et al.*, 1989). In most cases, RNA editing produces proteins more homologous to sequences from other species than would be predicted from the genomic DNA (Gualberto *et al.*, 1989; Hiesel *et al.*, 1989; Schuster *et al.*, 1990a; however see Graves *et al.*, 1990).

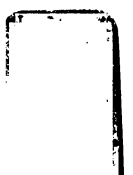
Almost all RNA editing in plants has been shown to involve substitutions of C for U (Lamattina *et al.*, 1989; Mulligan, 1991; Schuster *et al.*, 1991), although a few cases of U to C conversions have been reported (Gualberto *et al.*, 1990; Schuster *et al.*, 1990b). This is similar to C to U conversions in apolipoprotein B-48 mRNA from human intestine (Powell *et al.*, 1987; Chen *et al.*, 1987) but different from the RNA editing of paramyxovirus P, in which a run of G's is inserted (Vidal *et al.*, 1990), or trypanosome kinetoplasts, in which U's are added (Simpson and Shaw, 1989). "Guide" RNA's in the kinetoplasts are used to direct modification (Blum *et al.*, 1990). No mechanism for RNA editing has yet been identified in plants; it is possible that guide RNA's or enzymatic deamination is involved. Some transcripts are completely edited as isolated (Schuster and Brennicke, 1990) while others are only partially edited in an apparently random fashion (Schuster *et al.*, 1990a; Covello and Gray, 1990). It is not known if partially edited sequences are translated into proteins. Protein sequencing of the N-terminus of ATP synthase subunit 9 from wheat has confirmed that RNA editing completely changes the amino acid sequence predicted by the DNA (Graves *et al.*, 1990); however, the mRNA for this protein also showed complete editing (Begu *et al.*, 1990).

1.5 Research Interests

Interest in CMS has increased since Southern corn leaf blight destroyed a large portion of the 1970 corn crop in the United States. The fungal blight affected CMS but not fertile plants; therefore, the toxin produced by the fungus provides researchers with a tool for studying CMS. Physiological studies on isolated mitochondria can produce valuable information on the mechanisms of toxin sensitivity and possibly of male sterility.

Since CMS may involve defects in respiratory chain proteins, these proteins themselves have become the subject of increased research efforts. Cytochrome oxidase in particular has been theorized to be involved in CMS on the basis of a number of lines of evidence (see section 5.5). Moreover, DNA sequence analysis indicated radical substitutions of conserved residues in the subunit II gene that were corrected by RNA editing (see section 1.4.4). These findings have focused attention on the need to develop efficient and relatively large-scale purification procedures which will allow studies aimed at clarifying these issues.

The initial focus of this thesis project was to characterize the effects of the CMS-specific fungal toxin on normal and CMS maize mitochondria (chapters 2 through 4); a second and more intensive effort was the purification and characterization of cytochrome *c* oxidase from wheat seedling and wheat germ (chapters 5 through 8).



Chapter 2 - Literature review of cytoplasmic male sterility

2.1 Overview

Male sterility is a condition in plants characterized by the inability to shed viable pollen, whether because pollen production is aborted or anthers are underdeveloped. When the trait is maternally inherited, as is often the case, it is called cytoplasmic male sterility (CMS) because cytoplasmic factors (chloroplast or mitochondria) must therefore be responsible. Most CMS lines arose spontaneously under natural conditions; however, the trait can be artificially produced by either protoplast fusion or recurrent backcrossing (crossing the female offspring of each generation with the original male parent) between different species. These techniques produce offspring which contain the mitochondria and chloroplasts of one original parent (female) and the nuclear DNA of the other original parent (male). Thus, the evolved coordination of the cytoplasmic and nuclear genomes is lost. The important role of the nucleus in organellar protein synthesis is emphasized by the existence of nuclear restorer genes (*Rf*s), which restore fertility when crossed into CMS plants.

CMS has been exploited for the commercial production of hybrid seed. Since male-sterile plants still function normally as the seed-parent (female), any seed made by the CMS plant cannot be the result of self-pollination and is necessarily a hybrid. If rows of CMS plants are alternated with male-fertile or restored plants, the correct hybrid will always be produced. This procedure avoids the laborious and often impossible task of hand emasculation of the anthers. Since the CMS trait also does not affect vegetative growth of the plant, the use of CMS in maize

became so common that by the late 1960's over three-quarters of all acreage planted in the United States was the CMS Texas (T) type. Most of the crop was lost to Southern corn leaf blight in 1969 and 1970 (Ullstrup, 1972), but only the CMS-T plants were affected. The mechanism of the effects of the toxin produced by the fungus *Bipolaris maydis* race T (BmT; formerly called *Helminthosporium maydis*) on the plant as well as the mechanism of CMS became a focus of plant research.

Since CMS is maternally inherited, the trait could be encoded by either the chloroplast or the mitochondria since both contain their own DNA. Many studies found differences between male-sterile and male-fertile plants in mitochondrial but not the chloroplast DNA restriction endonuclease digestion patterns (Levings and Pring, 1976; Pring and Levings, 1978) and translation products (Forde *et al.*, 1978; Forde and Leaver, 1980; Borck and Walbot, 1982; Dixon and Leaver, 1982; Boutry and Briquet, 1982; Boutry *et al.*, 1984). Moreover, mitochondria but not other organelles were observed to degenerate in the tapetum and middle layer of CMS-T anthers (Warmke and Lee, 1977), and the BmT fungal toxin was shown to affect isolated mitochondria but not chloroplasts (Gregory *et al.*, 1978). Thus it is clear that the mitochondrial DNA (mtDNA) encodes CMS.

2.2 CMS in different plants

CMS has been found in over 140 plant species, and since most of these have arisen naturally, the underlying condition causing CMS must be easily generated. The hallmark of many of the types of CMS studied is mtDNA rearrangements which lead to altered or novel proteins. The large mitochondrial genomes of plants contain direct and inverted repeats which cause homologous recombination, producing different size DNA circles

(Palmer and Shields, 1984; Lonsdale *et al.*, 1984). It is therefore likely that these mtDNA rearrangements are also the result of homologous recombinations involving these repeats.

2.2.1 Sorghum

CMS has been generated several times in sorghum by backcrossing (Stephens and Holland, 1954; Webster and Singh, 1964). One unique type of CMS possessed small plasmid-like DNA's, N1 and N2, which were homologous to the S1 and S2 elements implicated in CMS-S in maize (Pring *et al.*, 1982; see below). The other types of CMS in sorghum were shown to have different mtDNA restriction patterns (Conde *et al.*, 1982), suggesting mtDNA rearrangements typical of CMS. Dixon and Leaver (1982) and Bailey-Serres *et al.* (1986) demonstrated that the CMS cytoplasms produced variant translation products when in nuclear backgrounds other than their own. One cytoplasm, designated 9E, synthesized a polypeptide of 42 kDa while every other cytoplasm (and other plants) produced a 38 kDa polypeptide. Antibodies againt yeast cytochrome oxidase subunit I immunoprecipitated both proteins (Dixon and Leaver, 1982). The subunit I gene (*coxI*) was later shown to have undergone a gene rearrangement in 9E which results in a C-terminal extension of 75 amino acids (Leaver *et al.*, 1985; Bailey-Serres *et al.*, 1986). Activity of cytochrome oxidase in seedlings with the altered *coxI* gene does not appear to affected (Bailey-Serres *et al.*, 1986), and a line with 9E nucleus *and* cytoplasm synthesized the larger *coxI* protein but was male-fertile (Leaver *et al.*, 1985). Therefore, the alteration of the *coxI* gene may not be the cause of CMS, although Bailey-Serres *et al.* (1986) have pointed out that the fertile line synthesizing the altered *coxI* protein may have nuclear genes that have co-evolved with the

mitochondrial genome rearrangements to compensate for the variant polypeptide.

2.2.2 Petunia

One unique restriction fragment was found in all naturally-occurring CMS petunia plants (Boeshore *et al.*, 1985), suggesting that only one mtDNA rearrangement is responsible for CMS in petunia. The ***pcf*** (for petunia CMS-associated fusion gene) locus of the unique restriction fragment was sequenced and found to contain fused fragments of the 5' flanking and coding regions of subunit 9 of the ATPase (*atp9*, which naturally contains a short region highly homologous to chloroplast *atpB*, the β subunit of the ATPase), in-frame coding regions of subunit II of cytochrome oxidase (*coxII*), and a sequence of unknown origin called *urf-S* (Young and Hanson, 1987). A spliced RNA intermediate is proposed for this DNA rearrangement because the intron normally found in the petunia *coxII* gene (Pruitt and Hanson, 1989) is not in the *pcf* sequence (Young and Hanson, 1987). Full length genes of both *atp9* and *coxII* appear elsewhere in the genome of CMS petunia (Young and Hanson, 1987). Downstream from the *pcf* locus is the only genomic copy of subunit 3 of the NADH dehydrogenase (*nad3*) and ribosomal protein small subunit 12 (*rsp12*), and these genes are co-transcribed with *pcf* (Rasmussen and Hanson, 1989). Transcripts of the *pcf* gene were found to be four to five times more abundant in anthers than in the leaves of CMS plants (Young and Hanson, 1987). Possibly the *pcf* locus is under the promoter of the *atp9* gene since the 5' flanking region which would contain the promoter is included in *pcf*; however, the levels of *atp9* transcripts do not appear to vary (Young and Hanson, 1987). A diagram of the *pcf* locus is shown in Figure 2.1A.

Nivison and Hanson (1989) produced antibodies to a number of oligonucleotides predicted from the *pcf* gene to verify the presence of a translation product. However, in the petunia mitochondria the peripheral membrane protein reacting to the antibody was only 25 kDa instead of 39 kDa as predicted from the open reading frame (Nivison and Hanson, 1989). Since antibodies to *coxII*-derived sequences did not react with the 25 kDa protein, no part of the *coxII*-derived sequence appears to be present in the mature protein. A single dominant nuclear restorer gene eliminates production of this protein, although the levels of the *pcf* transcript are not reduced (Rasmussen and Hanson, 1989), suggesting that the *Rf* protein is operating post-transcriptionally. Antibody to the *urf-S* sequence (the third segment of the fusion gene) also reacted with a 20 kDa integral membrane protein produced in both CMS and fertile petunia mitochondria (Nivison and Hanson, 1989).

Alternative oxidase activity was found to be lower in suspension culture cells of CMS plants than in isonuclear fertile or fertility-restored cells as well as in their mitochondria (Connett and Hanson, 1990). When specific tissues were analyzed, immature anthers, but not leaves and mature anthers, showed a statistically significant lower level of AO activity in CMS compared to fertile or restored lines. These differences were not due to either substrate saturation or ATP inhibition. Therefore one result of *pcf* expression may be to lower alternative oxidase activity. Musgrave *et al.* (1986) have previously reported a correlation between low AO activity and CMS in several different plants (but see Obenland *et al.*, 1988; Goyal *et al.*, 1991). The strong correlation in anthers but not leaves between *pcf* transcripts and lowered AO activity is suggestive of a connection between AO and CMS which may be developmentally regulated.

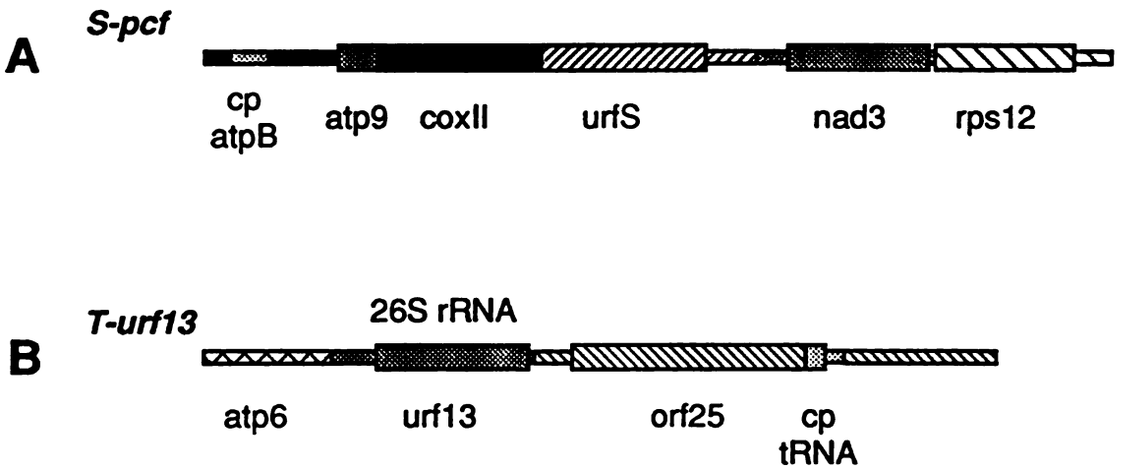


Figure 2.1: Schematic of the chimeric structures of the CMS-specific gene loci in petunia, *S-pcf* (**A**) and maize, T cytoplasm, *T-urf13* (**B**).

2.2.3 Maize

Maize has been the plant of choice for CMS research because of its agricultural importance and its convenience of use. Three CMS cytoplasm types have been identified in maize on the basis of their fertility restoration (Beckett, 1971): C (Charrua), S (USDA), and T (Texas). All three types were shown to contain mtDNA rearrangements (Levings and Pring, 1976; Pring and Levings, 1978) and to encode novel proteins (Forde *et al.*, 1978; Forde and Leaver, 1980) relative to normal (N) fertile mitochondria.

2.2.3.1 CMS-C

Rearrangements in the only genomic copy of three mitochondrial genes (*atp9*, *atp6*, and *coxII*) have been detected in all cytoplasmic types of the C group tested (Dewey *et al.*, 1991). The arrangements of these gene loci in CMS-C and -T or N are compared in Figure 2.2. No differences in hybridization patterns were found using probes to a number of other genes. Both the 5' and 3' regions of the *atp9* gene have undergone rearrangements, while the coding region remains unchanged (Figure 2.2A). Furthermore, the *atp6* and *coxII* genes have unusual chimeric organizations which may affect the size of their coding regions (Figure 2.2 B, C).

Antibodies to *coxII* oligopeptides reacted with 29 kDa polypeptides in both CMS-C and -T mitochondria, indicating that the *coxII* translation product is processed or that translation is initiated after the point of rearrangement (Figure 2.2C). Attempts to prepare antibodies to *atp6* sequences were unsuccessful; therefore, it cannot be determined whether CMS-T and -C produce identically-sized translation products for this gene. If the entire ORF 5' to the *atp6* coding region in both CMS-C and -T is translated and then processed at a site near where similarity to other *atp6*

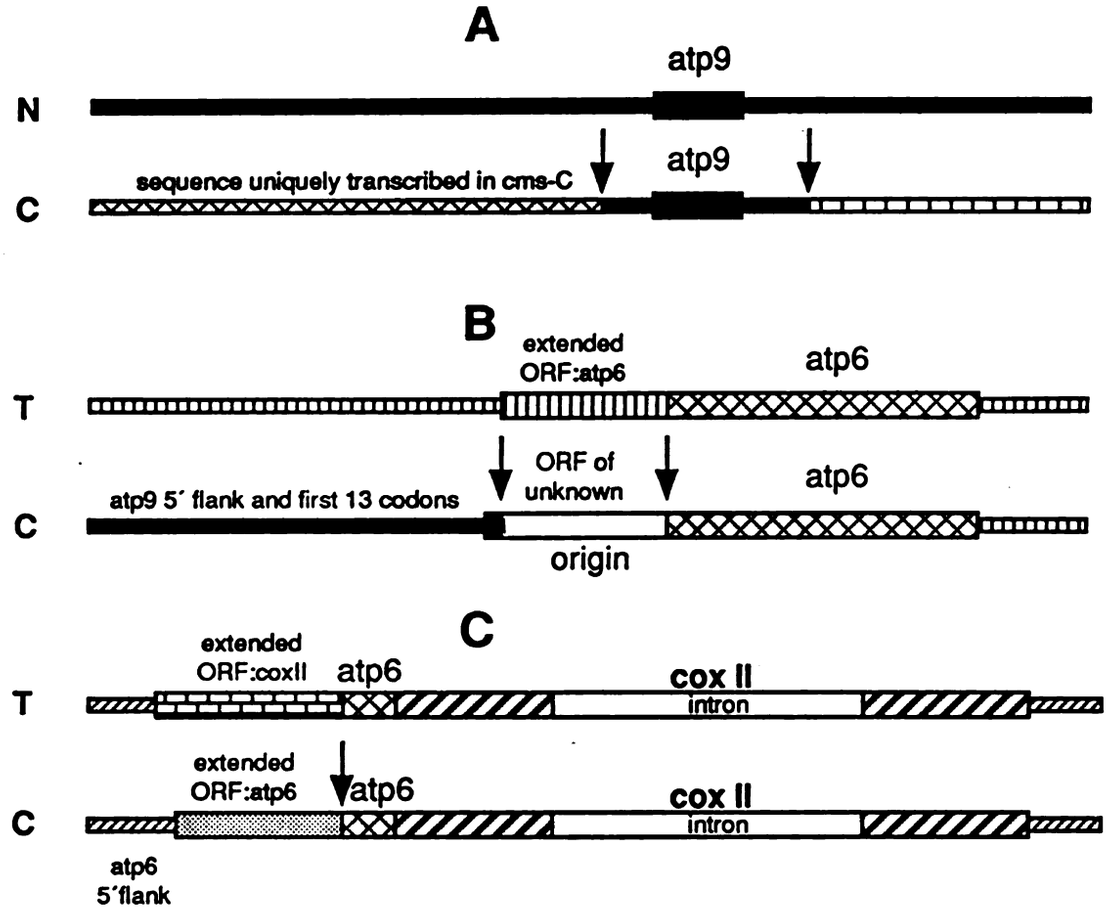


Figure 2.2: Schematic comparison of structures of *atp9* (**A**), *atp6* (**B**), and *coxII* (**C**) gene loci in mitochondrial DNA from maize with N, T, and C cytoplasms. The vertical arrows indicate probable sites of recombination.

sequences begins, then amino-terminal cleavage products of approximately 15.5 kDa in CMS-T and 17.8 kDa in CMS-C would be produced. Interestingly, Forde and Leaver (1980) found that CMS-C mitochondria synthesize a unique 17.5 kDa protein, while they are missing a 15.5 kDa protein found in other maize cytoplasms. However, this pattern of protein synthesis, as well as transcription of these three genes, is unaffected by the single nuclear restorer gene Rf_4, suggesting that these rearrangements may not be the cause of CMS in the C cytoplasm of maize.

2.2.3.2 CMS-S

Gel electrophoresis and electron microscopy of mtDNA demonstrated that mitochondria with the S cytoplasm possess 2 small linear DNA's (S1 and S2) in addition to the normal mtDNA (Pring *et al.*, 1977). S1 and S2 both possess 208-bp terminal inverted repeats (Kim, 1982; Levings and Sederoff, 1983; Palliard *et al.*, 1985) and terminally-attached proteins (Kemble and Thompson, 1982). Thus S1 and S2 appear similar to several double-stranded DNA viruses, such as adenovirus (Rekosh *et al.*, 1977) or *Bacillus subtilis* phage ø29 (Salas *et al.*, 1978) which both contain terminal inverted repeats and terminally-attached proteins. The viral proteins' role in DNA replication (Rekosh *et al.*, 1977; Mellado *et al.*, 1980) has prompted the suggestion that the terminal proteins in S1 and S2 prime the replication of these elements (Kemble and Thompson, 1982).

Upon spontaneous reversion to fertility (which is very common in the field for CMS-S, unlike CMS-C and -T), the S1 and S2 elements are lost (Levings *et al.*, 1980), suggesting that they are responsible for CMS. However, fertility restoration by the single nuclear gene Rf_3 did not affect either the presence of S1 and S2 (Kemble and Bedbrook, 1980) or the production of unique high molecular weight proteins by CMS-S

mitochondria (Forde and Leaver, 1980). Moreover, a number of fertile races of Latin American maize were found to have linear DNA's similar to S1 and S2, called R1 and R2. R1 shares considerable homology to S1 but contains additional unique sequences, and R2 is identical to S2 (Weissinger *et al.*, 1982). One report has suggested that S1 and S2 may even be retained as free elements in some revertants (Escote *et al.*, 1985). Therefore, the relation of these elements to male-sterility is not clear.

One theory for the involvement of S1 and S2 in CMS relates to the mitochondrial reorganization their presence induces. In their presence, the normally circular mitochondrial genome is linearized (Schardl *et al.*, 1984). Recombination between S1 and S2, and genomic sequences homologous to the terminal inverted repeat, produce many linear fragments terminated by S1 or S2 sequences. Upon reversion to fertility and loss of the free elements, the mitochondrial DNA is recircularized, although S1 and S2 sequences are integrated into the mitochondrial genome and retained (Schardl *et al.*, 1985). Therefore CMS may be the result of new DNA arrangements in the presence of S1 and S2, although they do not specifically encode CMS.

S2 contains 5452 bp and two long open reading frames, URF1 and URF2 (Levings and Sederoff, 1983). S1 is 6397 bp with 1462 bp of nearly perfect homology with S2; there are three long open reading frames, URF2, URF3, and URF4, with URF2 occurring in the region of homology with S2 (Paillard *et al.*, 1985). By using antibodies directed to oligonucleotides predicted from the DNA sequence, a 130 kDa protein was confirmed to be the gene product of URF1 in S2 (Zabala *et al.*, 1987) while a 103 kDa protein is produced by URF3 in S1 (Zabala and Walbot, 1988). Both antibodies detected protein when fertility was restored (spontaneously or by Rf_3), but

did not react with protein from CMS-T or CMS-C mitochondria. In N mitochondria, low levels of the 130 kDa protein but not the 103 kDa protein were detected (Zabala *et al.*, 1987; Zabala and Walbot, 1988), in line with the earlier findings that S1 and S2 sequences are present in the mtDNA of N but not CMS-C and -T cytoplasms (Thompson *et al.*, 1980; Lonsdale *et al.*, 1981).

2.2.3.3 CMS-T

The fungal toxin causing Southern corn leaf blight (BmT) which is specific for CMS-T plants has been a useful tool in identifying the defects involved in CMS. The traits of CMS and toxin sensitivity appear to be linked since CMS-T tissue culture revertants are almost always both male fertile and toxin insensitive (Bretell *et al.*, 1979; Bretell *et al.*, 1980; Dixon *et al.*, 1982; Kemble *et al.*, 1982; Umbeck and Gengenbach, 1983; Rottman *et al.*, 1987; Wise *et al.*, 1987b). Exposure to the BmT toxin affects isolated mitochondria of T corn, inducing swelling (Miller and Koeppe, 1971; Gengenbach *et al.*, 1973b; Gregory *et al.*, 1978; York *et al.*, 1980; Klein and Koeppe, 1985) and collapsing the potential across the inner membrane (Berville *et al.*, 1984; Holden and Sze, 1987a). NAD^+ and CoA leak from the matrix (Matthews *et al.*, 1979; Berville *et al.*, 1984) and Ca^{2+} transport into the mitochondria is reduced (Holden and Sze, 1984; Kimber and Sze, 1984). Respiration is inhibited when NAD^+-linked substrates (such as glutamate/malate) are used, while succinate respiration is unchanged in the presence of KCl but stimulated in its absence (Miller and Koeppe, 1971). Inhibited respiration can be restored by the addition of exogenous NAD^+, presumably by replenishing the NAD^+ lost by leakage (Berville *et al.*, 1984; Matthews *et al.*, 1979). ADP/O ratios (the amount of ADP phosphorylated

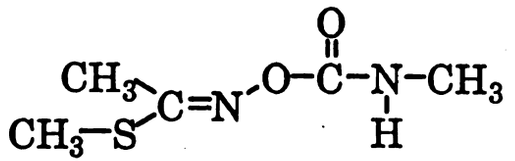
per oxygen atom consumed) are reduced by the toxin (Miller and Koeppe, 1971).

Sensitivity to BmT toxin remains one of the few major differences between N and T mitochondria. Similar respiratory control ratios (RCR: the state 3 rate, when ADP is present, divided by the state 4 rate, when ADP is absent) and ADP/O ratios are found (Dutch *et al.*, 1987). There are small differences in sensitivities to ionophores such as valinomycin and gramicidin, and a significant difference in sensitivity to nigericin (Gengenbach *et al.*, 1973a), but only the toxin shows complete selectivity. Methomyl (Figure 2.3A), a carbamate insecticide which inhibits acetylcholinesterase, shows identical selectivity for T corn and mimics toxin action, although at higher concentrations than required for the toxin (Koeppe *et al.*, 1978). There is no apparent structural rationale for the similar effects of the toxin and methomyl.

The mode of toxin action has been studied since 1971. Kono and Daly (1979) purified the toxin (Figure 2.3B) from a fungal extract and determined the toxin's 41-carbon polyketol structure (Kono *et al.*, 1980). The toxin could be washed out of affected mitochondria with fresh suspension media, reversing its inhibitory effects, indicating that the toxin did not bind strongly (Bednarski *et al.*, 1977). One report has indicated that removal of the outer membrane of N mitochondria (by the swell-shrink method) and subsequent toxin addition induced effects similar to that seen in T mitochondria (Watrud *et al.*, 1975). This suggested that the site of action might be on the inner membrane or in the matrix with selectivity conferred by the outer membrane or the inter-membrane space.

A number of toxin analogs and derivatives have also been tested for their effectiveness in order to determine the structural elements necessary

A



B

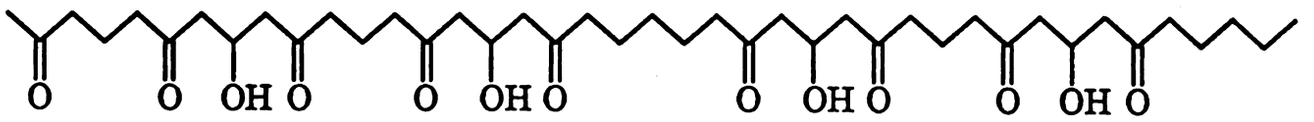


Figure 2.3: Molecular structures of methomyl (A) and Bm T toxin (B)

for toxin action. Chain length (Suzuki *et al.*, 1982) and methylene bridge length (Suzuki *et al.*, 1983) determine the potency of the toxin, with the native toxin most effective. The acetylated derivative demonstrated specificity and effectiveness for NADH and succinate but not malate oxidation, suggesting independent mechanisms for the two effects (Payne *et al.*, 1980). Borohydride-reduced toxin also retained biological activity and specificity, although binding of a tritiated form was non-specific and equal between N and T mitochondria (Frantzen *et al.*, 1987), questioning the existence of a discrete toxin binding site. Similar structural studies have been performed on methomyl (Aranda and Gauvrit, 1986).

Some evidence suggests that dicyclohexylcarbodiimide (DCCD) blocks toxin action (Holden and Sze, 1987b; Bouthyette *et al.*, 1985). DCCD is known to covalently bind to many mitochondrial proteins including the outer membrane porin, the K^+/H^+ exchange carrier, cytochrome *c* oxidase, and sites on both F_0 and F_1 of the ATP synthase (Solioz, 1984). Additionally, kaempferol, also an ATP synthase inhibitor, has been shown to block some effects of the BmT toxin and methomyl (Koeppe *et al.*, 1978). This evidence strongly suggests that a protein is involved in the toxin's effects and selectivity. However, the toxin has also been shown to be a channel former in black lipid membranes (Holden *et al.*, 1985), suggesting that no protein binding site is necessary. If the toxin forms channels it could leak large molecules such as NAD^+ and other coenzymes, as well as dissipate the membrane potential. Such behavior, however, would be expected to be observed in any membrane, not just those in T mitochondria, unless there were a significant difference in lipid composition of N and T mitochondrial membranes.

There are two pieces of evidence suggesting that T corn mitochondria have a higher concentration of sterol in their membranes. First, Gregory *et al.* (1977) found that four times the digitonin concentration needed in T was needed in N to maximally uncouple succinate respiration in corn root mitochondria. Since digitonin is a detergent that at low concentrations is selective for sterol-rich membranes, they proposed that T mitochondrial membranes contained more sterol than N mitochondrial membranes. York then attempted to chemically quantitate the sterol content differences between N and T membranes. He found that while the relative proportions of the major sterol types were the same, there was a greater overall amount of sterol in T than N by about 20% (York, 1980). York did not, however, attempt to distinguish between the inner and outer membranes, and he provided no statistical analysis of his data.

Mor recent data has indicated that CMS-specfic proteins provide the specificity of the toxin for CMS mitochondria. Mitochondrial DNA rearrangements correlating with CMS-T were identified by restriction endonuclease analysis (Levings and Pring, 1976; Pring and Levings, 1978). Forde *et al.* (1978) demonstrated a unique 13 kDa translation product in CMS-T mitochondria, while a 21 kDa protein found in other cytoplasms was missing. An open reading frame encoding a 12.9 kDa protein (Dewey *et al.*, 1986) was found on a CMS-T restriction fragment that is lost in male-fertile toxin-insensitive revertants (Umbeck and Gengenbach, 1983) and thus is proposed to be the unique 13 kDa peptide. The chimeric gene, called *urf13-T*, contains sequences from the *atp6* 5' flank, the 26S rRNA 3' flank, sequence of unknown origin, and 26S rRNA coding regions (Dewey *et al.*, 1986). A downstream open reading frame coding for a 25 kDa protein (ORF25) contains sequence homology to tRNA-Arg from tobacco

chloroplast, and is the only copy of this sequence in the genome. While the identity of ORF25 is unknown, sequences homologous to it have been identified in other plants. The structure of the *urf13-T* locus is illustrated in Figure 2.1 B. Antibodies to oligopeptides predicted by the *urf13-T* sequence confirmed that the translation product of the gene is a 13 kDa membrane protein (Wise *et al.*, 1987b; Dewey *et al.*, 1987). Presence of the Rf_1 restorer gene decreased production of the 13 kDa protein (Dewey *et al.*, 1987) but *urf13-T* transcripts were reduced to a lesser extent, suggesting that Rf_1 affects translational control rather than RNA processing (Kennell *et al.*, 1987; Kennell and Pring, 1989). Loss of the 13 kDa peptide by either frameshift mutation (Wise *et al.*, 1987a) or elimination by homologous recombination of the *urf13-T* gene (Rottman *et al.*, 1987) resulted in male-fertile and toxin-insensitive plants. These data, taken together, strongly suggests that the 13 kDa peptide encodes both toxin sensitivity and CMS.

The *urf13-T* gene has been cloned and expressed in other organisms to verify its role in toxin binding. In *E. coli* expressing the 13 kDa peptide, toxin causes inhibition of whole cell respiration and swelling of spheroplasts (Dewey *et al.*, 1988). In yeast, the gene was introduced into the nucleus and its translation product directed to the mitochondria by fusion of the gene with the targetting presequence portion of the gene for *Neurospora crassa* ATPase subunit 9. Toxin and methomyl inhibit growth and uncouple NADH-stimulated respiration in yeast expressing the 13 kDa peptide (Huang *et al.*, 1990; Glab *et al.*, 1990). Most recently, the gene has been introduced into tobacco, causing toxin-sensitivity (vonAllmen *et al.*, 1991).

The site of toxin and methomyl interaction with the 13 kDa peptide has been investigated by site-directed mutagenesis and binding studies. An

amino terminal deletion of residues 2-11 eliminated toxin sensitivity (Dewey *et al.*, 1988) while deletions of up to one-quarter of the amino acids at the carboxyl end had no effect (Braun *et al.*, 1989). Mutants of acidic residues identified Asp-12 and Asp-39 as DCCD-binding sites (Braun *et al.*, 1989). Asp-39 seems to be the more important of the two for toxin interaction since toxin sensitivity is eliminated when this residue alone is mutated or covalently modified by DCCD.

Despite the negative results of Frantzen *et al.* (1987, see above), Braun *et al.* (1990) were able to demonstrate binding of ^{3}H-reduced toxin to the 13 kDa peptide in competition and displacement studies. Furthermore, toxin and methomyl could compete out each other for binding, suggesting an identical or overlapping binding site. Binding of toxin in *E. coli* is cooperative, possibly indicating that the 13 kDa peptide exists in an oligomeric structure as suggested by preliminary cross-linking studies (Williams *et al.*, 1990). Most toxin-insensitive mutants of the 13 kDa protein bind little or no toxin except for the N-terminal 2-11 deletion mutant, which binds a normal amount of toxin, indicating that these residues may be important for toxin interaction. While these experiments convincingly define the 13 kDa peptide as theBmT toxin binding site, the role of the 13 kDa peptide in producing the CMS phenotype remains unclear.

2.3 Hypotheses for the mechanism of CMS

In the absence of any direct evidence for a mechanism of CMS, a number of theories have been put forth. Although a few of the theories were proposed prior to the discoveries of the *urf13-T* and *pcf* genes, they can still account for the experimental data on pollen abortion. Flavell (1974) first proposed that a substance produced only in the anther interacts with the

altered organelles in CMS plants, preventing normal anther development. He pointed out that the involvement of a regulatory substance under developmental control can account for the variation in the extent of sterility in different anthers and the dependence of sterility on the environment. Nuclear restorer genes are postulated to correct the altered organelle structure, making the organelle insensitive to the anther-specific substance, or to interfere with the substance's transport in the anther. Flavell's anther-specific substance may have a structure similar to the BmT toxin, binding to the 13 kDa peptide (or *urfS* peptide in petunia) and causing mitochondrial dysfunction and therefore pollen abortion.

The theory of Warmke and Lee (1978) involves their observation of a massive mitochondrial proliferation in the tapetal and sporangeous cells during early growth stages of anthers in normal plants (Lee and Warmke, 1979). The number of mitochondria can increase 20 to 40 times in these tissues while only moderate increases in the cell dimensions occur. The mitochondrial volume was shown to decrease concomitantly 12 to 15 times. This mitochondrial proliferation immediately precedes the observed degeneration in anthers with T cytoplasm. The mitochondria of the female reproductive tissues, however, were found to remain relatively constant in size and number during gametogenesis. Warmke and Lee (1978) therefore proposed that the altered DNA observed by restriction endonuclease digestion may lead to a change in the energy-generating ability of mitochondria such that they cannot support the rapid mitochondrial proliferation in the anthers. To update this model, one may theorize that the 13 kDa or urfS peptides may cause only a slight impedence to mitochondrial respiration under normal growth conditions; however, this

small decrease in the efficiency of respiration may be sufficient to prevent the rapid respiration necessary for proliferation and lead to pollen abortion.

Since CMS-linked mtDNA rearrangements often involved genes of respiratory complex subunits, alterations in the protein structures themselves have been suggested to be the cause of the CMS trait. Cytochrome oxidase was suspected because its genes are involved in the rearrangements of petunia, maize-C, sorghum, and several other plants. Additionally, the anthers of CMS-T maize were found to have lower cytochrome oxidase concentrations than normal fertile or restored plants (Watrud *et al.*, 1976). Moreover, immunoprecipitates of the oxidase contain the 13 kDa peptide unique to CMS-T (C. Leaver, personal communication). However, N and T maize mitochondria have similar respiratory rates and equal efficiencies of oxidative phosphorylation (Gengenbach *et al.*, 1973a; Dutch *et al.*, 1987).

The ATP synthase has been another candidate for involvement in CMS since the chimeric genes of both petunia and CMS-T contain pieces of ATPase genes and may be under control of ATPase gene promoters. The *atp6* gene is also disrupted in CMS Ogura radish (Makaroff *et al.*, 1989). Inhibitors of the ATPase (DCCD and kaempferol) also block toxin action, and ATPase subunits have been found to coprecipitate with antibodies to the 13 kDa protein (Wise *et al.*, 1987a). However, recent studies demonstrating direct DCCD and toxin binding to the 13 kDa peptide have cast some doubt on the possibility that altered respiratory proteins are involved in CMS.

More recently, Hanson *et al.* (1989) have pointed out that a common feature of CMS in both T maize and petunia is the presence of single copy genes downstream from the chimeric gene encoding the unique protein. In the case of petunia, the only genomic copies of the *nad3* (NADH

dehydrogenase subunit 3) and *rsp12* (ribosomal protein small subunit 12) genes are co-transcribed with *pcf*. Similarly, ORF25 (whose identification remains unknown) is cotranscribed with *urf13-T* in CMS-T. Since both single copy genes are likely transcribed from different promoters than in N mitochondria, their transcription and expression could be affected. When fertility is restored by *Rf* genes, transcription and translation may be altered for these genes, allowing a more "normal" expression and thus fertility.

Further studies are needed to determine which, if any, of these theories may explain male sterility. To assess the Hanson theory, the chimeric genes could be introduced into other plants such that no other single copy genes are co-transcribed. vonAllmen *et al.* (1991) have expressed *urf13-T* under the control of the cauliflower mosaic virus 35S promoter in tobacco. The transformed plants are toxin sensitive, but appear to be fertile. However, since the gene is now located in the nucleus and the protein is therefore not inserted in the mitochondrial inner membrane, it is not possible to determine whether CMS is caused by the 13 kDa peptide. Newly developed methods of mitochondrial transformation (Sanford, 1990) should be useful for performing this experiment.

Chapter 3 - A search for significant structural and functional differences between mitochondria from normal and Texas male-sterile maize

3.1 Introduction

Bipolaris maydis race T, the fungus which caused the Southern corn leaf blight epidemics of 1969-71, produces a toxin (BmT) which affects Texas cytoplasmic male-sterile maize but not normal-fertile maize. The toxin causes physiological and ultrastructural changes in the mitochondria of CMS-T maize which presumably are responsible for the damage to the plant. The toxin provides a valuable tool in studying the phenomenon of cytoplasmic male-sterility.

Previous studies of CMS-T lead to three main questions: 1) What is the mode of selectivity of toxin for CMS-T maize? 2) What is the action of the toxin on mitochondria? 3) How does the selectivity of toxin relate to the male-sterile condition? Despite the discovery of a 13 kDa peptide unique to CMS-T maize which binds toxin and DCCD, many years of investigation have so far failed to conclusively demonstrate what differences between N and T mitochondria account for male-sterility. Moreover, the actual mechanism of the toxin-induced effects remains unexplained.

Some difference in the sterol content of the mitochondrial membranes of normal and CMS-T maize has been documented and suggested to be responsible for the selectivity of the toxin. We have attempted to determine whether these differences are significant and relevant. Other studies have suggested that the ATP synthase complex (F_o-F_1) of the inner mitochondrial membrane may be involved. A rearrangement of the mitochondrial genome

that is unique to T involves the *atp6* gene, while ATPase inhibitors DCCD and kaempferol were found to reduce some toxin-induced effects in T mitochondria. Although the 13 kDa peptide has been shown to be sufficient to bind toxin (Braun *et al.*, 1990), the above data suggests that the respiratory complexes may be involved in toxin binding or CMS in some as yet undefined way. We therefore hoped to verify the effects of DCCD and test other known ATPase inhibitors, as well as to assess whether any differences exist between N and T mitochondrial ATPase activity. These studies have attempted to determine if the ATP synthase complex, or subunits thereof, may form part of the binding site for the BmT toxin or be involved in its mechanism of action.

3.2 Materials and methods

3.2.1 Materials

Methomyl (> 99% pure) was obtained from the United States Environmental Protection Agency Pesticides & Industrial Chemicals Repository. BmT toxin was the gift of Dr. Robert Scheffer. Two microliters of toxin extract produced effects equal to 2 μL of 2*M* methomyl. Maize lines W64ATMS (male-sterile) and W64AHT (fertile) were obtained from the Nebraska Foundation Seed Company.

3.2.2 Isolation of mitochondria

Maize seeds were soaked in water overnight, planted in vermiculite and grown in darkness at 27°C and 80% relative humidity. Mitochondria are isolated from 4-day old etiolated maize seedlings by the sucrose cushion centrifugation method of Day and Hanson (1977). Respiratory control ratios

(RCR) of 5–7 and ADP/O ratios of 2.7–2.9 are typically obtained using glutamate/malate as substrate in a buffer ("RCR buffer") containing 0.25 *M* sucrose, 10 m*M* TES, 5 m*M* KH_2PO_4, 5 m*M* $MgCl_2$, 1 m*M* EGTA, and 0.1% BSA, pH 7.2 ar 23°C (Figure 3.1). RCR values were also tested using a buffer ("KCl buffer") containing 0.4 *M* KCl, 40 m*M* HEPES, 8 m*M* KH_2PO_4, 2 mg/mL BSA, pH 7.4 at 23°C. The mitochondria maintain good respiratory control for up to 6 hours suspended in 0.4 *M* sucrose at 20 mg/mL protein on ice. They are shown to be greater than 95% intact by their response to exogenously supplied cytochrome *c*. Cytochrome *c* cannot cross the outer membrane and thus cannot be oxidized by cytochrome *c* oxidase unless the membrane is "leaky" (Wikström and Casey, 1985). The ratio of the respiration rate induced by cytochrome *c* to the rate induced by subsequent addition of lauryl maltoside (which completely disrupts the membrane allowing free access of cytochrome *c* to oxidase) gives a measure of the degree of intactness (Figure 3.2).

3.2.3 Determination of a difference in sterol concentration

We repeated the digitonin-sensitivity experiments of Gregory *et al.* (1977) using RCR buffer instead of KCl buffer and incubation conditions which support greater mitochondrial stability. Digitonin, whose interaction with membranes is dependent on their sterol content, was dissolved at 10 mg/mL by adding hot 0.25 *M* sucrose, sonicating, and continued heating. Mitochondria and varying concentrations of digitonin were mixed in 0.25 *M* sucrose and incubated on ice for five minutes prior to assay. In each run, two glutamate/malate RCR values and one succinate RCR value are measured, and the intactness of the outer membrane is determined by the cytochrome *c*

Figure 3.1: Polarographic measurement of the respiratory control ratio in maize mitochondria. Mitochondria (0.3 mg protein) are added to a buffer containing 0.25 *M* sucrose, 10 m*M* TES, 5 m*M* KH_2PO_4, 5 m*M* $MgCl_2$, 1 m*M* EGTA, 0.1% BSA, pH 7.2 at 23° C with 25 µmol glutamate/25 µmol malate. ADP (0.2 or 0.3 µmol) is added as indicated to induce a burst of O_2 consumption (state 3 rate). When the ADP has been consumed, the rate returns to a lower value (state 4). The RCR is the ratio of the state 3 rate to the state 4 rate.

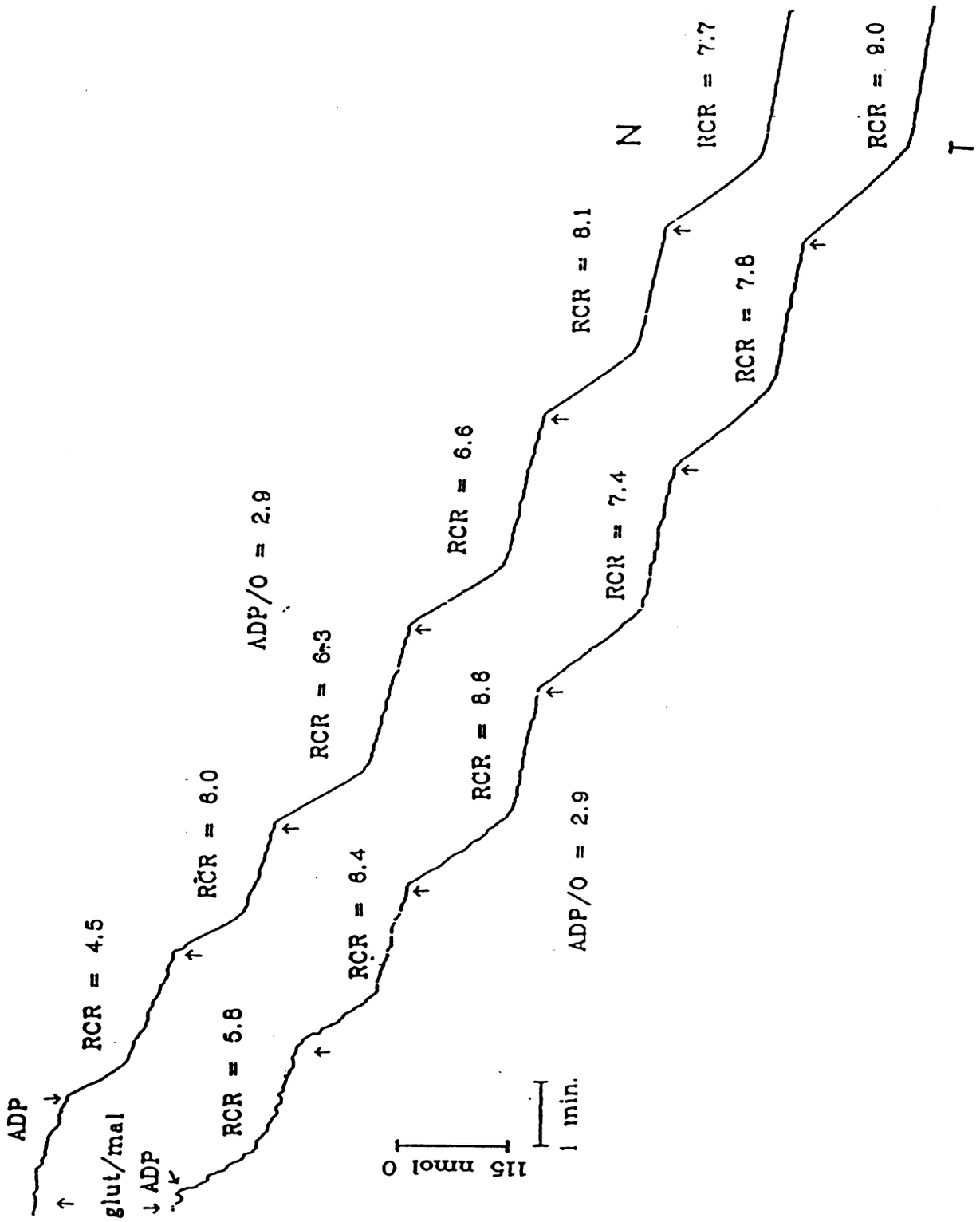

ADP
glut/mal
↓ ADP
RCR = 4.5
RCR = 5.8
RCR = 6.0
RCR = 8.4
ADP/O = 2.8
RCR = 6.3
RCR = 8.8
RCR = 6.6
ADP/O = 2.9
RCR = 8.1
RCR = 7.4
N
RCR = 7.7
RCR = 7.8
RCR = 8.0
T
115 nmol O
1 min.

Figure 3.1

Figure 3.2: Polarographic assay of the intactness of the outer mitochondrial membrane. Ascorbate (0.2 μmol) and cytochrome *c* are added to a mitochondrial suspension (0.3 mg protein) in RCR buffer at 23°C. After a respiratory rate has been established, lauryl maltoside (LM, final concentration 0.1%), is added to disrupt the membrane, producing a higher rate. The ratio of the two rates gives an estimate of the intactness of the outer membrane. The upper trace represents the typical intactness of the outer membrane of untreated mitochondria while the lower trace represents digitonin-treated mitochondria (0.50 mg digitonin/mg protein).

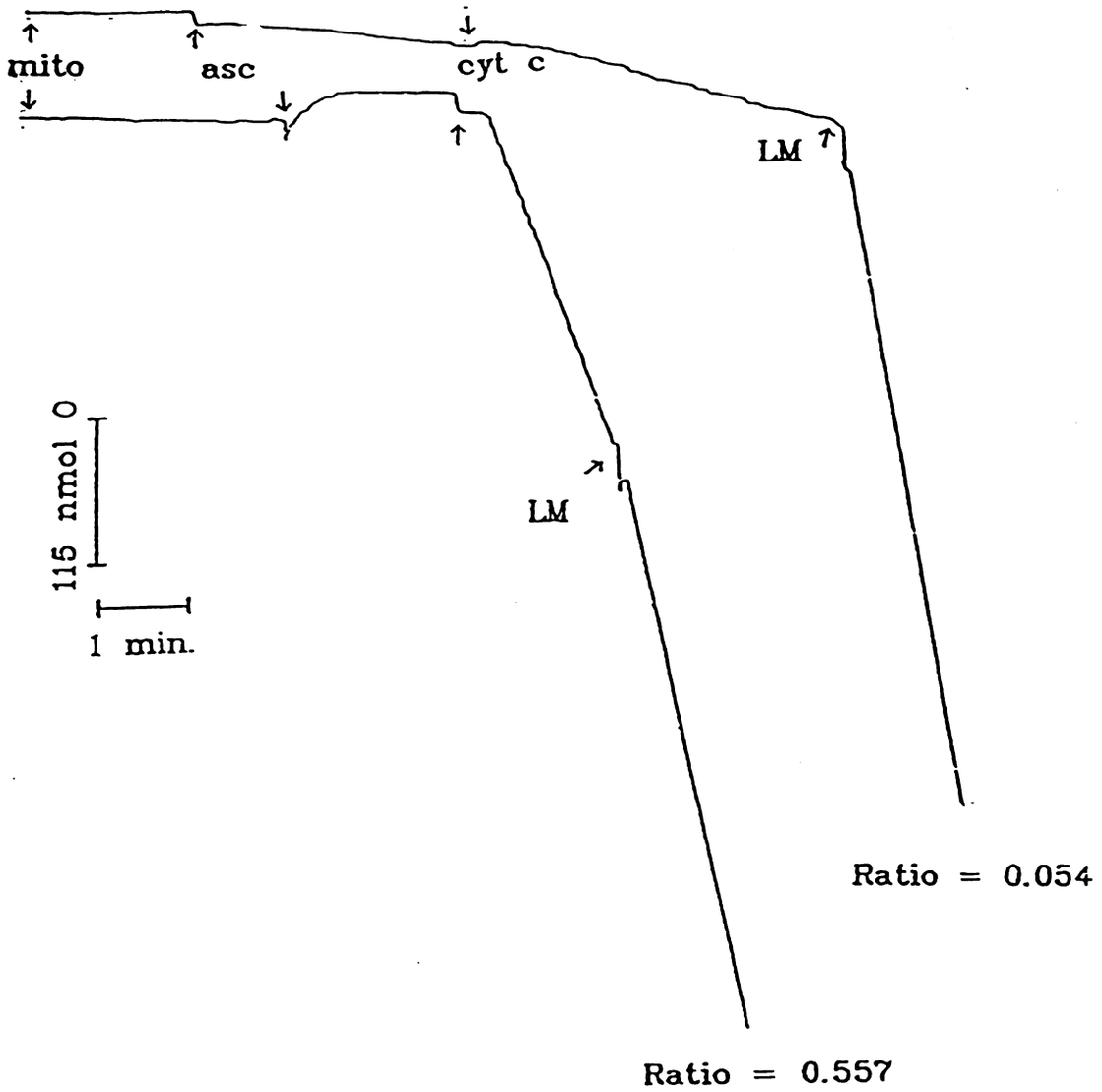


Figure 3.2

Figure 3.3: Polarographic trace of a typical assay for digitonin sensitivity of mitochondria. Mitochondria (0.3 mg) are mixed with digitonin and 0.25 *M* sucrose to a total volume of 150 µL and incubated on ice for five minutes. Glutamate/malate (50 µmol), succinate (25 µmol), ascorbate (25 µmol), ADP (0.2 µmol) and 130 µL of the mitochondrial mixture were added as indicated to the RCR buffer at 23° C. Cytochrome *c* leakage is calculated as the ratio of the O_2 consumption rate induced by cytochrome *c* and ascorbate and the rate induced by lauryl maltoside (LM, final concentration 0.1%).

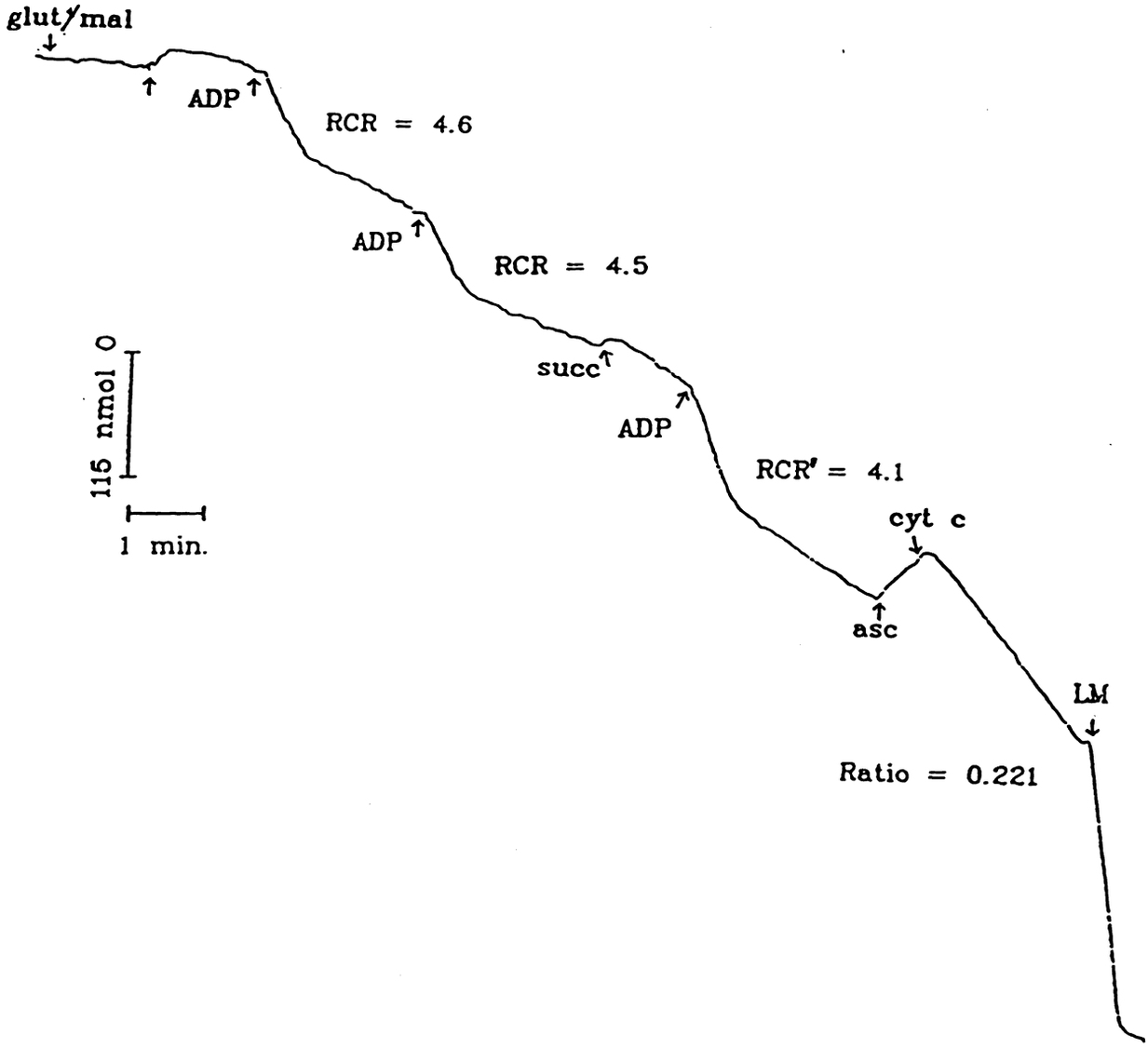

glut/mal
ADP
RCR = 4.6
ADP
RCR = 4.5
succ
ADP
RCR' = 4.1
cyt c
asc
LM
Ratio = 0.221
115 nmol O
1 min.

Figure 3.3

leakage test. (Figure 3.3) A Clark-type oxygen electrode (Yellow Springs Instruments) was used to measure oxygen consumption catalyzed by cytochrome *c* oxidase.

3.2.4 Measurement of mitochondrial swelling

Swelling of mitochondria is detected spectrophotometrically by a decrease in absorbance at 520 nm (Beavis *et al.*, 1985). Passive swelling of N and T mitochondria in a KCl buffer was measured to detect differences in sensitivity to KCl. In RCR buffer after addition of an energy substrate such as glutamate/malate or succinate to a mitochondrial suspension to establish a membrane potential, toxin or methomyl was added and swelling observed. Effectors were added either before the run was begun (as with DCCD) or before toxin was added (as with oligomycin and quercetin) to determine their effect on the toxin-induced swelling of T mitochondria.

3.2.5 Assay of ATPase activity

Mitochondria (0.15 mg protein) were incubated with succinate (2.5 µmol) as an energy source for five minutes to facilitate uptake of varying concentrations of exogenously supplied ATP (Abou-Kahlil and Hanson, 1977; Jung and Hanson, 1975). The dissipation of the proton gradient by 0.5 nmol carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, five minutes after ATP addition) induces ATP hydrolysis. After ten minutes the mitochondria are treated with oligomycin A (0.33 µg/mg protein) to inhibit the reaction and lysed in twenty volumes of water to release the contents of the mitochondria. After thirty minutes the mixture was centrifuged at 12000*g* for 10 minutes to remove mitochondrial debris. ATPase activity is then assayed as released inorganic phosphate in the supernatant by the method of Bencini *et al.* (1983)

which uses an ammonium molybdate reagent under mild conditions (pH 5.0) which promote minimal ATP hydrolysis. The absorbance of each sample was read at 350 nm. All procedures were carried out on ice. When DCCD inhibition of the ATPase was tested, the reaction was carried out at room temperature and terminated with mersalyl (67 nmol/mg protein). Oligomycin A inhibition of ATPase activity was measured by adding oligomycin (0.33 μg/mg protein) before ATP was added to the mitochondrial mixture. All other effectors were added prior to CCCP.

3.2.6 Determination of Protein Concentration

Mitochondrial protein concentration was measured by the bicinchoninic acid (BCA) method (Smith *et al.*, 1985) using reagents purchased from Pierce Chemical Company. Bovine serum albumin (BSA, Fraction V) was used as a standard.

3.3 Results

3.3.1 Differences in sterol concentrations between N and T mitochondria

To examine the reproducibility of reported differences in sterol content of N and T mitochondria, we repeated the experiments of Gregory *et al.* (1977) using RCR buffer instead of KCl buffer since the former seemed to better maintain the mitochondria's structural and functional integrity. Various parameters of the mitochondria were measured as a function of concentration of digitonin, a sterol-specific detergent. While Gregory tested only the integrity of the inner membrane (respiration of malate or succinate), we tested parameters related to the permeability of both the inner and outer

membranes so that the locations of any sterol differences could be determined. Inner membrane intactness was judged by the RCR values with glutamate/malate and succinate, while outer membrane integrity was judged by the oxidation of exogenously supplied cytochrome *c*. (Figure 3.3) The digitonin concentration required to maximally disrupt the outer membrane after five minutes was at most 10–20% lower in T compared to N mitochondria. (Figure 3.4) There was a 10–30% difference in deterioration of the RCR values with glutamate/malate or succinate between N and T, but this was probably not significant in view of the differences in sensitivity observed between different preparations. (Figure 3.5) Gregory saw a greater difference between N and T incubating his mitochondria with digitonin for ten minutes, while we used only five minute incubations. Ten minute incubations, when assayed in RCR buffer, show no greater difference in digitonin sensitivity than five minute incubations, and longer incubations interfere with the outer membrane intactness test. It may be that the difference in sensitivity of N and T mitochondria to digitonin is due to a differential sensitivity to the high KCl buffer used by Gregory, leading to a differential interaction with the digitonin that is not directly related to a sterol difference. KCl buffer consistently produces lower RCR values in control mitochondria than those observed with the RCR (sucrose) buffer (Figure 3.6), but no difference in passive swelling in the KCl buffer (Figure 3.7) was observed between N and T mitochondria. (Table 3.1) This argues against a difference in sensitivity to KCl between N and T. Using a simplified assay which measures only the outer membrane intactness in RCR buffer, N mitochondria appeared more sensitive to digitonin than T mitochondria (Figure 3.8), suggesting that any differences observed in digitonin sensitivity assays are due to variations between mitochondria preparations. The simplified assay is probably a more

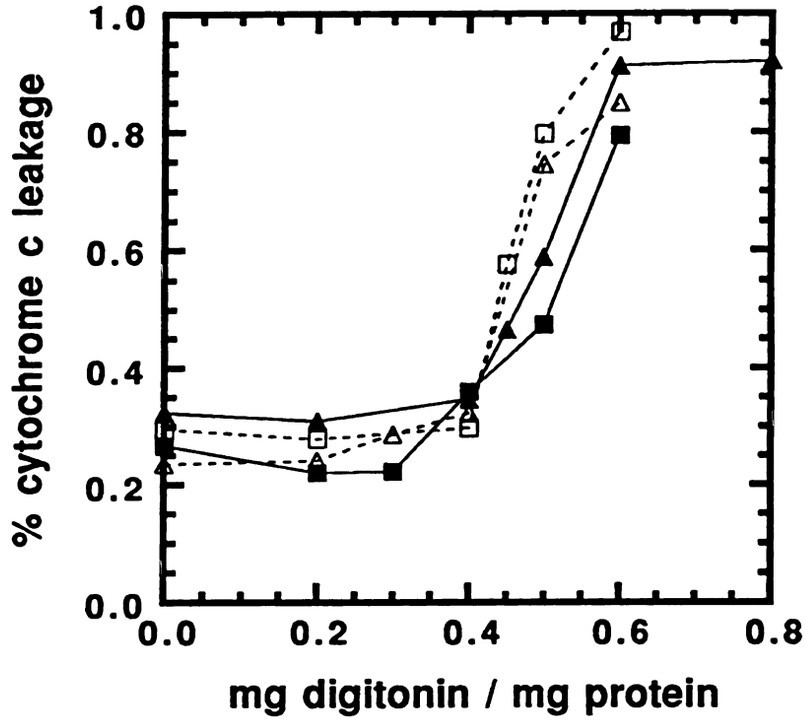


Figure 3.4: Comparison of digitonin sensitivity of two N and T mitochondria preparations each as measured by the cytochrome *c* leakage test for intactness of outer membranes. The assay is described in section 3.2.3 and illustrated in Figure 3.3. Dashed lines and open symbols represent T mitochondria, and solid lines and solid symbols represent N mitochondria.

Figure 3.5: Comparison of digitonin sensitivity of N and T mitochondria as measured by respiratory control ratios. The assay is described in section 3.2.3 and illustrated in Figure 3.3. RCR's were measured using glutamate/malate (**A**) and succinate (**B**) as substrate. Each curve represents a separate mitochondrial preparation. Dashed lines and open symbols represent T mitochondria, and solid lines and solid symbols represent N mitochondria.

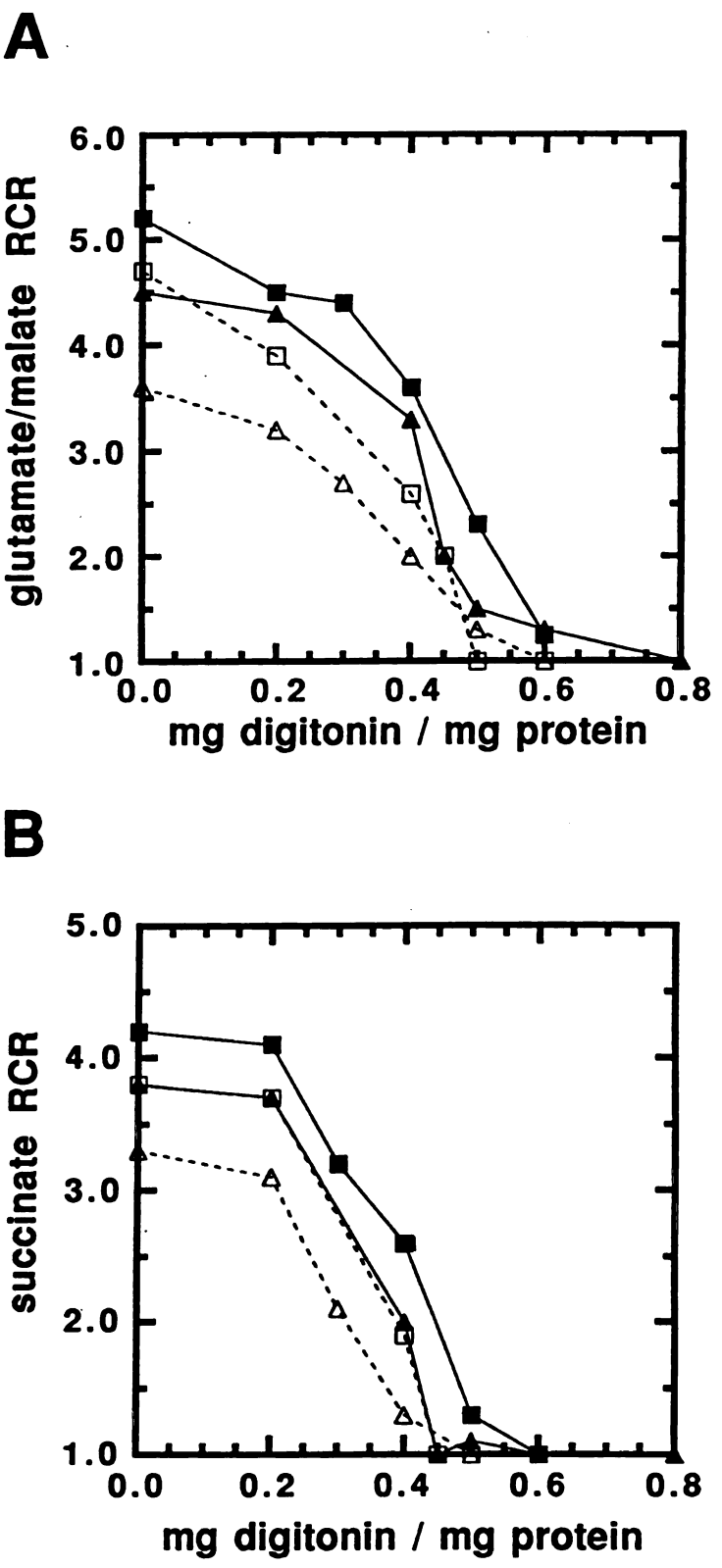

A
glutamate/malate RCR
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0.2
0.4
0.6
0.8
mg digitonin / mg protein
B
succinate RCR
5.0
4.0
3.0
2.0
1.0
0.0
0.2
0.4
0.6
0.8
mg digitonin / mg protein

Figure 3.5

Figure 3.6: Comparison of RCR values of T mitochondria measured in KCl or sucrose buffers. The same mitochondrial preparation was used for the two measurements. Glutamate/malate (25 µmol) was added to a mitochondrial suspension (0.3 mg) in the indicated buffer at 23° C. ADP (0.2 µmol) was added as indicated to induce coupled respiration. ***Upper trace*:** Sucrose buffer containing 0.25 *M* sucrose, 10 m*M* TES, 5 m*M* KH_2PO_4, 5 m*M* $MgCl_2$, 1 m*M* EGTA, 0.1% BSA, pH 7.2. ***Lower trace*:** KCl buffer containing 0.4 *M* KCl, 40 m*M* HEPES, 8 m*M* KH_2PO_4, 0.2% BSA, pH 7.4.

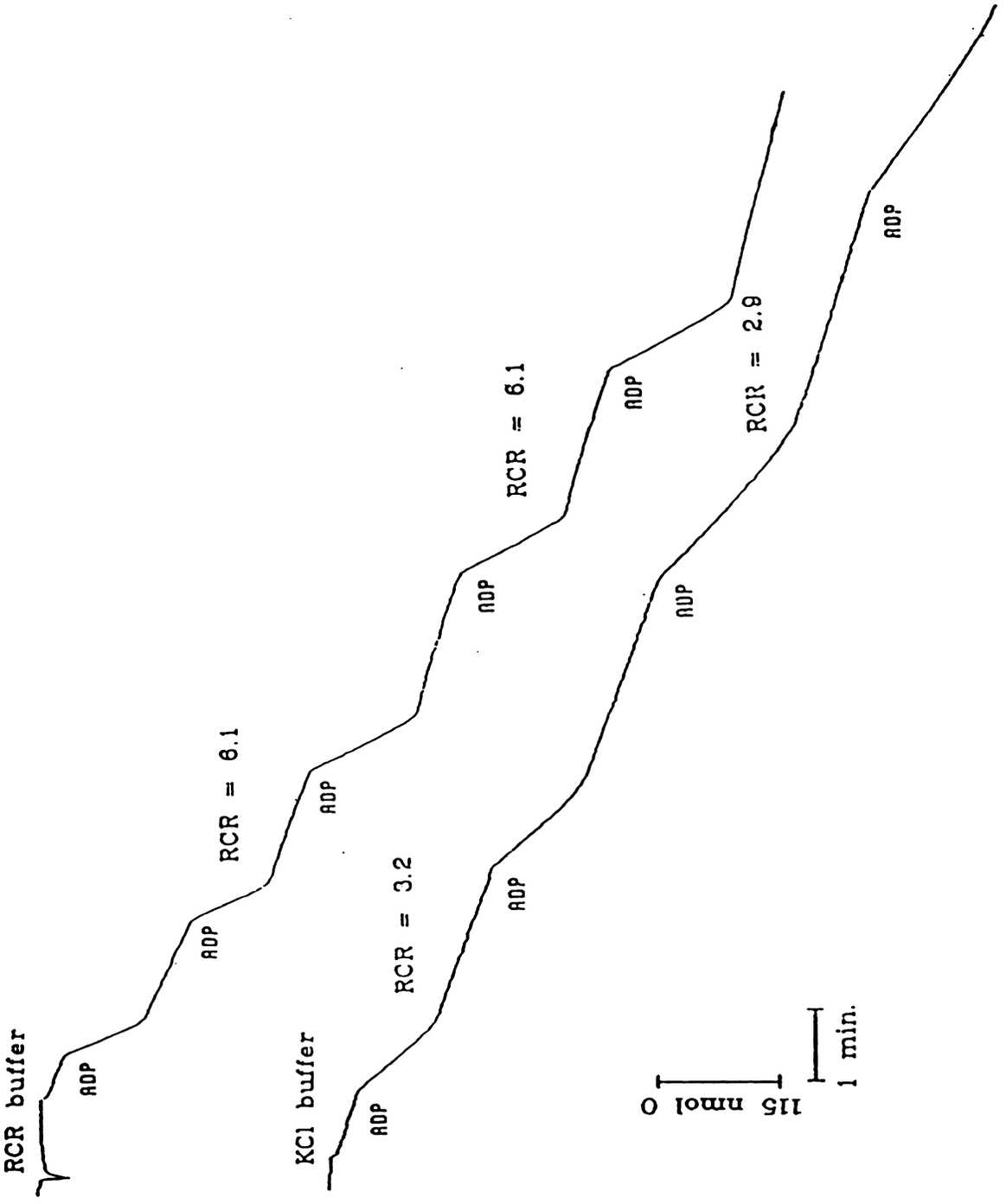

RCR buffer
ADP
ADP
RCR = 6.1
ADP
ADP
RCR = 6.1
ADP
ADP
RCR = 2.9
ADP
KCl buffer
ADP
RCR = 3.2
ADP
ADP
115 nmol O
1 min.

Figure 3.6

Figure 3.7: Passive swelling of T maize mitochondria in a KCl buffer. Swelling is measured as a change in absorbance at 520 nm over 20 minutes. Mitochondria (final concentration 0.1 mg/mL) were added at time = 0 min. to a buffer containing 0.4 *M* KCl, 40 m*M* HEPES, 8 m*M* KH_2PO_4, 0.2% BSA, pH 7.4 at 23° C.

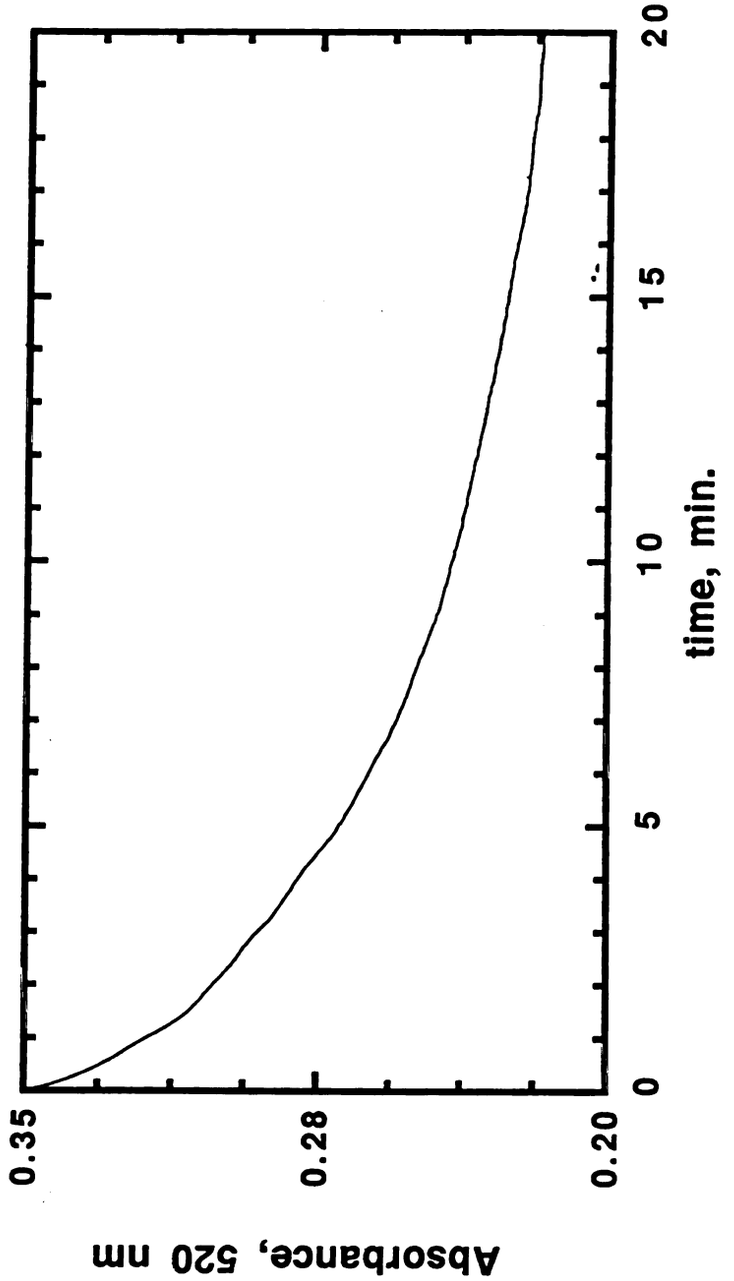


Figure 3.7

Table 3.1: Comparison of passive swelling of N and T maize mitochondria in a KCl buffer. Mitochondria (final concentration 0.1 mg protein/mL) were added at time = 0 and percentage of the initial absorbance at 520 nm was measured after 6 minutes and 10 minutes. sd = standard deviation.

	% change in absorbance, 520 nm	
	6 min.	10 min.
N	24.5 (sd=0.8, n=3)	30.6 (sd=0.7, n=3)
T	24.7 (sd=0.4, n=5)	30.0 (sd=0.6, n=4)

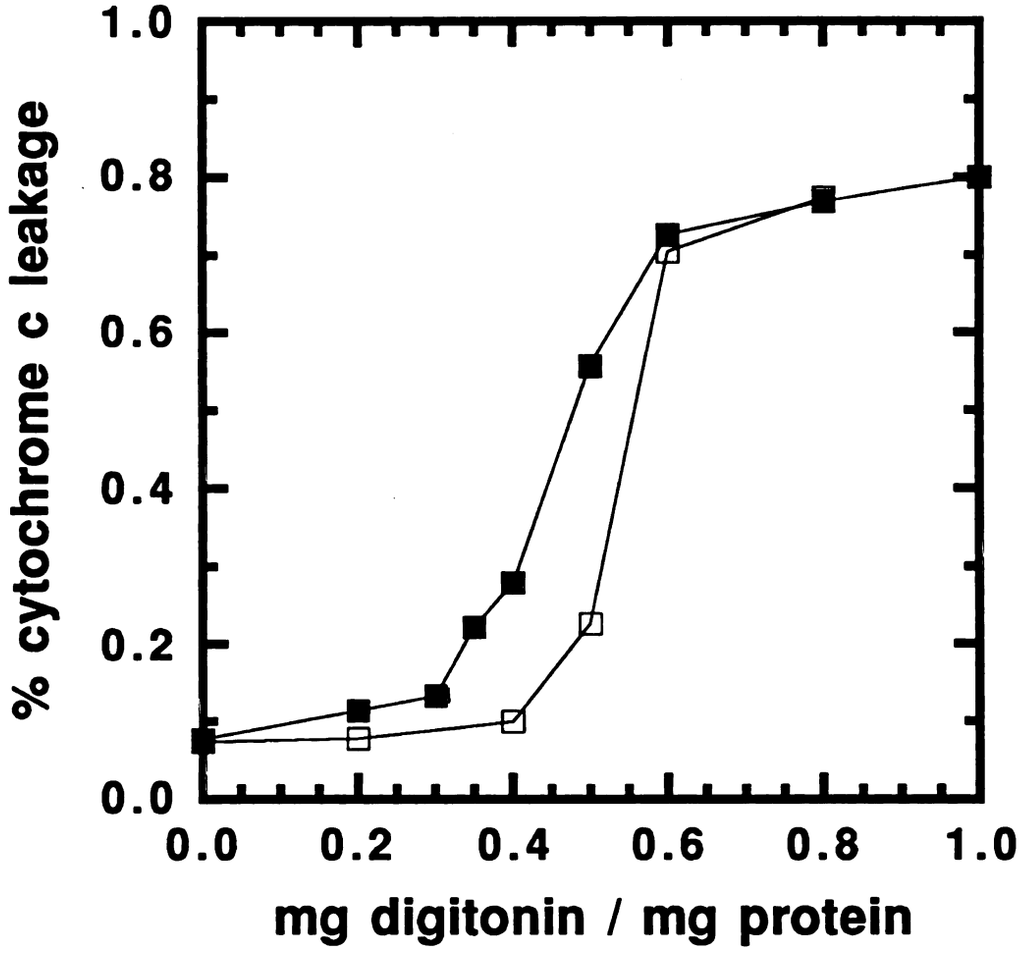


Figure 3.8: Comparison of digitonin sensitivity of N (■) and T (❑) mitochondria as measured by the simplified cytochrome *c* leakage test for intactness of outer membranes. The assay is described as described in section 3.2.2 and illustrated in Figure 3.2. Each curve is from one mitochondrial preparation.

reliable measure of determining outer membrane intactness because mitochondria spend less time in the diluted state before the assay is performed.

3.3.2 Characterization of N and T mitochondria

Because of the variety of conflicting results in the literature regarding the comparative respiratory parameters of the N and T mitochondria, and their differential sensitivity to toxin, some of these properties were re-examined.

3.3.2.1 Respiration in N and T mitochondria

Both N and T mitochondria exhibit good respiratory control with glutamate/malate in the RCR buffer (described in section 3.2.2) with average RCR values of 5–7 and ADP/O ratios of 2.7–2.9. (Figure 3.1) Using the KCl buffer (described in section 3.2.2) the RCR values are much lower. (Figure 3.6) It should be noted that most of the RCR values reported in the literature are considerably lower than those reported here. No significant differences in RCR values or ADP/O ratios are found under our assay conditions between N and T mitochondria. Both N and T mitochondria are 95% intact as determined by the cytochrome *c* leakage test (Figure 3.2).

3.3.2.2 Methomyl and toxin-induced swelling of isolated maize mitochondria

Using as substrates both glutamate/malate (Figure 3.9) and succinate (Figure 3.10), which feed into the electron transport chain at Complex I and II respectively, toxin and methomyl were shown to swell T mitochondria as measured by a decrease in absorbance at 520 nm. This swelling is not due simply to the toxin and methomyl-induced collapse of the proton gradient since prior addition of CCCP, which dissipates the gradient and thereby uncouples respiration, does not affect the extent of swelling. Addition of

Figure 3.9: Effect of toxin (**A**) and methomyl (**B**) on the swelling of T and N maize mitochondria using glutamate/malate as substrate. A decrease in absorbance (520 nm) of a mitochondrial suspension is correlated with swelling of the mitochondria. Glutamate/malate (10 μmol) and CCCP (0.5 nmol) were added as indicated to a mitochondrial suspension (0.09 mg protein) in RCR buffer at 23° C. Methomyl (2 μmol) or toxin (1 μL) was added as indicated.

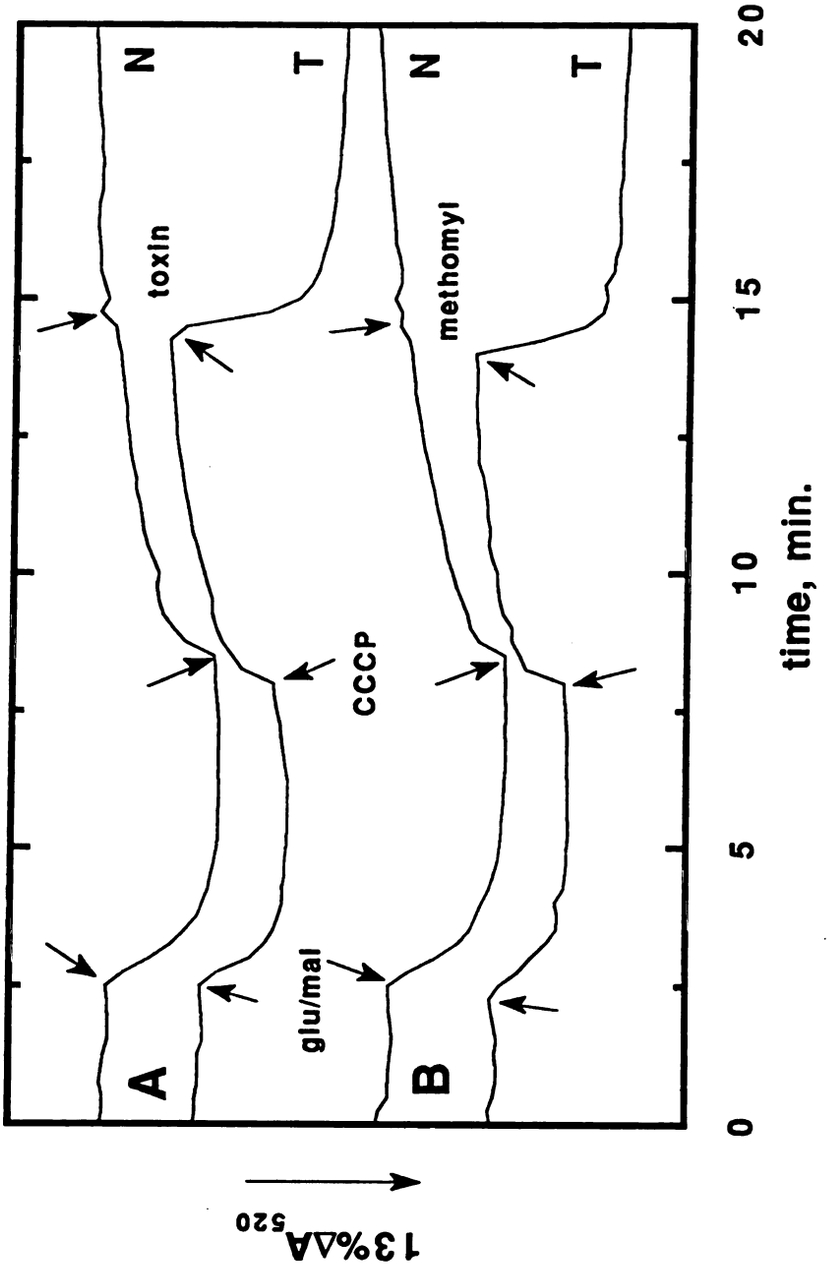

N
T
N
T
toxin
methomyl
CCCP
glu/mal
A
B
0
5
10
15
20
time, min.
13%ΔA520

Figure 3.9

Figure 3.10: Effect of toxin (A) and methomyl (B) on the swelling of T and N maize mitochondria using succinate as substrate. A decrease in absorbance (520 nm) of a mitochondrial suspension is correlated with swelling of the mitochondria. Succinate (10 μmol) and CCCP (0.5 nmol) were added as indicated to a mitochondrial suspension (0.09 mg protein) in RCR buffer at 23° C. Methomyl (2 μmol) or toxin (1 μL) was added as indicated.

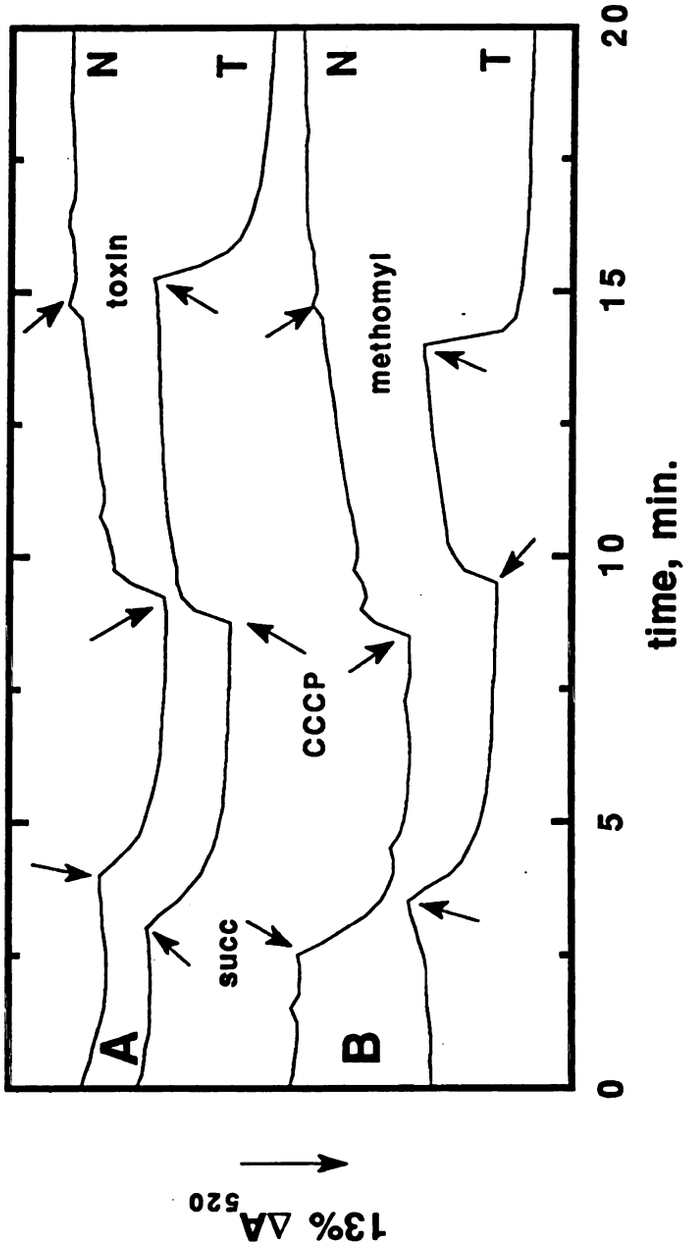


Figure 3.10

CCCP after toxin or methomyl has no effect, showing that the proton gradient has been collapsed. Neither toxin nor methomyl has any effect on N mitochondria using either substrate.

3.3.3 Effects of inhibitors on toxin and methomyl-induced swelling

Reports that DCCD and kaempferol, both ATP synthase inhibitors, blocked some effects of the toxin suggested that the ATP synthase might be involved in toxin binding or action. We have attempted to extend these previous studies by testing the ability of these and other ATP synthase inhibitors to interfere with toxin-induced swelling, an easily quantifiable effect that is unique to T mitochondria.

3.3.3.1 Effect of DCCD

DCCD covalently labels many membrane proteins of the mitochondria, including sites on F_0 and F_1 of the ATP synthase, and has been shown to block the toxin-induced collapse of the membrane potential in T mitochondria. We have shown that incubation of T mitochondria with DCCD in RCR buffer reduced methomyl-induced swelling using both succinate (Figure 3.11) and glutamate/malate (Figure 3.12) as substrates. This effect was optimal at approximately 250 nmol DCCD/mg mitochondrial protein incubated for twelve minutes (the time required to perform the assay). At higher concentrations of DCCD, a variety of inhibitory and uncoupling effects occur, as indicated by the lack of response to the uncoupler CCCP (Figure 3.11D, Figure 3.12D).

3.3.3.2 Effect of quercetin

Because quercetin, which inhibits the ATP synthase by binding to F_1, is structurally similar to kaempferol, we tested its ability to block methomyl-induced swelling. Inhibition of methomyl-induced swelling of T mitochondria

Figure 3.11: Effect of DCCD on methomyl-induced swelling of T maize mitochondria using succinate as substrate. DCCD was added at time = 0 to a mitochondrial suspension (0.09 mg protein) in RCR buffer at 23° C. Succinate (0.1 µmol), CCCP (0.5 nmol),and methomyl (2 µmol) were added as indicated. ***A.*** 5 µL MeOH, no DCCD. ***B.*** 149.7 nmol DCCD/mg mitochondrial protein. ***C.*** 449.1 nmol DCCD/mg protein. ***D.*** 598.8 nmol DCCD/mg protein.

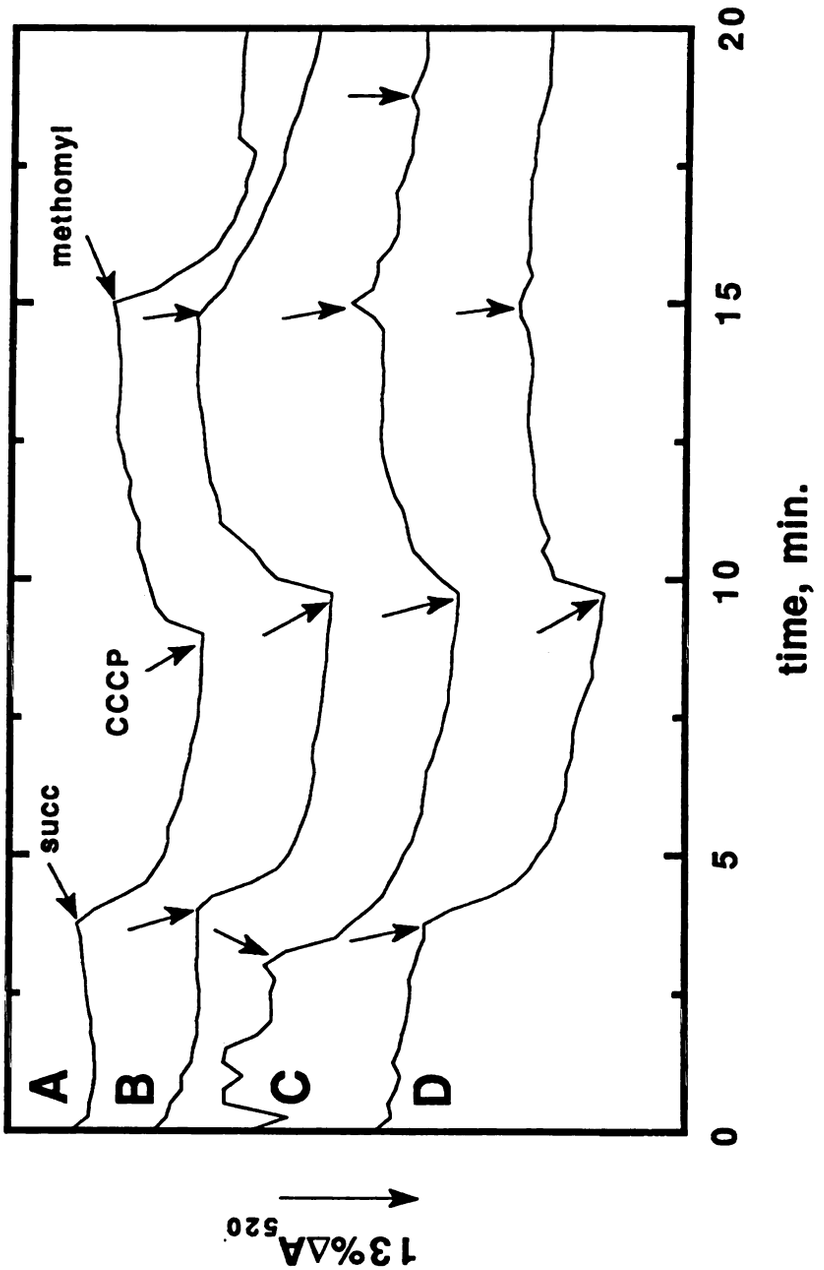


Figure 3.11

Figure 3.12: Effect of DCCD on methomyl-induced swelling of T maize mitochondria using glutamate/malate as substrate. DCCD was added at time = 0 to a mitochondrial suspension (0.09 mg protein) in RCR buffer at 23° C. Glutamate/malate (0.1 μmol), CCCP (0.5 nmol), and methomyl (2 μmol) were added as indicated. ***A.*** 5 μL MeOH, no DCCD. ***B.*** 106.9 nmol DCCD/mg mitochondrial protein. ***C.*** 213.9 nmol DCCD/mg protein. ***D.*** 320.8 nmol DCCD/mg protein.

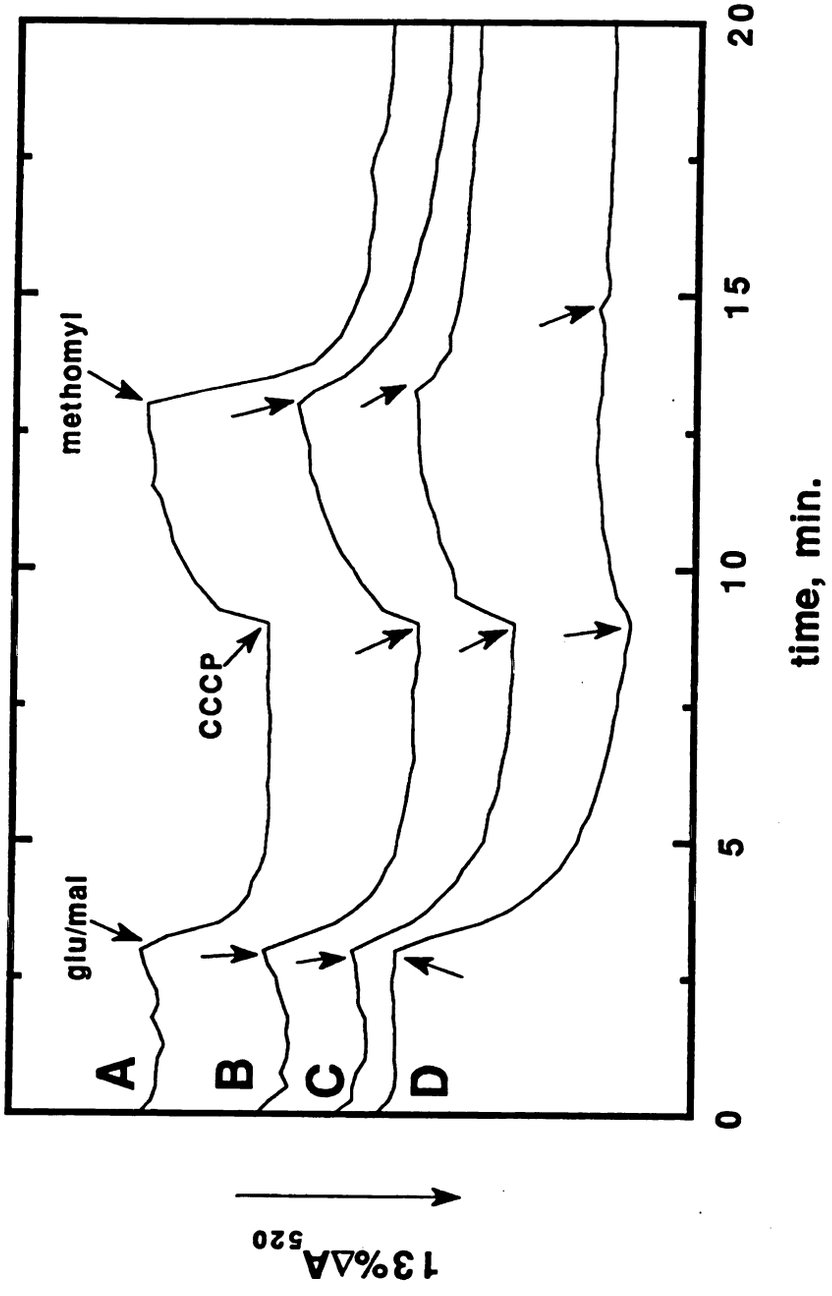


Figure 3.12

by quercetin was observed when succinate was used as a substrate (Figure 3.13). Addition of more methomyl was able to elicit a weak swelling response while the quercetin concentration was unchanged (Figure 3.13C).

3.3.3.3 Effect of oligomycin and diethylstilbestrol

Oligomycin A, an inhibitor of the ATP synthase which binds to the F_o channel, had no effect on the toxin or methomyl-induced swelling of T mitochondria at a concentration (0.5 μg/mg protein) which blocked ADP-stimulated respiration (Figure 3.14B). Addition of higher concentrations of oligomycin had no effect. Diethylstilbestrol (DES), recently shown to be an ATP synthase inhibitor (McEnery and Pedersen, 1986), also had no effect on methomyl-induced swelling in T mitochondria (Figure 3.14E).

3.3.3.4 Effect of carboxyatractyloside and mersalyl

Since the processes of ADP/ATP translocation and P_i transport are closely linked to ATP synthesis, inhibitors of these processes were also tested for their ability to interfere with toxin and methomyl-induced swelling in T mitochondria. Carboxyatractyloside, a competitive inhibitor of the ADP/ATP translocase, was unable to block toxin-induced mitochondrial swelling at a concentration (0.3 μmol/mg protein) which effectively blocks ADP-stimulated respiration (Figure 3.14D). Mersalyl, an inhibitor of phosphate transport and adenine nucleotide accumulation in mitochondria (Abou-Kahlil and Hanson, 1977), increased the swelling normally induced by toxin and methomyl as much as two-fold (Figure 3.15) at a concentration (0.6 μmol/mg protein) which blocked ADP-stimulated respiration.

3.3.4 ATPase activity in N and T mitochondria

Since several lines of evidence have suggested that some aberration in the ATP synthase complex could be related to both toxin susceptibility

Figure 3.13: Effect of quercetin on the methomyl-induced swelling of T maize mitochondria. A decrease in absorbance (520 nm) of the mitochondrial suspension is correlated with swelling of the mitochondria. Succinate (10 µmol) was added to the mitochondrial suspension (0.09 mg protein) in RCR buffer at 23° C as indicated. ***A***. DMSO only, 2 µmol methomyl. ***B***. 0.1 µmol quercetin, 2 µmol methomyl added twice. ***C***. 0.1 µmol quercetin, 4 µmol methomyl. ***D***. 0.2 µmol quercetin, 2 µmol methomyl added twice.

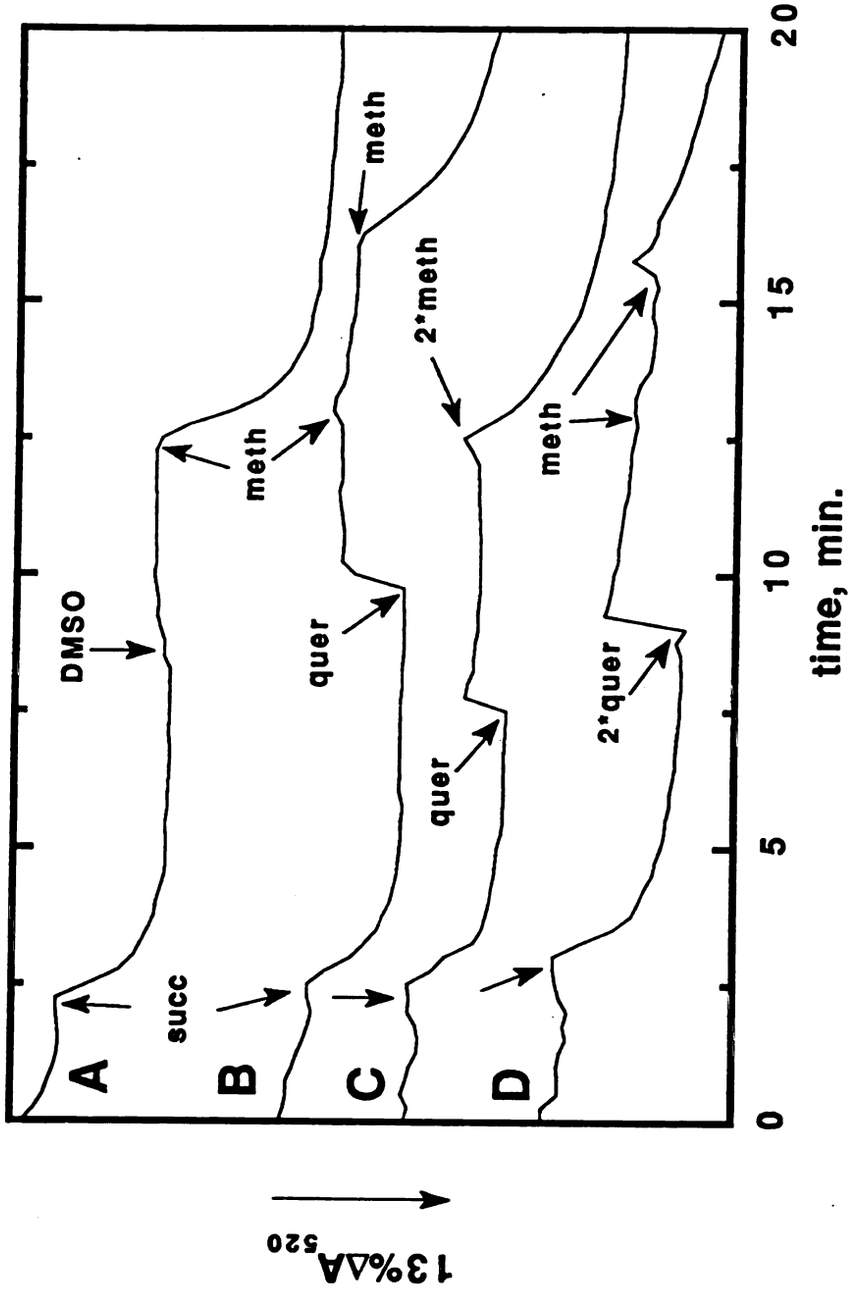

A
succ
B
C
D
DMSO
meth
quer
meth
2*meth
quer
meth
2*quer
0
5
10
15
20
time, min.
13%ΔA520

Figure 3.13

Figure 3.14: Effects of various ATP synthase inhibitors on the toxin or methomyl-induced swelling of T maize mitochondria. Glutamate/malate (10 μmol) or succinate (10 μmol), inhibitors, and toxin (1 μL) or methomyl (2 μmol) were added as indicated to a mitochondrial suspension (0.09 mg protein) in RCR buffer at 23° C. ***A.*** Toxin only ***B.*** Oligomycin A (0.28 μg/mg protein) ***C.*** Methomyl only ***D.*** Carboxyatractyloside (10 nmol) ***E.*** Diethylstilbestrol (DES, 20 nmol)

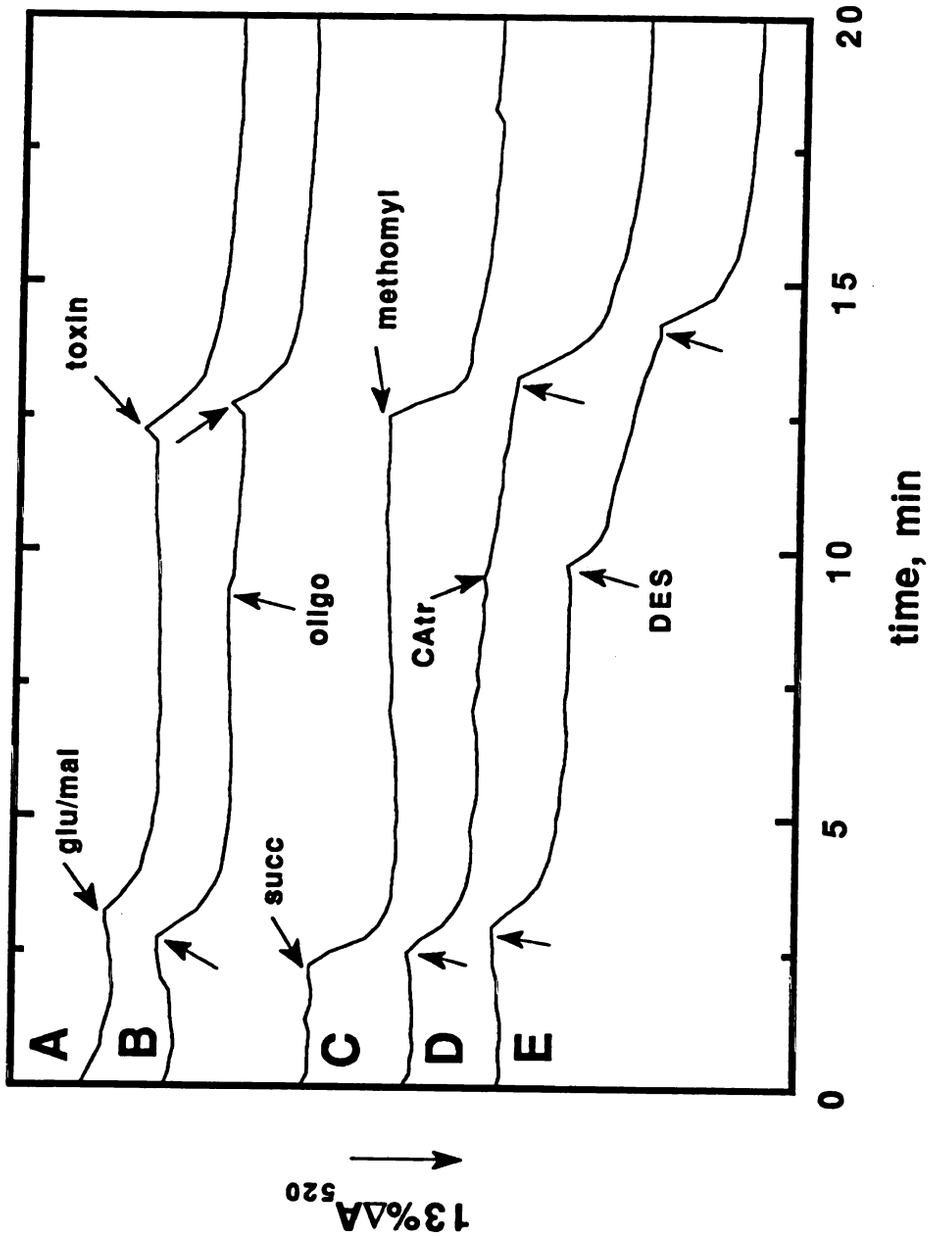

A
B
C
D
E
glu/mal
succ
oligo
toxin
methomyl
CAtr
DES
0
5
10
15
20
time, min
13%ΔA520

Figure 3.14

Figure 3.15: Effect of mersalyl on the toxin and methomyl-induced swelling of T maize mitochondria. Succinate (0.1 μmol), CCCP (0.5 nmol) and mersalyl (20 nmol) were added as indicated to a mitochondrial suspension (0.09 mg protein) in RCR buffer at 23° C. ***A.*** toxin (1 μL) ***B.*** methomyl (2 μmol)

duced swelling
l), CCCP (0.
indicated to a
RCR buffer a

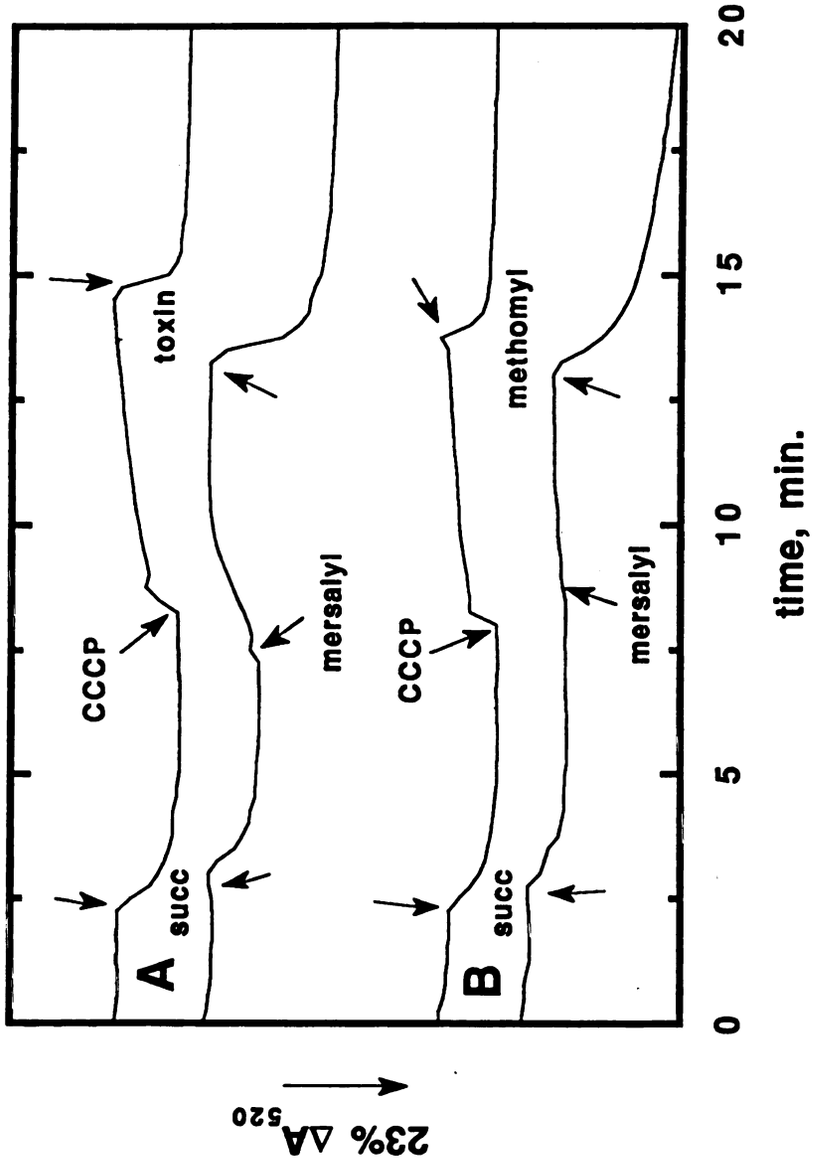


Figure 3.15

and male sterility, a more direct assay of ATP synthase activity was attempted by testing ATP hydrolysis (ATPase activity).

3.3.4.1 Activity of the N and T ATPase

A structural alteration in the T ATP synthase, perhaps due to its association with the 13 kDa peptide, may be expected to affect its activity in hydrolyzing ATP; therefore, maximal activity of N and T ATPase was tested to determine if a difference existed. Increasing concentrations of ATP (up to 6.7 μmol/mg protein) were added to N and T mitochondria to reach a maximal ATP hydrolysis velocity. Total inorganic phosphate liberated in the incubation period was calculated and corrected for small differences in protein concentration. Four replicates (each from separate mitochondrial preparations) of each N and T were performed (Figure 3.16), and the data analyzed using Wilman4, a computer program which uses a weighted linear regression to calculate K_m and V_{max} values. The N and T averages were shown to be significantly different using the Student t-test at the 95% confidence level (Table 3.2). A difference of 24% in maximal activity is indicated. When the assay was performed at room temperature, 1.3 μmol P_i/mg protein/10 minutes was released by N mitochondria, a value comparable to those obtained in similar studies (Jung and Hanson, 1973, 1975). The K_m values for ATP did not differ significantly (Table 3.2), and were similar to values previously reported (Jung and Hanson, 1973) for cauliflower mitochondria.

3.3.4.2 Effect of DCCD on ATPase activity

To determine whether the ATP synthase binds DCCD at a concentration similar to that which inhibits methomyl-induced swelling in T mitochondria, we tested the ability of DCCD to inhibit activity of the ATPase (Figure 3.17). The concentration producing maximal inhibition was 35 nmol DCCD/mg protein for ten minutes. This is somewhat lower than the

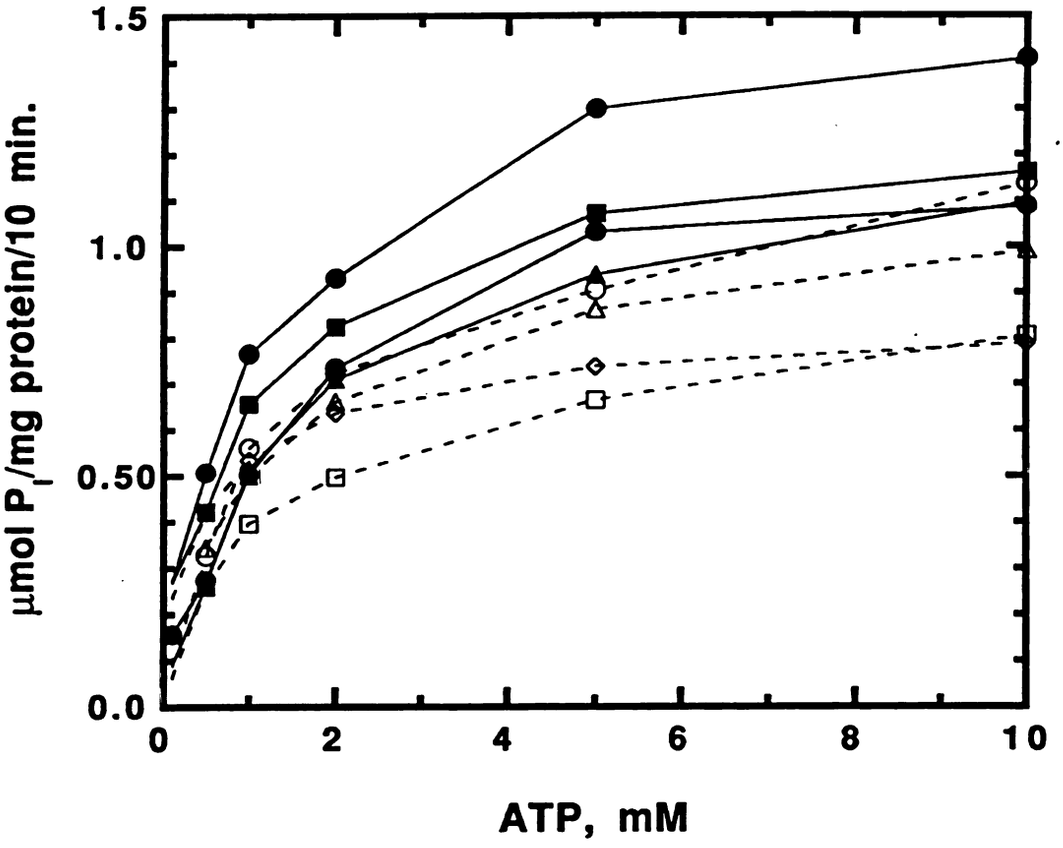


Figure 3.16: ATPase activity vs. ATP concentration for N and T maize mitochondria. Four preparations of each N and T mitochondria were assayed for ATPase activity as described in section 3.2.5. Dashed lines and open symbols represent T mitochondria, and solid lines and solid symbols represent N mitochondria.

Table 3.2: Comparison of N and T maize mitochondria ATPase V_{max} and K_m values. ATPase assays were performed on four N and T mitochondrial preparations as described in section 3.2.5. The difference between the N and T V_{max} averages was shown to be significant using the Student t-test at the 95% confidence level.

V_{max} μmol P_i/min/10 min		K_m m*M* ATP	
N	T	N	T
1.28	0.78	1.46	0.36
1.22	1.24	0.77	1.37
1.26	1.09	1.57	1.18
1.51	0.88	0.94	1.32
ave=1.32	ave=1.00	ave=1.18	ave=1.06
sd=0.13	sd=0.21	sd=0.39	sd=0.41

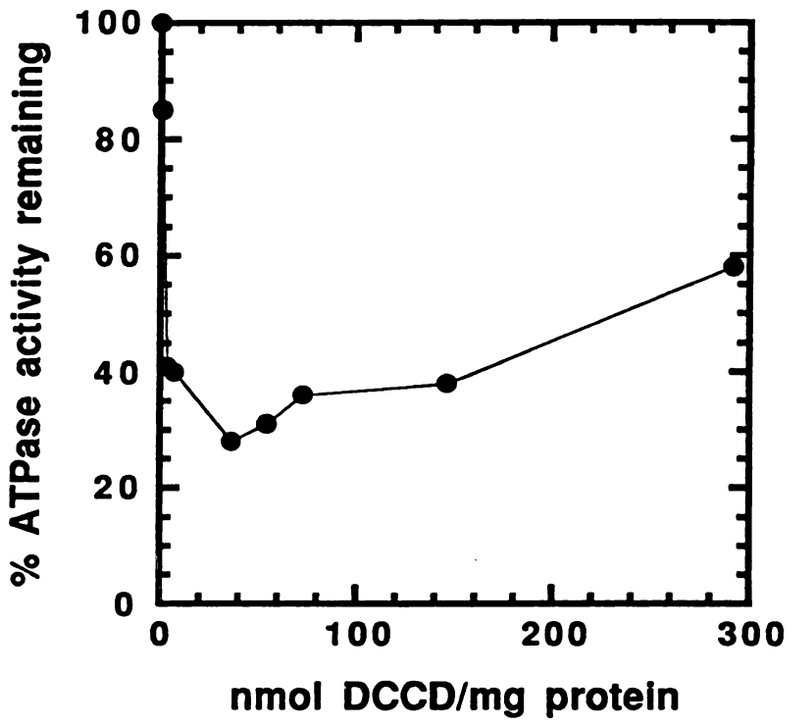


Figure 3.17: Effect of DCCD on uncoupler-induced ATPase activity in T maize mitochondria. ATPase activity was measured as described in section 3.2.5 except that assays were performed at room temperature and were terminated by the addition of 67 nmol mersalyl/mg protein. ATPase activity not inhibitable by mersalyl (added at the beginning of the assay to prevent uptake of ATP) was subtracted from each value. These data from one mitochondrial preparation are represenative of values obtained from many experiments.

concentration producing optimal effects on methomyl-induced swelling (250 nmol/mgprotein); however, conditions were slightly different in the ATPase assay from the swelling assay.

3.3.4.3 Effect of toxin and methomyl on ATPase activity

Because toxin or methomyl binding to the ATP synthase complex might be expected to affect its normal function, methomyl and toxin were tested for their ability to inhibit ATPase activity. Toxin and methomyl inhibited uncoupler-induced ATPase activity in T mitochondria but had no effect in N mitochondria (Table 3.3). However, the results of these assays were variable and wide ranges of effects by oligomycin, toxin, and methomyl on ATPase activity were observed. The data presented here represent assays performed consecutively in one week on separate mitochondrial preparations.

3.4 Discussion

The work of York and Gregory suggested that there is a difference in the sterol concentration of N and T mitochondrial membranes that could be responsible for the susceptibility of T mitochondria to the BmT toxin. However, many questions were left unanswered by their work. No statistical analysis of York's N and T sterol content values was performed. Since inner and outer membranes were not separately analyzed, the values reported are weighted averages of the two (as well as microsomal contamination), being more reflective of the inner membrane composition, since there is more inner than outer membrane. However, we have been able to demonstrate only a small or no difference in outer membrane sterol content (by the digitonin-induced cytochrome *c* leakage test) and inner membrane sterol content (by digitonin-induced deterioration of RCR values). The discrepancy between the

TABLE 3.3: Inhibition of ATPase activity by oligomycin, methomyl, and BmT toxin. ATPase assays were performed on three mitochondrial preparations of each N and T as described in section 3.2.5. Values reported are percentages of inhibition of the CCCP-induced ATPase activity for each mitochondrial preparation by each inhibitor.

Percent Inhibition of ATPase Activity

	N maize				T maize			
	(1)	(2)	(3)	ave.	(1)	(2)	(3)	ave.
Oligomycin	83	65	69	72	81	67	74	74
Methomyl	16	17	26	20	62	42	31	45
Toxin	0	0	7	2	71	32	44	49

results obtained by ourselves and Gregory may be due to the fact that we used maize shoots while Gregory used maize roots. It has been shown that the lipid composition of the two tissues varies (Mackender and Leech, 1974); therefore, sterol composition may also vary. However, since mitochondria from both shoot and root are equally sensitive to the toxin, it would seem unlikely that any differences found between the two tissues would be relevant to toxin sensitivity.

Although toxin has been shown to bind to the 13 kDa peptide in both T maize mitochondria and *E. coli* expressing *urf13-T* (Braun *et al.*, 1990), it is not clear yet whether additional proteins are necessary to mediate the toxin effect. Other proteins may be involved indirectly as part of a cascade of events which leads to swelling and uncoupling upon toxin binding to its receptor. Alternatively, the normal functioning of a respiratory protein may be altered by its physical association with the 13 kDa peptide, causing the observed effects of the toxin. Co-immunoprecipitation of the 13 kDa peptide with respiratory proteins (Wise *et al.*, 1987a; C. Leaver, personal communication) suggests the latter. The toxin sensitivity of *E. coli* expressing the 13 kDa peptide does not rule out either of these possibilities. Many proteins in mitochondria are highly homologous to their counterparts in bacteria; therefore, the 13 kDa peptide may be associating with an *E. coli.* protein in analogous manner, leading to toxin sensitivity. Until it is shown that the purified 13 kDa peptide can be reconstituted into artifical lipid vesicles which then become sensitive to toxin effects, such as leakage of cofactors pre-loaded in the vesicles, it remains a possibility that the 13 kDa peptides associates with a membrane protein important for respiratory function.

We suggest that the ATP synthase complex (or some of its subunits) may be involved in toxin action. Three pieces of evidence support this hypothesis. First, DCCD and quercetin, both inhibitors of the ATP synthase, block toxin-induced swelling. Although DCCD has recently been shown to bind directly to the 13 kDa peptide (Braun *et al.*, 1989), given the relative non-specificity of DCCD (Solioz, 1984), this binding site may not be the only one which affects toxin action. Second, the V_{max} of the ATPase differs between N and T mitochondria in the *absence* of toxin. Third, we have shown that toxin and methomyl inhibit T but not N mitochondrial ATPase activity.

The effects of the toxin, however, need not be due to the ATP synthase directly. If the toxin directly affected ATP/ADP translocation, or P_i or Mg^{2+} transport, the activity of the ATPase would also be affected. However, inhibition of the ADP/ATP translocase by carboxyatractyloside, a competitive inhibitor of ADP translocation in plants, did not block toxin-induced swelling, so the possibility that the translocase is involved is less likely. Interestingly, mersalyl, which binds to the phosphate transporter, actually enhanced the toxin-induced swelling effect. These results might suggest a toxin or 13 kDa peptide interaction with the phosphate transporter, but mersalyl also binds to other mitochondrial proteins which have available and reactive sulfhydryls, including F_1 of the ATPase. Therefore, we cannot conclude from the above data that the phosphate transporter is important in mediating the increase in swelling, but it is a possibility worthy of consideration. Thus, the observed effects of toxin on mitochondria may involve a protein which supports activity of the ATP synthase through supply of substrate, rather than the ATP synthase itself.

Our finding of toxin and methomyl-induced inhibition of ATPase activity has recently been verified by Moussa *et al.* (1990). They found that

the activity of the partially purified soluble ATPase (F_1) from T, but not N and C mitochondria, is inhibited 75% by both toxin and methomyl. These results, along with those presented herein, provide strong support for involvement of the ATPase in toxin action and suggest a direct effect rather than mediation by an auxiliary protein.

While the evidence indicates a role for the ATPase in the action of the toxin, the role of the ATPase in the CMS trait remains unclear. Our results indicate that the V_{max} of the ATPase differs between N and T mitochondria, suggesting that the ATPase may in fact be involved in CMS as well. If the 13 kDa peptide were to physically associate with the ATPase, then the complex's native conformation and therefore its kinetic properties could be affected, possibly reducing V_{max} as we have observed.

Several mechanisms can be postulated to explain how a physical association of the 13 kDa peptide with a respiratory protein causes CMS. The 13 kDa peptide–ATPase complex would be present in relatively low amounts in most CMS plant tissues, and therefore only a small effect on respiration would be expected under normal growth conditions in the absence of toxin. This explains why RCR values and ADP/O ratios are similar in T and N mitochondria. Perhaps only under the special growth conditions in the anthers, or in the presence of an anther-specific, toxin-like substance, would an aberrant activity of the new complex be induced.

Another possibility is that higher levels of a 13 kDa peptide–respiratory protein complex are necessary to interfere with energy production such that pollen production is aborted. In petunia, greater amounts of the *pcf* mRNA (the rearranged segment of the mitochondrial genome present only in male-sterile plants) are produced in anthers compared to other tissues during pollen production (Young and Hanson, 1987). If the 13 kDa peptide of T

maize is also expressed at higher levels during pollen production, two effects could result. First, newly synthesized subunits of respiratory or transport complexes could instead associate with the 13 kDa peptide, reducing the energy-producing capacity of the plant. However, the increase in *pcf* expression in petunia is probably insufficient to produce this effect. Alternatively, the presence of higher levels of the aberrant complex might increase membrane permeability to a point where respiration is uncoupled, and therefore not enough energy is available for rapid growth.

In summary, our evidence suggests that there may be a small difference in the maximal activity of the ATPase in CMS-T maize mitochondria, in accordance with the theory that the 13 kDa peptide is physically associated with a mitochondrial membrane complex. This data, together with the effects of ATPase inhibitors on toxin action, as well as inhibtion of ATPase activity by the toxin and methomyl in T mitochondria, suggests a role for the ATPase in both toxin action and CMS.

Chapter 4 - Analysis of possible physical association of CMS-specific proteins with other mitochondrial proteins

4.1 Introduction

Cytoplasmic male sterility (CMS) has been correlated with mitochondrial DNA rearrangements which often generate open reading frames directing synthesis of novel proteins. The mechanism by which these CMS-specific proteins cause pollen abortion while allowing female gametophyte production and normal vegetative growth is not understood. The inability of mitochondria to provide sufficient energy to support the rapid mitochondrial proliferation in pollen production (Warmke and Lee, 1978) has been suggested to be caused by the presence of anther-specific substances which bind to the novel proteins (Flavell, 1974), the over-expression of the novel proteins in the anther (Young and Hanson, 1987), the mis-expression of non–CMS-specific genes affected by mtDNA rearrangements (Hanson *et al.*, 1989), and the alteration of respiratory proteins (Bailey-Serres *et al.*, 1986). In the previous chapter, we have provided evidence for the possible involvement of the ATPase in the action of the fungal toxin causing Southern corn leaf blight in CMS-T maize. While toxin may bind to or near the ATPase, or its action may involve a cascade of events which require the ATPase, there is not yet sufficient evidence to conclude that the ATPase is involved in the mechanism of CMS.

One explanation for the participation of the ATPase in toxin action and CMS is its association with the CMS-T maize-specific 13 kDa peptide. Even though the toxin binds directly to the 13 kDa peptide, association of the 13 kDa peptide with other proteins (such as the ATPase) may be necessary

for full toxin effects. The role of the 13 kDa peptide in CMS can also be explained in terms of its association with other proteins. Anther-specifc hormones may have a toxin-like effect, leading to mitochondrial degeneration, or simply induce more 13 kDa peptide production, resulting in a higher level of its associated form. Although the new complex may cause only slightly altered activity under normal growth conditions, its presence at higher levels in the anthers may cause sufficient change in energy output that the rapid mitochondrial proliferation necessary for pollen production is not possible.

Therefore, in order to understand how the presence of the 13 kDa peptide might cause the CMS trait, it is desirable to determine whether it associates with other mitochondrial proteins. This experiment first involves separation of the proteins. We have already developed an FPLC procedure to separate the mitochondrial membrane proteins for the purpose of purifying cytochrome oxidase (see chapter 6). The proteins are first solubilized with lauryl maltoside (a mild non-ionic detergent which allows cytochrome oxidase to retain high electron transfer activity), then rapidly chromatographed on an anion-exchange column (MonoQ). A modification of this procedure is a logical choice to use for this experiment. Another choice would be native gel electrophoresis; however, the difficulty of this technique when membrane proteins are used (see section 7.3.1.1) makes this an undesirable option.

Detection of the CMS-specific peptides is the second problem in performing this experiment. One option is the use of radiolabelled toxin, produced by growing the fungus on radiolabelled substrates or reducing the keto groups with tritiated borohydride. However, a previous experiment

failed to demonstrate toxin binding (Frantzen *et al.*, 1987); therefore, this option would not appear to be the best choice. A second possibility is the use of radiolabelled DCCD; DCCD binds to the toxin and prevents toxin action (Braun *et al.*, 1989). DCCD has the advantage of binding covalently, so it cannot be washed away under the diluting conditions used in chromatography. However, this approach has the problem that DCCD labelling is somewhat unspecific, and many proteins would become labelled, making the results difficult to interpret. In particular, labelling of the ATPase proteolipid, which occurs at very low concentrations of DCCD, is likely to interfere since we may expect to see association with the ATPase. The third approach, one which can be applied to other CMS systems, is the of use antibodies against the CMS-specific proteins. The concern with this method is the difficulty of generating antibodies against hydrophobic proteins like those associated with CMS-T maize and petunia. However, other laboratories have successfully produced antibodies which are suitable for this experiment.

Therefore, we have separated lauryl maltoside-solubilized mitochondrial protein from fertile and male-sterile maize and petunia using anion-exchange FPLC, and probed the fractions separated on Western blots with antibodies prepared by our collaborators against the CMS-specific peptides, 13 kDa and urfS. The work on CMS-T maize has been done in cooperation with Ken Korth from the laboratory of Dr. C.S. Levings, III (North Carolina State University), and the petunia work with Marie Connett and Helen Nivison from the laboratory of Dr. Maureen Hanson (Cornell University).

4.2 Materials and Methods

4.2.1 Materials

Antibodies against the 13 kDa peptide, ATPα, and cytochrome oxidase subunit II were kindly provided by Dr. C.S. Levings, III. Antibody against the alternative oxidase was the gift of Dr. Lee McIntosh, and antibody against the urfS peptide was kindly provided by Dr. Maureen Hanson. All other reagents were from the sources indicated in chapter 6 or from Sigma.

4.2.2 Preparation of mitochondria

Mitochondria from wheat and maize germ were prepared by the method described in section 6.2.3. Mitochondria from CMS (10636 or 3688), fertile (3699), and restored (3337) petunia cell lines prepared by the method of Connett and Hanson (1990) were the gift of Dr. Maureen Hanson. Protein concentrations were determined by the BCA method (Pierce).

4.2.3 Separation of proteins by FPLC

Mitochondrial membranes in 0.35 *M* sucrose, 50 m*M* KCl, 5 m*M* TES, 1 m*M* EGTA, pH 7.2 buffer at near 20 mg/mL were solubilized in 2 mg lauryl maltoside per mg mitochondrial protein and approximately 10 μg DNase per mg protein with 1 m*M* $MgCl_2$ at 4°C for 30 minutes. The mixture was centrifuged at 40000*g* for 20 minutes and the supernatant filtered through a 0.45 μm pore size membrane (Gelman Acrodisc for large volumes, Millipore HA filter syringe unit for small volumes) and loaded onto a Pharmacia MonoQ 10/10 (maize) or 5/5 (petunia) anion-exchange (MonoQ) or 5/5 cation-exchange (MonoS) column on the Pharmacia FPLC

system at 0.0–0.15 *M* KCl. Up to one hundred milligrams of maize mitochondrial protein were loaded onto the 10/10 column and two to six milligrams of petunia mitochondria protein were loaded onto the 5/5 column. A gradient of 0 to 1.0 *M* salt in buffer (FPLC buffer: 5% sucrose, 20 m*M* KH_2PO_4, 4 m*M* lauryl maltoside, pH 7.5) was applied to the column at 0.5 mL/min (5/5 column) or 1.0 mL/min (10/10 column).

4.2.4 Gel electrophoresis and Western blotting

Protein from maize FPLC fractions was precipitated in ice cold trichloroacetic acid (final concentration 4.5%) and centrifuged. Rinsed pellets were sonicated with sample buffer (described in Kadenbach *et al.*, 1983 except that 0.04% bromophenol blue was added) for 30 minutes at 15°C prior to loading onto gels. SDS-urea polyacrylamide gel electrophoresis was performed according to Kadenbach *et al.* (1983) using the Bio-Rad mini gel system. Blotting onto nitrocellulose (0.45 μm pore size, Bio-Rad) was performed in 12.5 m*M* Tris, 96 m*M* glycine, and 20% methanol (in which the gel was pre-equilibrated for 10 minutes) at 100 V for 1 hour. The filter paper on the cathode side of the gel was soaked in this buffer with 0.5% SDS for ten minutes before blotting. Proteins were visualized by Amido black stain (0.025% in 7.5% acetic acid and 22.5% ethanol) and blots blocked overnight in 5% dry milk protein in 20 m*M* Tris, 0.5 *M* NaCl, and 0.05% Tween-20, pH 7.5. Antibodies were incubated with blots for at least 4 hours, followed by incubation with goat anti-rabbit antibody conjugated with alkaline phosphatase (Bio-Rad). Either 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium, or the fast red system (fast red TR and napthol AS-MX phophate) was used for immunodetection.

4.3 Results

4.3.1 Separation and identification of maize proteins

N and T maize germ mitochondria were fractionated by the method used to purify cytochrome oxidase from wheat germ mitochondria. Lauryl maltoside-solubilized mitochondria are treated with DNase I to break the mitochondrial DNA into small enough pieces so as not to clog the column filter which would increase column backpressure. Centrifuged and filtered mitochondria were made 0.0 to 0.15 *M* KCl (depending on the experiment) and loaded onto the MonoQ 10/10 column. Up to half of the total protein did not bind to the column matrix and eluted in the flow-through (1–3 in Figure 4.1), as detected by continuously monitored absorbance at 280 nm. A discontinuous KCl gradient was used to elute protein from the column. KCl was increased from 0 to 0.32 *M* , eluting a large peak, or sometimes group of peaks (5–8, Figure 4.1). Antibodies to the α subunit of the complex reacted strongly with these fractions on Western blots (see below), although almost all fractions reacted somewhat. This observation is common in fractionations of plant mitochondria (T. Elthon, personal communication; Hawkesford *et al.*, 1990). If the gradient was held at 0.22 *M* KCl, as in Figure 4.1, a peak (9) elutes after the ATPase at about 0.25 *M* KCl. We tentatively identify it as the NADH dehydrogenase because of its high number of bands on SDS-polyacrylamide gels. Subsequently, the identity of this peak was verified (in the petunia system, see below) by using a standard assay for NADH dehydrogenase activity which follows the reduction of ferricyanide spectrophotometrically (Hatefi, 1978) and by using a native gel activity stain (Connett, 1991). The gradient pause at 0.22 *M* was

Figure 4.1: Separation of maize mitochondrial proteins by anion-exchange FPLC. Membranes made 0.15 *M* KCl were loaded onto a MonoQ10/10 column and washed in FPLC buffer (5% sucrose, 20 m*M* KH_2PO_4, 4 m*M* lauryl maltoside, pH 7.5). Bound protein was eluted at 1.0 mL/min with a discontinuous KCl gradient: 0–0.22 M in 10 mL, 0.22–0.32 *M* in 5 mL, 0.32 *M* for 10 mL, 0.32–0.39 *M* in 1.5 mL, 0.39 *M* for 7.5 mL, 0.39–1.0 *M* in 8 mL. The solid line indicates the A_{280} and the dashed line indicates the KCl gradient.

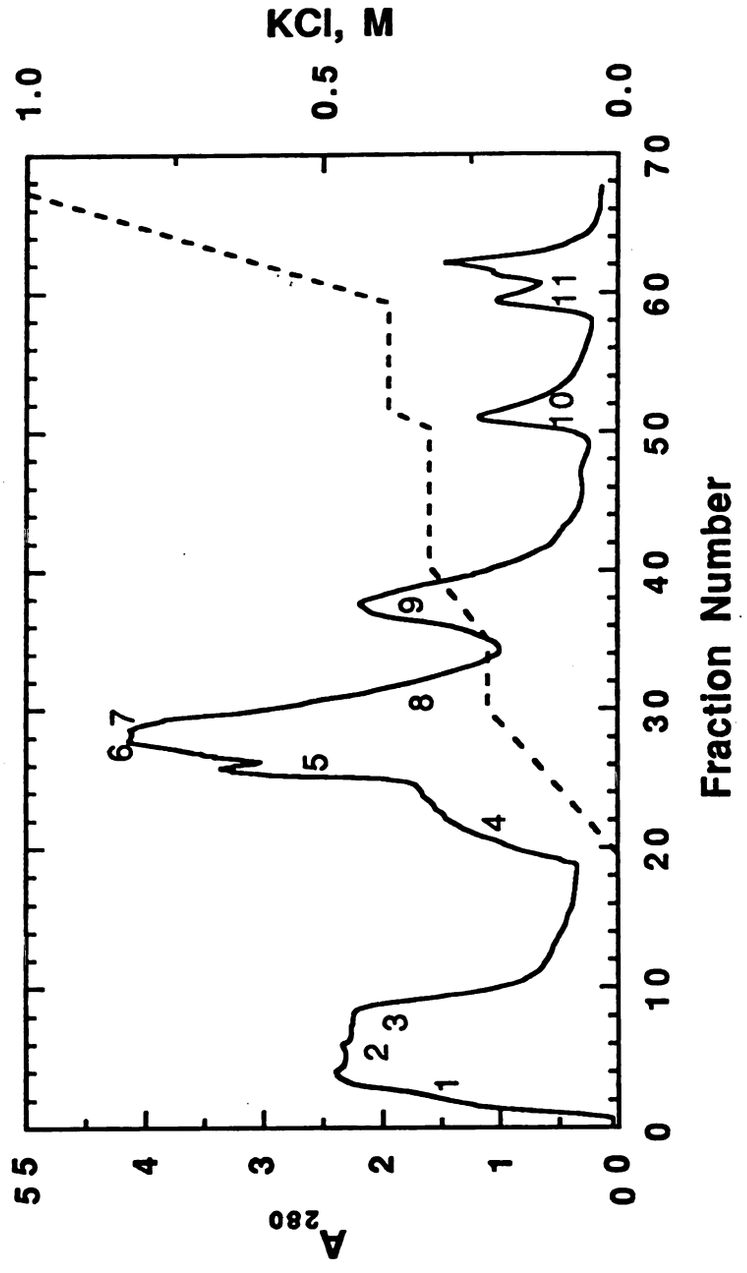

KCl, M
1.0
0.5
0.0
Fraction Number
0
10
20
30
40
50
60
70
A280
0
1
2
3
4
5
1
2
3
4
5
6
7
8
9
10
11

Figure 4.1

used only for initial peak separation and identification, and was not routinely employed.

The salt concentration is held constant at 0.32 *M* KCl for a period of time (10–15 mL) to allow clearing of all the protein from the previous peaks before continuing the gradient to elute the cytochrome oxidase at about 0.35 *M* KCl. The gradient is again held at 0.39 *M* KCl to delay the elution of the bc_1 complex, which otherwise seriously contaminates the oxidase. The oxidase and bc_1 peaks have been identified and characterized in chapter 6. Following the bc_1 elution, peaks of varying size elute which are identified as nucleic acid by precipitation in ammonium acetate and ethanol.

4.3.2 Detection of the 13 kDa peptide

Maize protein from T germ was separated by the standard gradient for purifying cytochrome oxidase, that is, with gradient holds at 0.32 and 0.39 *M* KCl. The first run was performed with no KCl added before loading on the column, so that the total KCl concentration during loading of the samples is 0.05 *M* KCl, the concentration in the last buffer of the mitochondria preparation from germ (chapter 6). This run is referred to as the "low salt" run (Figure 4.2A). Because of the reduced salt concentration, a greater percentage of the loaded protein is retained by the column. The first group of eluted peaks (6–8) are therefore much larger, while the later-eluting peaks remain relatively unchanged compared to the "high salt" (0.15 *M* KCl) run in Figure 4.1. The "low salt" loading procedure allows for maximal separation of all mitochondrial proteins.

Approximately equal amounts of protein from each of the numbered peaks in Figure 4.2, as well as total N and T mitochondria as a control, were run on gels and blotted to nitrocellulose for reaction with the antibody

Figure 4.2: Separation of CMS-T maize mitochondrial proteins by anion-exchange FPLC under "low salt" conditions (**A**), and reaction of fractions with antibody against the 13 kDa peptide (**B**). Membranes which were 0.05 *M* KCl were loaded onto a MonoQ10/10 column and washed in FPLC buffer (5% sucrose, 20 m*M* KH_2PO_4, 4 m*M* lauryl maltoside, pH 7.5). Bound protein was eluted at 1.0 mL/min with a discontinuous KCl gradient: 0–0.32 *M* in 16 mL, 0.32 *M* for 10 mL, 0.32–0.39 *M* in 1.5 mL, 0.39 *M* for 7.5 mL, 0.39–1.0 *M* in 8 mL. The solid line indicates the A_{280} and the dashed line indicates the KCl gradient. Western blots were performed as described in section 4.2.4.

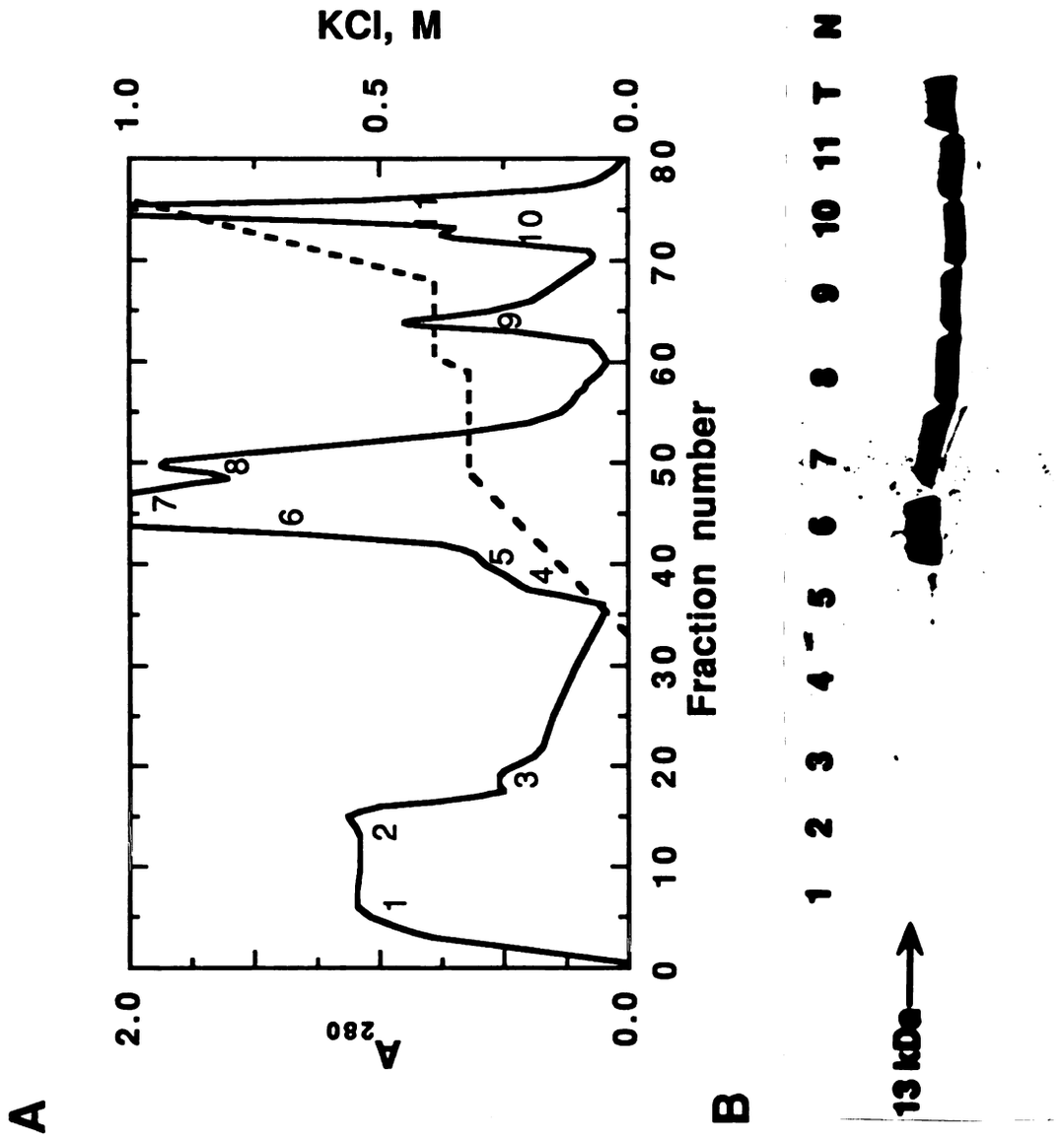

KCl, M
1.0
0.5
0.0
A
2.0
A_{280}
0.0
0
10
20
30
40
50
60
70
80
Fraction number
1
2
3
4
5
6
7
8
9
10
B
N
T
11
10
9
8
7
6
5
4
3
2
1
13 kDa

Figure 4.2

against the 13 kDa peptide. The antibody reacted with relatively equal intensity with peaks 6 through 11 (Figure 4.2B). These peaks represent the ATPase (6–8), the NADH dehydrogenase (8), cytochrome oxidase (9) and the bc_1 complex (10, 11 mixed with nucleic acid) as deduced above and verified by reactions of duplicate blots with antibody against COXII and ATPα (Figure 4.3). The COXII antibody reacted with fraction 9 (Figure 4.3A) while the ATPα antibody reacted with fractions 2 through 8. As stated above, it is common for ATPα to appear in many fractions of plant mitochondria protein separations.

Because the 13 kDa peptide appeared to be in every fraction that contained significant amounts of protein, it seemed possible that it was associating with all of the proteins, or alternatively, that the hydrophobic nature of the peptide causes it to "smear" across the column, appearing in every fraction. Therefore, a second run was performed in which, prior to loading, the mitochondrial fraction was made 0.15 *M* KCl, in order to lower the amount of protein binding to the column and perhaps reduce smearing. The elution profile of this "high salt run" is shown in Figure 4.4A.

The 13 kDa peptide antibody again reacted with protein from every fraction of the "high salt" run, this time including the the flow-through (Figure 4.4B). Although there appears to be no reaction with fraction 4, it is likely that there was too little protein in this fraction to appear strongly on the blot, even though we attempted to load equal amounts of protein in each lane (based on the A_{280} value). Alternatively, it is possible that there is no 13 kDa peptide in this fraction because there is no other protein in the fraction to which the 13 kDa peptide could stick. Fraction 7 also reacted less strongly, probably for the same reasons.



Figure 4.3: Western blot analysis of fractions from "low salt" FPLC (Figure 4.2A) using antibodies against ATPα and cytochrome oxidase subunit II. Blots were performed as described in section 4.2.4.

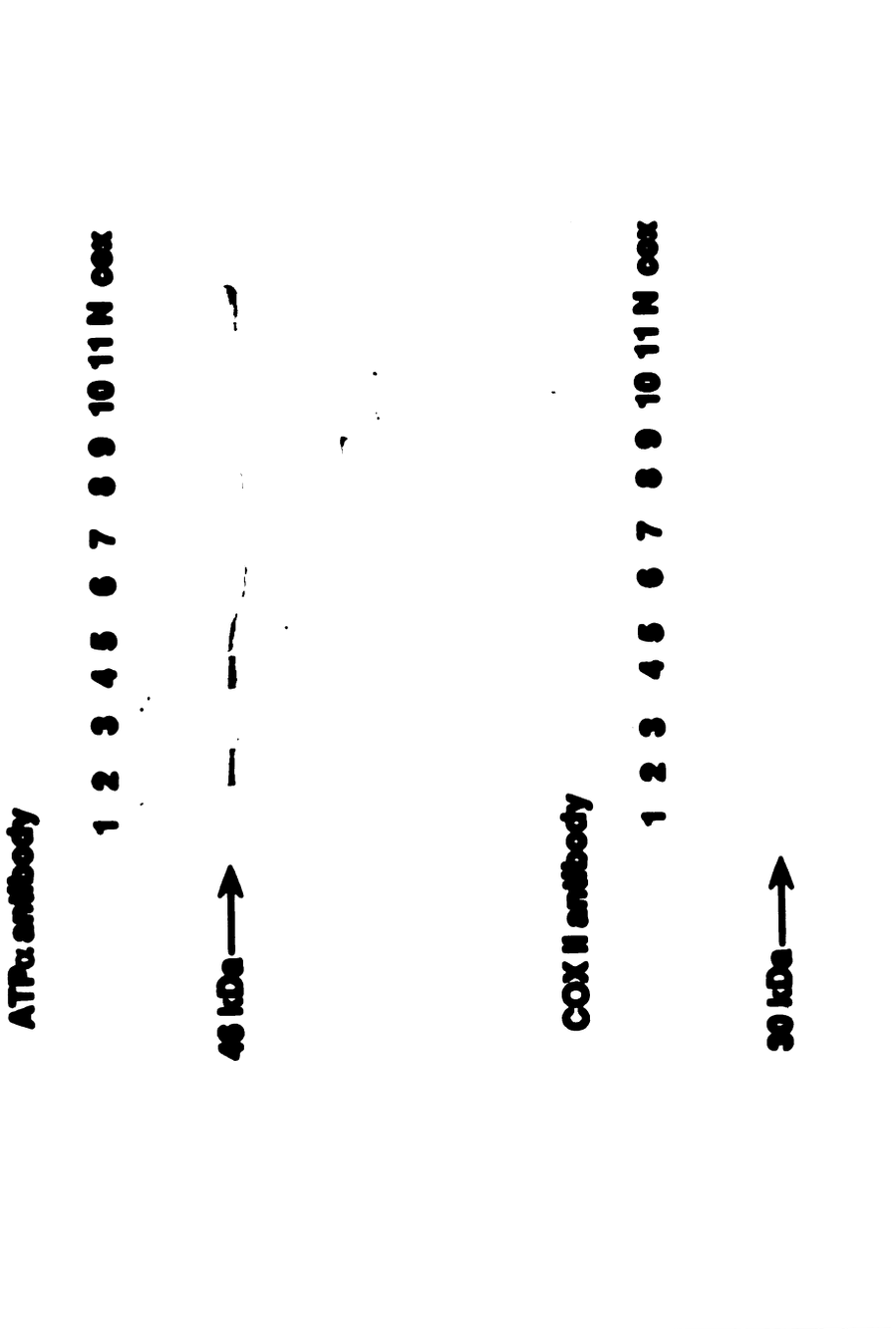

ATPα antibody
1 2 3 4 5 6 7 8 9 10 11 N cox
46 kDa
COX II antibody
1 2 3 4 5 6 7 8 9 10 11 N cox
30 kDa

Figure 4.3

Figure 4.4: Separation of CMS-T maize mitochondrial proteins by anion-exchange FPLC under "high salt" conditions (**A**), and reaction with antibody against the 13 kDa peptide (**B**). Membranes were made 0.15 *M* KCl were loaded onto a MonoQ10/10 column and washed in FPLC buffer (5% sucrose, 20 m*M* KH_2PO_4, 4 m*M* lauryl maltoside, pH 7.5). Bound protein was eluted at 1.0 mL/min with a discontinuous KCl gradient as described in the legend of Figure 4.2. The solid line indicates the A_{280} and the dashed line indicates the KCl gradient. Western blots were performed as described in section 4.2.4

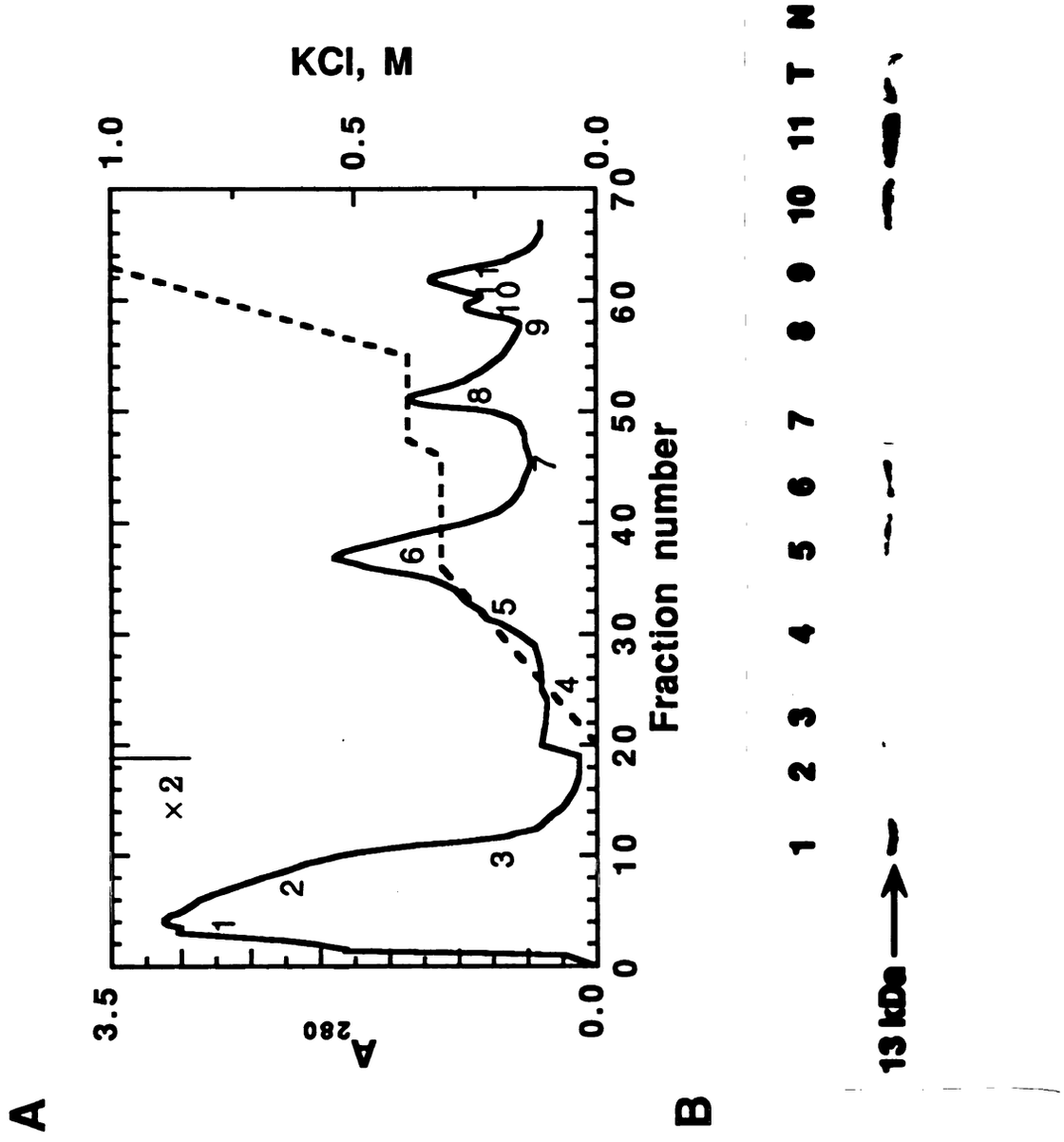

A
KCl, M
1.0
0.5
0.0
3.5
A_{280}
0.0
× 2
1
2
3
4
5
6
7
8
9
10
11
0
10
20
30
40
50
60
70
Fraction number
B
1 2 3 4 5 6 7 8 9 10 11 T N
13 kDa

Figure 4.4

If the 13 kDa peptide is simply "smearing" across the column due to hydrophobic interaction, additional detergent in the buffers might be able to overcome this effect. Therefore we repeated the run using 15 m*M* rather than 4 m*M* lauryl maltoside in the buffers. (In the solubilization step, the mitochondria are already at much higher detergent concentration than 15 m*M*, and are loaded onto the column at this high detergent.) The protein for the "high detergent run" was loaded onto the column at 0.1 *M* KCl, high enough to overcome any electrostatic adhesion to the column, but low enough to prevent too much of the protein from flowing through the column without binding. Although the elution profile was slightly different than previous ones, the basic features and order of elution remained the same (Figure 4.5A). Again, all fractions reacted with the antibody (Figure 4.5B), indicating that either the 13 kDa peptide was truly associating with the mitochondrial proteins and not simply "smearing" on the column, or that far greater detergent is required to prevent this latter situation.

If the 13 kDa peptide is really interacting only with the column and not with the proteins, then re-chromatographing a fraction should redistribute the 13 kDa peptide throughout the gradient, and reduce the amount of it in the fraction containing the re-run protein. We pooled a series of 4–1 mL fractions near peak 6, and re-ran the protein on a smaller column using the same buffers and a continuous gradient (Figure 4.6). Equal amounts of protein (as determined by the BCA protein assay) from the original pool and from the second run peak were precipitated for gel electrophoresis and blotting. The intensity of the reaction of these two fractions with the 13 kDa peptide antibody appeared nearly identical (Figure 4.5, lanes C1 and C2). Therefore, the 13 kDa peptide does not redistribute on the column and is probably associating with protein and not the column.

Figure 4.5: Separation of CMS-T maize mitochondrial proteins by anion-exchange FPLC under "high detergent" conditions (**A**), and reaction of fractions antibody against the 13 kDa peptide (**B**). Membranes were made 0.1 *M* KCl were loaded onto a MonoQ10/10 column and washed in high detergent FPLC buffer (5% sucrose, 20 m*M* KH_2PO_4, 15 m*M* lauryl maltoside, pH 7.5). Bound protein was eluted at 1.0 mL/min with a discontinuous KCl gradient as described in the legend of Figure 4.2. The solid line indicates the A_{280} and the dashed line indicates the KCl gradient. The fractions between the arrows were pooled for rechromatography. Western blots were performed as described in section 4.2.4. Lanes C1 and C2 refer to the pooled protein from the initial (Figure 4.5A) and second (Figure 4.6) FPLC runs respectively.

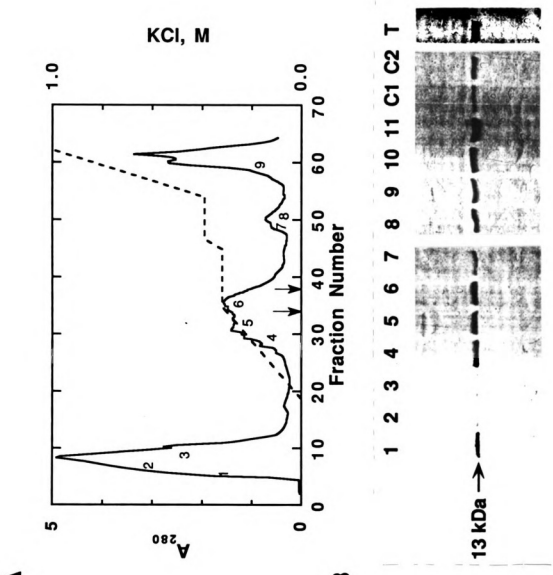

A
B
KCl, M
1.0
0.0
5
0
A_{280}
0 10 20 30 40 50 60 70
Fraction Number
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9 10 11 C1 C2 T
13 kDa

Figure 4.5

Figure 4.6: Re-chromatography of a CMS-T maize mitochondria fraction from the "high detergent" FPLC run on anion-exchange FPLC. The protein pooled from the initial run (Figure 4.5A) was diluted to 0.05 *M* and loaded onto a MonoQ 5/5 column at 0.75 mL/min. Bound protein was eluted at 0.5 mL/min by a gradient from 0 to 0.4 *M* in 15 mL and 0.4 to 1.0 *M* in 7 mL. The fractions between the arrows were pooled for Western blot comparison to initial run fractions (designated C2 in Figure 4.5B).

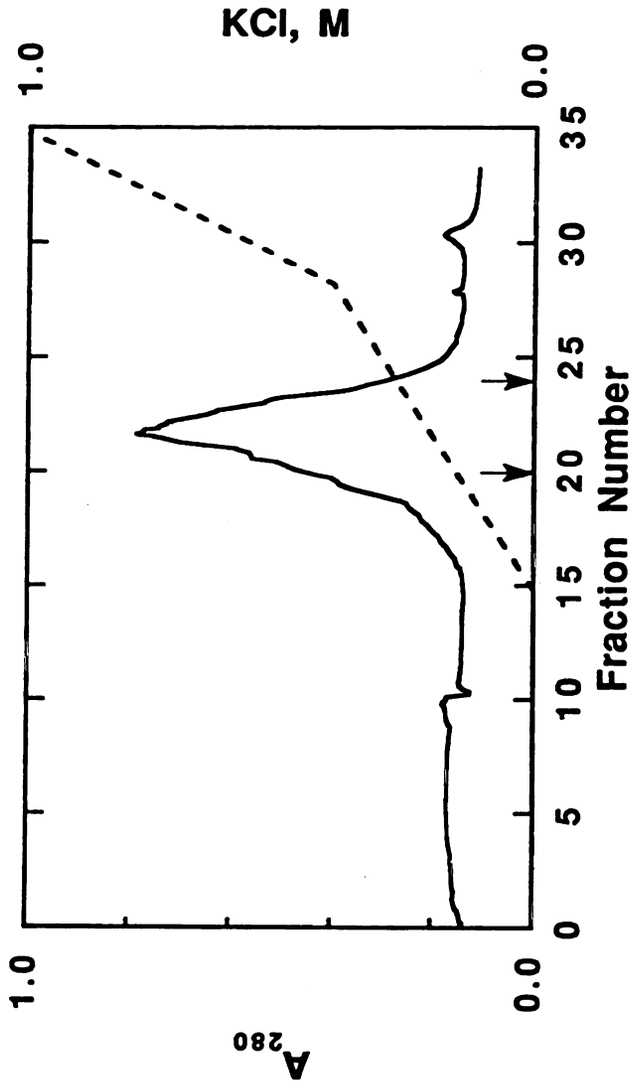


Figure 4.6

4.3.3 CMS petunia experiments

We have previously reported (Connett, 1991) that the urfS protein does not bind to MonoQ (anion exchange), but does bind to MonoS (cation exchange), as determined by immunoblots of eluted fractions. The MonoQ elution profile of petunia mitochondrial proteins for these fractionations was similar to those from wheat and maize mitochondria. The alternative oxidase (AO) also bound to MonoS but not MonoQ, and furthermore, was eluted in the same fractions as urfS. This result was very interesting in light of the observation that AO activity is significantly lower while cytochrome oxidase activity is higher in whole (suspension) cells and purified mitochondria from CMS lines than in male-fertile lines of petunia (Connett and Hanson, 1990). Difference spectra of one preparation each of lauryl-maltoside solubilized mitochondria from CMS, fertile, and restored petunia cell lines showed higher amounts of cytochrome oxidase in CMS compared to male-fertile and restored lines. We have suggested that this difference in oxidase content is related to the differences in activity observed, and, since these lines were isonuclear, might also be related to CMS.

4.3.3.1 FPLC fractionation of petunia mitochondria

We have attempted to repeat the fractionation experiments in order to determine whether the co-elution of urfS and AO reflects a true physical association. If it does, perhaps we can begin to explain the lower alternative oxidase activity and possibly even the mechanism of CMS in petunia. By fractionating mitochondria from CMS, fertile, and restored, and also *E. coli* expressing urfS, we might determine if the elution characteristics of the AO or urfS changed when the two proteins are not present together, which would suggest an association.

Mitochondria (about 5 mg per run) from CMS (3688), fertile (3699), and restored (3337) lines were solubilized (as for maize mitochondria above) and loaded directly onto MonoS for fractionation. Elution profiles were somewhat different from the previous experiment, with less sharp peaks and more "smearing" (Figure 4.7). In all three cell lines the AO did not bind to the column and was eluted in the early flow-through (Figure 4.8, lane Fl1) while urfS appeared throughout the flow-through (Figure 4.8, lanes Fl1 and Fl2) and the first few fractions (lanes 6–8). The antibody also reacted with a higher molecular weight band (upper arrow), possibly an urfS dimer which was the result of incomplete solubilization. The lower molecular weight band reacting with the urfS antibody (lower arrow) may be the 20 kDa band which also reacts with the antibody (see section 2.2.2). The original experiment suggesting association of the AO and urfS was not reproduced, since the column retained some urfS but no AO.

A second attempt was made to repeat this experiment using mitochondria (4 mg) from a different CMS line (10636). The elution profile more closely resembled the original in which AO and urfS associated (Figure 4.9); however, immunoblots of the fractions revealed that AO eluted equally in the flow-through and all of the fractions from the first group of peaks (data not shown). The blot was not probed for urfS.

4.3.3.2 Difference spectra of petunia mitochondria

In order to provide physical evidence to explain the lower AO but higher cytochrome oxidase activity in CMS compared to fertile and restored petunia lines, we attempted to verify the previous result that mitochondria from CMS lines contain more cytochrome oxidase than fertile or restored lines as measured by visible difference spectra. Different preparations and

Figure 4.7: Separation of mitochondrial protein from CMS petunia (3688) by cation-exchange FPLC. Lauryl maltoside-solubilized mitochondria at 0.01 *M* KCl were loaded onto a MonoS 5/5 column at 0.5 mL/min and washed in FPLC buffer (5% sucrose, 20 m*M* KH_2PO_4, 4 m*M* lauryl maltoside, pH 7.5) to remove unbound protein. Bound protein was eluted at 0.5 mL/min with a continuous gradient of 0–0.4 *M* KCl in 20 mL. The solid line indicates the A_{280} and the dashed line indicates the KCl gradient.

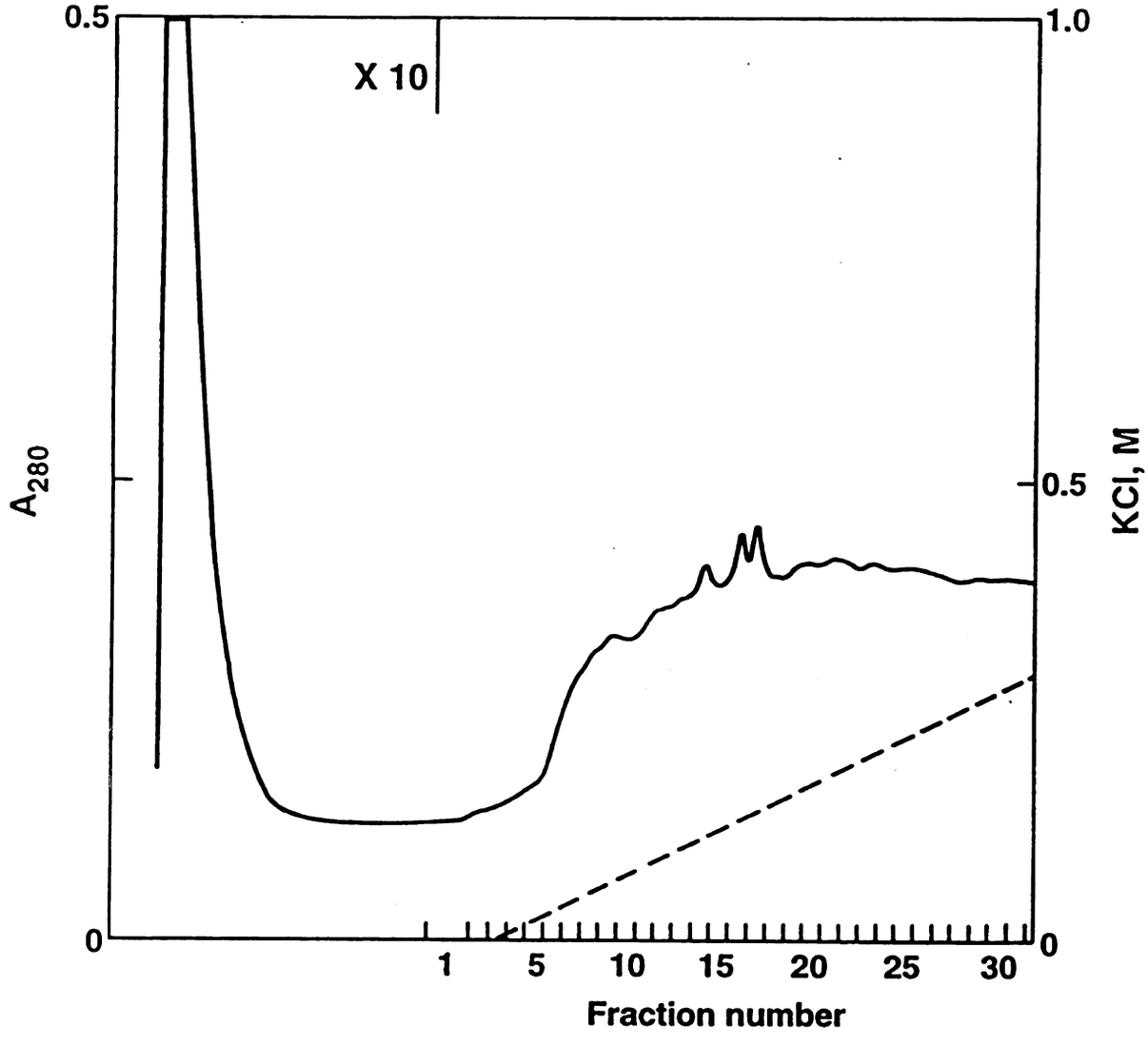


Figure 4.7

Figure 4.8: Western blot analysis of fractions from CMS petunia (3688) fractionation on cation-exchange chromatography. Western blots were performed as described in section 4.2.4. *Lanes:* mt, starting mitochondria; Fl1 and Fl2, early and late flow-through fractions; 6–14, fractions from the FPLC run shown in Figure 4.7. The AO band labelled with an arrow was the only band to react with the AO antibody, while the urfS antibody reacted with a band at its predicted molecular weight (labelled arrow) and one higher and lower molecular weight band (unlabelled arrows). The position of molecular weight standards (in kDa) are shown to the right of lane 14.

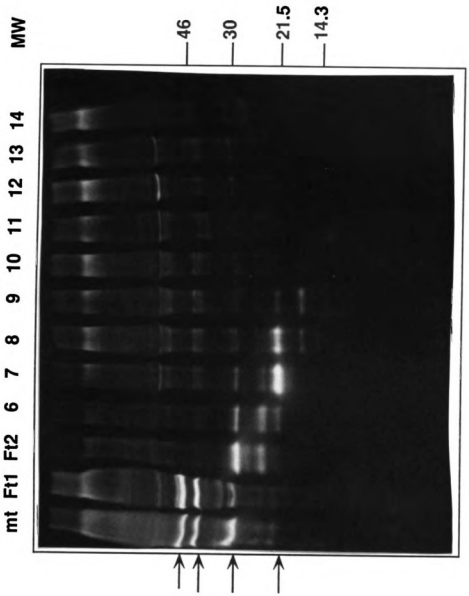


Figure 4.8

Figure 4.9: Separation of mitochondrial proteins from CMS petunia (10636) by cation-exchange FPLC. Lauryl-maltoside solubilized mitochondria at 0.01 M KCl were loaded onto a MonoS 5/5 column at 0.5 mL/min and washed in FPLC buffer (5% sucrose, 20 m*M* KH_2PO_4, 4 m*M* lauryl maltoside, pH 7.5) to remove unbound protein. Bound protein was eluted at 0.5 mL/min with a continuous gradient of 0–0.3 M KCl in 15 mL. The solid line indicates the A_{280} and the dashed line indicates the KCl gradient.

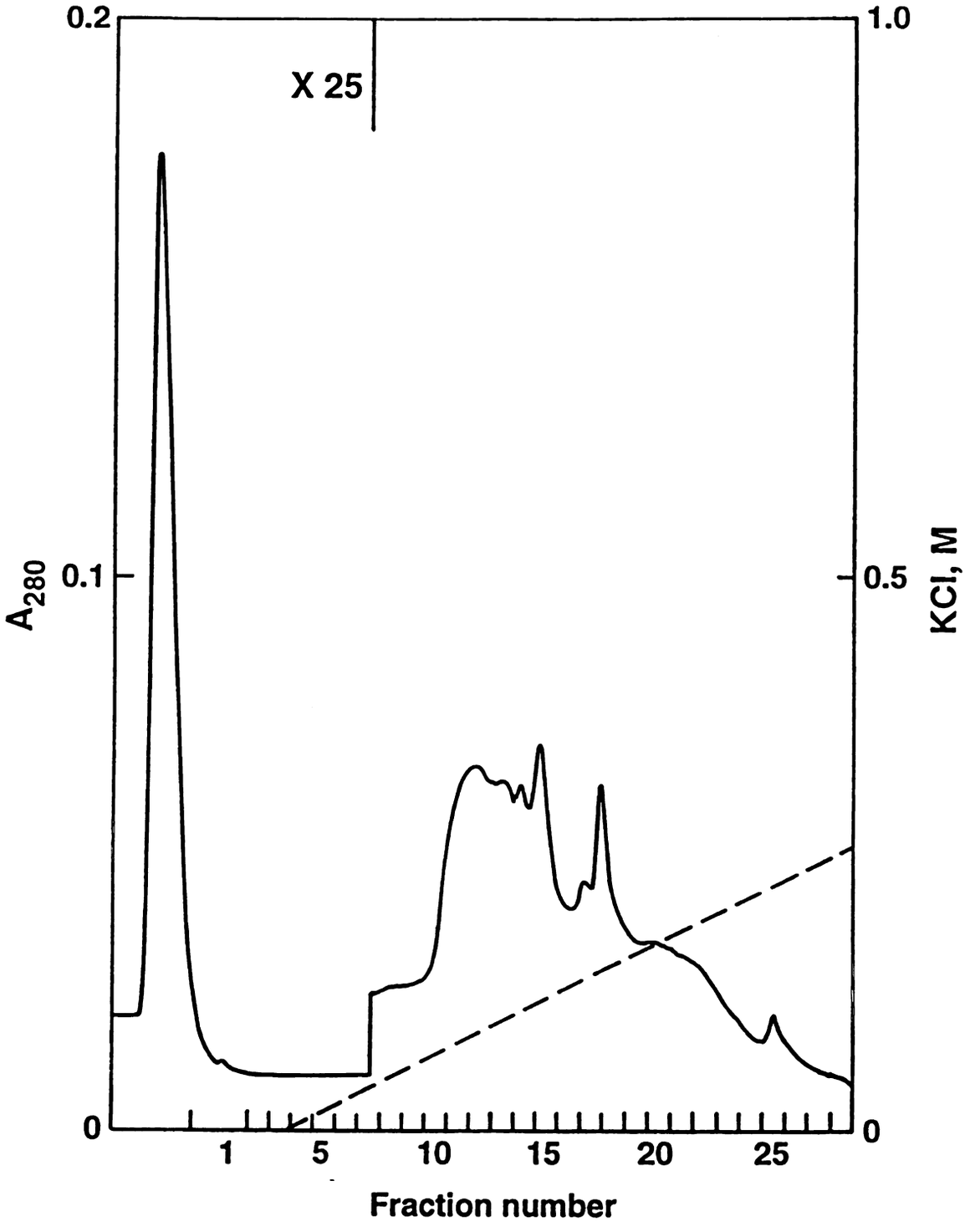


Figure 4.9

two CMS lines were analyzed this time. Since the oxidase content was being judged by the ratio of the *b* and *c* cytochrome peaks to aa_3 peaks, the outer membrane was removed in some preparations to deplete mitochondria of cytochrome *c*, which when present, increases the intensity of the bc_1 peak. The mitochondria were further purified by density gradient centrifugation to remove possible microsomal contaminants which might also contain *b* and *c* cytochromes. A composite of these spectra, normalized to the bc_1 peak, is shown in Figure 4.10. One CMS line (3699) had more oxidase than any other line, while the other CMS line (10636) contains less than a fertile line, but more than all the others. Clearly, there is a large variability in oxidase content between mitochondrial preparations, as there was in assays of oxidase activity (Connett and Hanson, 1990). Perhaps if both oxidase activity and content were tested for each preparation a correlation between them could be observed. Many more preparations would need to be tested to determine whether CMS in petunia is related to higher cytochrome oxidase, but lower alternative oxidase content and activity.

4.4 Discussion

4.4.1 Association of the 13 kDa peptide with maize mitochondrial protein

The 13 kDa peptide was found to co-elute with protein-containing fractions from MonoQ fractionation, confirming the observations of others that the 13 kDa peptide co-purifies with the ATPase (R. Dewey, personal communication) and cytochrome *c* oxidase (C. Leaver, personal communication). In the "low salt" run, several early fractions, including the flow-through, did not appear to contain any the the 13 kDa peptide. This

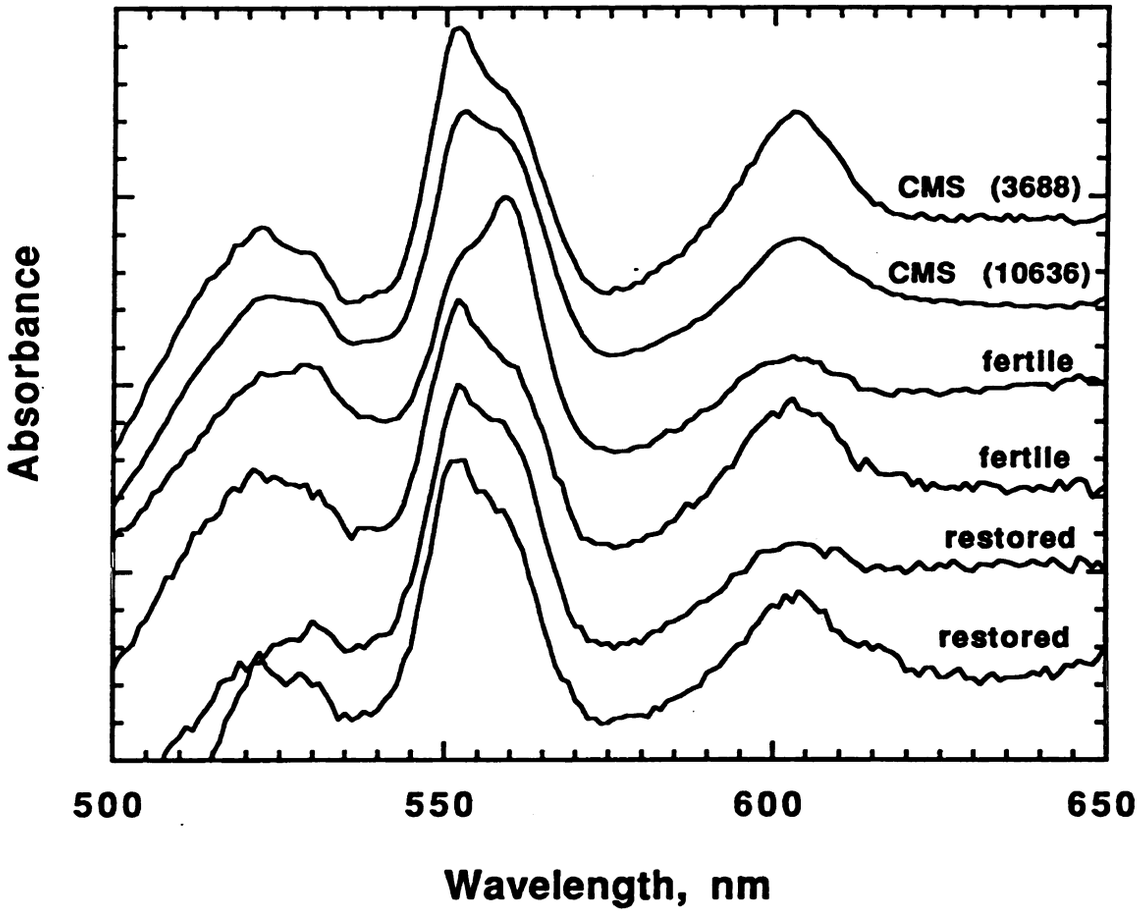


Figure 4.10: Visible difference spectra (dithionite-reduced minus air-oxidized) of lauryl maltoside-solubilized petunia mitochondria. The ratio of the bc_1 peak at 555-560 nm to the cytochrome oxidase peak at 602 nm may differ between the CMS, fertile, and restored lines.

result suggests one of two possibilities: *1)* The 13 kDa peptide is truly associated with many mitochondrial proteins by virtue of hydrophobic interaction; all the identified proteins are sufficiently hydrophobic to account for this possibility while presumably the early flow-through fractions are less hydrophobic. *2)* The hydrophobic nature of the 13 kDa peptide may cause it to bind strongly to the column matrix and "smear" non-specifically, such that it elutes as a continuum, with or without the presence of protein.

When higher salt loading conditions were used, such that some proteins which would bind to the column under low salt conditions would elute in the flow-through, the 13 kDa peptide was also detected in the flow-through fractions, suggesting a true association with those proteins that had previously been retained by the column. Higher detergent, which might disrupt hydrophobic interactions that might be causing the 13 kDa peptide to smear on the column, did not alter the elution pattern of the 13 kDa peptide, suggesting that non-specific hydrophobic interactions with the column are not a factor. Moreover, re-chromatographing a protein fraction did not "redistribute" the 13 kDa peptide since the same amount of it was associated with the fraction before and after the second column run. Therefore, the evidence supports Possibility 1, that the 13 kDa peptide is associating mainly with the mitochondrial protein and not simply the column.

Because the 13 kDa peptide seems to associate with all the major respiratory complexes, this experiment demonstrates that the 13 kDa protein is not associated specifically with a single complex. However, this does not mean that all of these many associations are meaningful, nor does it rule out that the 13 kDa peptide is acting independently (in terms of toxin

action or CMS). It is quite possible that only one of these associations is detrimental in a manner causing either toxin sensitivity or CMS. It is not necessary for a large proportion of one complex, for instance the ATPase, to have 13 kDa peptide associated with it in order to produce toxin sensitivity. Modification of only a few channels per mitochondrion is necessary to collapse the proton gradient and cause leakage of cofactors. A small number of 13 kDa peptide–ATPase complexes could cause a large enough reduction in the efficiency of respiration to prevent the huge mitochondrial proliferation necessary for pollen production, as discussed in chapter 2. Therefore, even though the 13 kDa peptide associates with many proteins, only one specific interaction, which therefore occurs at low levels, is necessary to produce both toxin sensitivity and CMS.

Does this association of the 13 kDa peptide with the mitochondrial proteins reflect what is really occurring in the mitochondria, or is it an artifact of the procedure? Preliminary studies have so far demonstrated that the 13 kDa peptide cross-links only to itself under a variety of conditions (Williams *et al.*, 1990), raising the possibility of oligomeric structures in the membrane which form an independent channel-like structure. However, if the 13 kDa peptide is really associating with many *different* proteins, no one protein would be likely to be identified by crosslinking techniques, although a large percentage of high molecular weight species would be expected to be formed. Until the purified 13 kDa peptide incorporated into lipid vesicles is shown to induce toxin sensitivity, it must be considered that the crosslinking study does not rule out that the effects of the 13 kDa peptide are dependent on a specific interaction with another protein.

Summary. In the previous chapter, we have proposed the invovlement of the ATPase in toxin sensitivity and CMS for several reasons:

1) ATPase inhibitors such as DCCD, kaempferol, and quercetin block toxin-induced swelling in T mitochondria, *2)* The toxin inhibits ATPase activity in T but not N mitochondria, and *3)* The V_{max} of the ATPase is different in N and T mitochondria. We hypothesized that the physical association of the CMS-specific 13 kDa peptide with the ATPase could mediates these effects. In this chapter, we demonstrate by partial fractionation of mitochondrial proteins that the 13 kDa peptide associates with many of the membrane complexes, including the ATPase. This association, even though present in low percentage in the mitochondria, could account for both the sensitivity and action of the BmT toxin as well as the mitochondrial breakdown leading to pollen abortion.

4.4.2 Association of urfS with petunia mitochondrial proteins

Despite the reproducible and highly similar MonoQ elution profiles of mitochondria from different plants, we have been unable to reproduce our previous result that urfS co-elutes with AO from a cation-exchange column (Connett, 1991). In fact, we obtained different A_{280} and AO/urfS elution profiles from the two fractionations using mitochondria from the two CMS lines. Interestingly, the elution profile of line 10636 more closely resembled the original run (Connett, 1991) which also used 10636; however, AO/urfS eluted differently. While we have been unable to definitively explain this, there are several possibilities. We have observed that contaminants in different batches of lauryl maltoside affect the activity of wheat cytochrome oxidase, and may possibly affect the ability of the detergent to solubilize mitochondria effectively. Poorly solubilized proteins could exist in aggregate forms or supercomplexes, possibly accounting for different degrees of "smearing" or non-binding observed in different experiments.

Alternatively, the column itself may be affected in adverse ways by the proteins chromatographed, such that subsequent experiments would give different results. For instance, the hydrophobic ADP/ATP translocase, a major protein component of mitochondria, appears to bind to the MonoS column, eluting as a "smear" once a certain salt concentration is reached (based on its predicted abundance and molecular weight). It is possible that not all the protein is removed during the gradient or by routine cleaning techniques, so the column remains "coated" with the protein when the next batch of sample is loaded. The column matrix would effectively be changed, and the binding and elution characteristics might be altered. This phenomenon could account for our unreproducible results.

In any case, the urfS and AO did not co-fractionate in our attempt to reproduce the original result. Whether or not some external problem caused the different elution behavior, the result that the two did not co-elute is meaningful. If slightly altered conditions are able to abolish co-elution, then co-elution may be coincidental, or the physical association between the peptides is not very strong. However, weak interactions between proteins can often be functionally significant, as in the case of cytochrome *c* and cytochrome oxidase, which do not remain associated under the conditions of FPLC. Therefore, while our results indicate that it is doubtful that there is a strong physical association between urfS and AO, we cannot completely rule out the possibility that some interaction does exist, and that it is significant for CMS. Ion-exchange chromatography is perhaps not well suited to clarify this issue.

Chapter 5 - Literature review of cytochrome *c* oxidase

5.1 Introduction

Cytochrome *c* oxidase (E.C. 1.9.3.1) is a multisubunit enzyme in the respiratory pathway of the inner mitochondrial membrane of eukaryotes and the cytoplasmic membrane of aerobic bacteria. It plays a central role in aerobic metabolism, functioning as the terminal electron sink for the oxidation of foodstuffs by reducing O_2 to H_2O. In fact, cytochrome oxidase is responsible for 90% of all O_2 consumed by living organisms on Earth (Wikström *et al.*, 1981).

Cytochrome oxidase accepts electrons from reduced cytochrome *c* and transfers them sequentially through a series of bound coppers and hemes A to O_2. Electron transfer is coupled to the vectorial transport of protons across the membrane, contributing with the other respiratory complexes to an electrochemical gradient which is used to generate ATP. Four electrons are needed to reduce the bound oxygen to two water molecules and pump four protons. A diagram of the components and reactions of the oxidase is shown in Figure 5.1. The overall reaction is:

$$4\ e^- + 8\ H^+\ (\text{matrix}) + O_2 \rightarrow 2\ H_2O + 4\ H^+\ (\text{cytoplasm})$$

5.2 Purification

Over the years there has been a great deal of controversy over the structural and even functional aspects of cytochrome oxidase, particularly the metal and subunit content and the enzyme's proton pumping activity. Many of the differences in observed properties have been found to be the

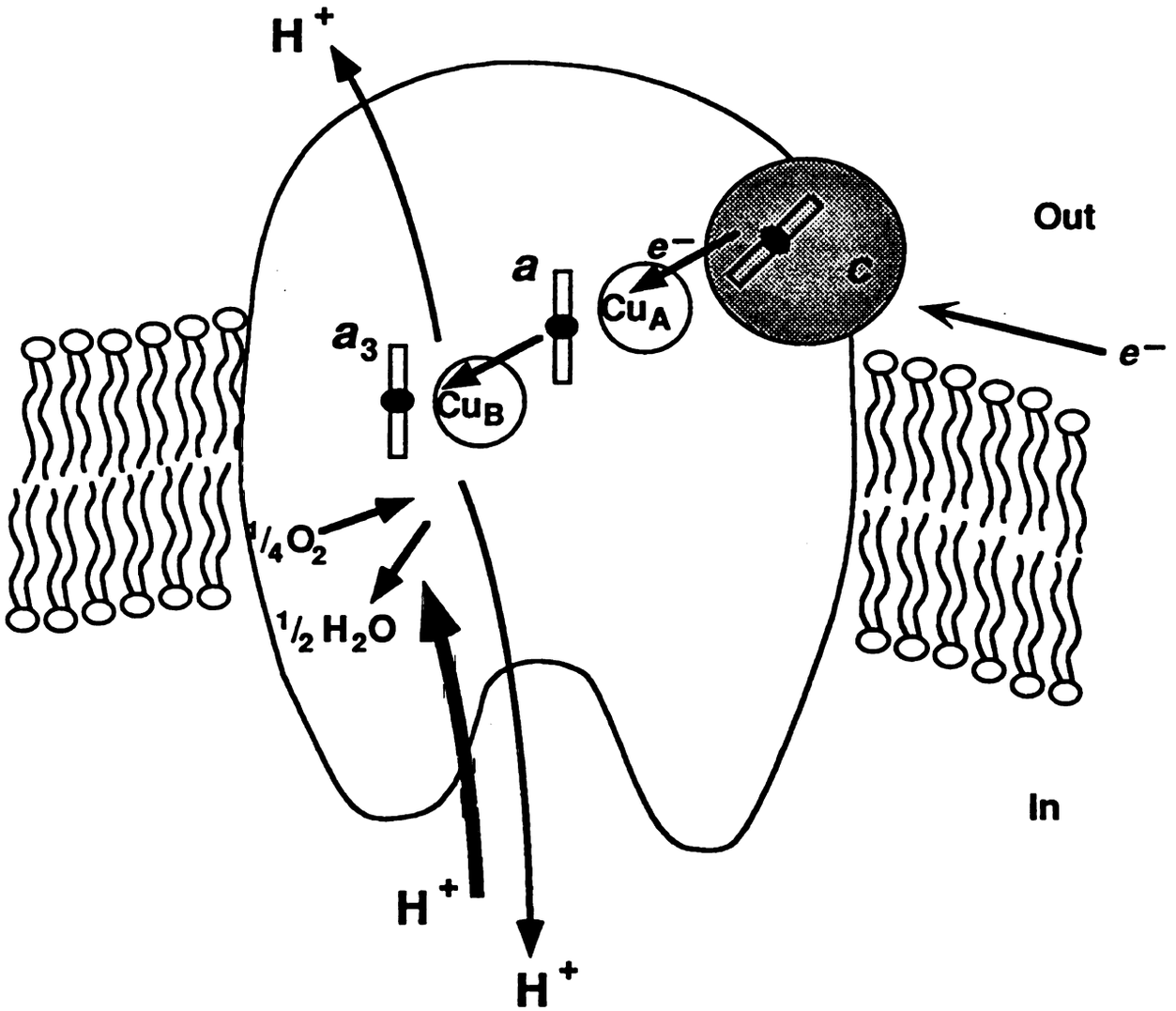


Figure 5.1: Diagram of cytochrome *c* oxidase.

result of variations in purification procedures. Therefore, an understanding of the diverse approaches to purifying this multisubunit integral membrane protein is critical to understanding the wealth of conflicting results in the field.

Since cytochrome oxidase is embedded in a lipid bilayer, a membrane fraction (the mitochondrial inner membrane in eukaryotes, the cytoplasmic membrane in bacteria) must be isolated and solubilized with detergent. One approach to solubilization in mitochondria takes advantage of the property of oxidase that it is less easily removed from the membrane by detergents than most other protein complexes. Bile salts such as deoxycholate or cholate (or Triton X-114; Hartzell and Beinert, 1974) are used with salt at a concentration which extracts other integral membrane proteins but leaves the oxidase in the membrane, resulting in a "red-green split" (because the more easily extracted cytochrome bc_1 complex is red and the oxidase is green); higher detergent concentrations solubilize the oxidase from the remaining membrane fraction, resulting in significant purification (Fowler *et al.*, 1962; Kuboyama *et al.*, 1972; Errede *et al.*, 1978). Alternatively, the entire membrane is dissolved in detergent. A brief dialysis lowers the concentration of detergent and salt to the point where the oxidase preferentially aggregates into a membranous form which can be removed by centrifugation and resolubilized (Suarez *et al.*, 1984). The fractions from these related methods are subjected to ammonium sulfate fractionation to yield a highly purified enzyme.

A second approach to purification uses column chromatographies relying on cytochome *c* affinity (Weiss *et al.*, 1979; Azzi *et al.*, 1982; Thompson and Ferguson-Miller, 1983; Bisson *et al.*, 1985; Hill and Robinson, 1986), anion exchange (Rubin and Tzagoloff, 1978, using DEAE;

Finel and Wikström, 1986; Steffens *et al.*, 1987; Haltia *et al.*, 1988, all using MonoQ), or hydrophobic interaction (Weiss and Sebald, 1978; Goto *et al.*, 1982; Ozawa *et al.*, 1982; Bisson *et al.*, 1985) as a first or last step. Non-ionic detergents such as Triton X-100, lauryl maltoside, and LDAO are generally used with these methods; however, as in many of the references just cited and others (Georgevich *et al.*, 1983; Heinrichs and Schönert, 1987) loss of subunits occurs under some conditions, and the activity of the enzyme isolated in different detergents varies widely. It is not clear what conditions are most responsible for loss of activity, but it is evident that the type of detergent used is critical. For many types of oxidases, lauryl maltoside appears to be the most activating (Rosevear *et al.*, 1980). Rapid purification and avoidance of the use of ammonium sulfate may allow some labile enzymes to retain activity.

The purity of the resulting preparation may be analyzed using SDS-polyacrylamide electrophoresis. Often, however, the resolving ability of the gel is a limiting factor in identifying both contaminants and true subunits, and electrophoresis alone cannot distinguish between true subunits and contaminants. Native gel electrophoresis (Ludwig *et al.*, 1979; Heinrichs and Schönert, 1987; Schägger and vonJagow, 1991) and immunoprecipitation (Poyton and Schatz, 1975; Ludwig *et al.*, 1979; Merle and Kadenbach, 1980a) have been used to provide additional evidence for purity and subunit composition of the preparation. Ultimately, complete sequence and stoichiometry determination is necessary to confirm the subunit composition.

5.3 Structure

5.3.1 Oxidases and their polypeptide composition

The oxidase from mammals has been the most extensively studied because of the high concentration of the enzyme in the mitochondrial membranes and the abundance of mitchondria in certain tissues, such as beef heart. As gel electrophoresis systems became more highly resolving, early estimates of seven subunits (Downer *et al.*, 1976) had to be updated. The mammalian oxidase complex is now thought to have thirteen subunits (Kadenbach *et al.*, 1983) with a molecular weight of 204,000 calculated from the protein sequences (Buse *et al.*, 1985). Although several subunits (including III) can be removed by relatively mild procedures (Penttilä, 1983; Thompson and Ferguson-Miller, 1983; Prochaska and Reynolds, 1986), they have been concluded to be true subunits based on their 1:1 stoichiometry with the others and their presence in immunoprecipitates and preparations from several mammals and tissue types (Merle and Kadenbach, 1980). Numerous systems of subunit nomenclature have been used throughout the literature; the system of Kadenbach *et al.* (1983) will be used here.

The idea of the seven subunit mammalian oxidase maintained support for a long time because yeast was at first also found to have seven subunits (Rubin and Tzagaloff, 1973; Poyton and Schatz, 1975). Yeast was later shown to have nine subunits on a higher resolving gel and by HPLC (Power *et al.*, 1984). This enzyme has been well-studied because of the ability of yeast to grow despite respiratory defects as well as the its ability to be genetically manipulated. Cytochrome oxidase from another fungus, *Neurospora crassa*, has been purified with eight subunits (Werner, 1977).

It was first discovered in fungi that the largest three subunits of all eukaryotic oxidases are encoded by mitochondrial DNA while the rest are encoded by nuclear DNA, synthesized on cytoplasmic ribosomes and imported into the mitochondria (Sebald *et al.*, 1972; Mason and Schatz, 1973b; see Hartl and Neupert, 1990 or Pfanner *et al.*, 1992 for a review).

Oxidases from many other sources have been analyzed for subunit composition. The relatively simple slime mold, *Dictyostelium discoideum*, has a seven-subunit oxidase (Bisson *et al.*, 1985). Plant oxidases have seven to nine subunits (Maeshima and Asahi, 1987; Hawkesford *et al.*, 1989; Peiffer *et al.*, 1990), while bird and fish oxidases have eleven to thirteen (Merle and Kadenbach, 1980b; Montecucco *et al.*, 1987).

Oxidases of the aa_3-type (see below) with similar catalytic and proton-pumping activities have been purified and studied from a number of aerobic bacteria, including *Paracoccus denitrificans* (Solioz *et al.*, 1982; Haltia *et al.*, 1988), *Rhodobacter sphaeroides* (Gennis *et al.*, 1982; Hosler *et al.*, 1992), *Bacillus subtilis* (Saraste *et al.*, 1991; Lauraeus *et al.*, 1991), the thermophilic bacteria PS3 (Sone and Yanagita, 1982; Sone *et al.*, 1990) and *Thermus thermophilus* (Fee *et al.*, 1980; Hon-nami and Oshima, 1984; Buse *et al.*, 1989), and the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius* (Anemüller and Schafer, 1989). The subunit composition of these bacterial enzymes is much simpler, containing only 2 or 3 subunits which are homologous to the mitochondrially-encoded subunits of eukaryotes. The PS3 (Sone *et al.*, 1990) and one of the *B. subtilis* oxidases (Saraste *et al.*, 1991) have been isolated with a fourth subunit; the PS3 sequence appears unrelated to the fourth subunit of eukaryotic oxidases but nevertheless crossreacts with mammalian subunit IV antibodies (Sone *et al.*, 1990).

Many aerobic bacteria contain multiple terminal oxidases, some of which use other heme types or accept electrons from ubiquinols instead of cytochrome *c*. The ubiquinol oxidase *bo* complex in *E. coli* also pumps protons (Puustinen *et al.*, 1989) and shows striking sequence homologies with the aa_3-type oxidases, particularly in subunit I. The heme O structure is similar to heme A (Puustinen and Wikström, 1991; Wu *et al.*, 1992) with a methyl group replacing the formyl, and it may replace a_3 in the aa_3 enzyme of *P. denitrificans* under certain environmental conditions (Sone and Fujiwara, 1991).

5.3.1.1 Mitochondrial subunits

The sequences of the mitchondrially-encoded subunits of oxidases from different sources are highly homologous, with 56% amino acid identity between mammalian and yeast subunit I (Capaldi *et al.*, 1986). Homology with the bacterial sequences is generally lower, although *R. sphaeroides* and *P. denitrificans*, members of the purple bacteria class from which mitochondria are thought to have arisen, show amino acid identity with the mammalian sequence almost as high as yeast, 51% (Shapleigh and Gennis, 1992) and 54% (Raitio et al., 1987; Mather *et al.*, 1990) respectively. All subunit I sequences together show only 15% identity with 35 strictly conserved amino acids. Subunit I from the cytochrome *bo* complex is homologous to those from other bacteria but has extensions at both the N and C-termini (Chepuri and Gennis, 1990), while *B. subtilus* aa_3 has only a C-terminal extension (Saraste *et al.*, 1991). *P. denitrificans* may contain isozymes of cytochrome oxidase; two genes for subunit I which are 90% homologous have been found (Raitio *et al.*, 1990).

Subunit II from some organisms has an N-terminal extension which is retained in bacteria but is removed in *Neurospora* (Machleidt and

Werner, 1979), yeast (Sevarino and Poyton, 1980), and perhaps plants (Maeshima *et al.*, 1989). Cytochrome *c* covalently bound to subunit II has been found in the subunit IV-containing *B. subtilis* (Saraste *et al.*, 1991) oxidase and the oxidases of the thermophilic bacteria *T. thermophilus* (Buse *et al.*, 1989; Mather *et al.*, 1990) and PS3 (Sone and Yanagita, 1982); this appears to be the result of gene fusion of subunit II and cytochrome *c*. There is high homology in the C-terminal region of most subunits II where cytochrome *c* and Cu_A are proposed to bind (see below). One exception is the DNA sequence of wheat subunit II which predicts an arginine in place of a strictly conserved cysteine (Bonen *et al.*, 1984). Recently, however, RNA sequencing has shown that the cysteine is restored (Covello and Gray, 1989). RNA editing now appears to be relatively common in the mitochondria of plants.

Subunit III has been suggested to have a role in proton pumping because DCCD, which binds acidic residues in hydrophobic regions of proteins, inhibits proton pumping under conditions where predominantly subunit III is labelled (Casey *et al.*, 1980). Prochaska *et al.* (1981) showed that the labelled residue is Glu-90. However, when this residue is mutagenized in *P. denitrificans*, proton pumping activity is retained (Haltia *et al.*, 1991). Moreover, subunit III-depleted mammalian oxidases (Püttner et al., 1985; Thompson et al., 1985; Sarti et al., 1985; Finel and Wikström, 1986) as well as many two-subunit preparations of bacterial oxidases (Solioz *et al.*, 1982) pump protons. After deletion of the subunit III gene in *P. denitrificans* free subunit I and II and only some I-II complexes are formed, suggesting that subunit III plays a role in enzyme assembly (Haltia *et al.*, 1989).

5.3.1.2 Nuclear-encoded subunits

The N-terminal amino acid sequences of all the nuclear-encoded subunits of bovine heart and yeast oxidase have been determined. All of the nine yeast subunits have homologues in the thirteen-subunit mammalian enzyme (with 13–28% homology; Capaldi *et al.*, 1986) while subunits VIa, VIb, VIc, and VIIb are unique to mammals. The homologies between the subunits mostly occur in short well-conserved domains (e.g. the N-terminus of subunit VIIa; Capaldi *et al.*, 1986) within otherwise dissimilar sequences. Subunit VIa contains a conserved cysteine which may bind the zinc associated with the oxidase (Naqui *et al.*, 1988).

5.3.2 Three-dimensional structure

A clear understanding of the the three-dimensional structure of cytochrome oxidase has remained elusive because of the difficulty in producing three-dimensional crystals from membrane proteins. In fact, only a few crystal structures have been deduced from membrane proteins, including the photosynthetic reaction center from *Rhodopseudomonas virdis* (Deisenhofer *et al.*, 1985) and from *Rhodobacter sphaeroides* (Chang *et al.*, 1986; Allen *et al.*, 1987), and the bacterial porin from *Rhodobacter capsulatus* (Weiss *et al.*, 1991). Therefore only the general shape of the oxidase has been deduced from two-dimensional crystal structures at 10–20 Å resolution, while other methods have had to be used to determine the aggregation state and orientations of the subunits.

5.3.2.1 General shape

Two methods have produced two-dimensional crystals. Deoxycholate extraction of mitochondria produces protein-rich sheets containing no continuous bilayer. The oxidase exists as a monomer lined up in

alternating orientation (Fuller *et al.*, 1979). Triton X-100 treatment of mitochondrial membranes can produce flattened vesicles with patches of ordered oxidase molecules. Electron microscopy of these vesicles with 30 Å resolution showed that the oxidase exists as a dimer with all molecules oriented the same in the membrane. The molecule appeared Y-shaped with most of the molecular mass inside the vesicles at the stem of the Y; subunit-specific antibodies showed that the inside of the vesicles corresponded to the cytoplasmic (C) side of the oxidase, while the outside was the matrix (M) side (Frey *et al.*, 1978). An improved image was produced by Deatherage *et al.* (1982) with 20 Å resolution, confirming that separate "M1" and "M2" domains protrude from the vesicles. Additionally a cleft was seen at the dimer interface in the "C" domain. Calculation of a volume for the molecule indicated a molecular weight of 110-165 kDa per monomer, which is much lower than the 204 kDa deduced from gene sequences.

More detail could be seen in the three-dimensional image reconstruction of Valpuesta *et al.* (1990). A negatively stained vesicle crystal preparation analyzed by electron cryo-microscopy provided 10 Å resolution. While the overall structure deduced from previous studies was verified, a hollow cavity at the center of the dimer was identified (corresponding to Deatherage's cleft) and is proposed to be the cytochrome *c* binding site. Additionally, the M1 and M2 domains appear not to separate until they are out of the membrane. As before, cross-sectional areas can account for only 12-16 α helices despite predictions of 21 for all the subunits combined. The authors point out that if only subunits I, II, and III are considered, the number of sequence-predicted helices is closer to the number allowed by the observed size. However, since molecular edges are

imprecisely determined in this type of image reconstruction, the actual number of helices fitting into the predicted space could be much larger.

5.3.2.2 Aggregation state

The aggregation state of a preparation can be determined by sedimentation equilibrium, gel filtration or radiation inactivation (Suarez *et al.*, 1984). As with the crystalline membrane preparations discussed above, cytochrome oxidase can be isolated as a dimer, although certain detergents (and high concentrations of others) can produce a monomer (Georgevich *et al.*, 1983; Suarez *et al.*, 1984; Robinson and Talbert, 1986; Finel, 1987). Conditions which remove subunit III often monomerize the oxidase, suggesting that this subunit plays a role in promoting dimerization (Georgevich *et al.*, 1983; Finel, 1987) although monomer containing subunit III can be prepared (Suarez *et al.*, 1984; Finel and Wikström, 1986). It is not clear what functional role the dimer might play, since for many of its proposed functions it has been shown that monomers are sufficient. Monomers are fully capable of electron transfer and biphasic kinetics (Thompson and Ferguson-Miller, 1983; Suarez *et al.*, 1984; Robinson and Talbert, 1986; Hakvoort *et al.*, 1987). It has also been proposed that dimerization is important for proton pumping (Finel and Wikström, 1986); however, shark oxidase, which appears to exist as a monomer under all conditions studied, pumps protons (Bickar *et al.*, 1985).

5.3.2.3 Subunit orientations

Hydropathy plots (Kyte and Doolittle, 1982) identify long stretches of predominantly hydrophobic residues which are likely to form transmembrane α helices in the membrane. On this basis, subunit I is predicted to have twelve helices, subunit II two and subunit III six to seven. In addition, the bovine subunits IV, VIa c, VIIa, b, c, and VIII are

predicted to have transmembrane helices, while subunits Va, Vb, and VIb do not have obvious membrane-spanning regions (Capaldi *et al.*, 1986) and do not react with lipophilic reagents (Cerletti and Schatz, 1979; Bisson *et al.*, 1979). This totals 28 transmembrane helices, and as mentioned above, this number of helices cannot fit into the area in the membrane that can be calculated from the current 3-D model (Valpuesta *et al.*, 1990).

Chepuri and Gennis (1990) examined the orientation of subunit I of the cytochrome *bo* complex in the membrane by random gene fusions with alkaline phosphatase or β-galactosidase. Extramembrane loops could then be localized to either side of the membrane by assaying for marker enzyme activity. Since the sequence of cytochrome *o* is so similar to subunit I of the aa_3 oxidases, their results have been interpreted to confirm the 12-helix model of subunit I. These gene fusion experiments also confirmed the basic topology of subunit II as well. Hydropathy plots predict 2 transmembrane helices (the second of which contains a large number of conserved residues) forming a hairpin near the N-terminus with a large extramembranous C-terminal domain which was demonstrated to be on the cytoplasmic side of the membrane by labelling experiments (reviewed in Capaldi *et al.*, 1983) of the cytochrome *c* binding site. The loop of the hairpin was shown to be in the mitochondrial matrix (inside the bacterial cell) by protease accessibility experiments (Finel, 1988). Subunit III, predicted to have seven transmembrane helices, is thought to form a somewhat independent domain based on thermal denaturation studies (Morin *et al*, 1990) and its ease of removal by mild conditions (see above).

The orientations of the mammalian subunits have been investigated by chemical labelling and protease digestion (Zhang *et al.*, 1984; Malatesta et al, 1983; Jarausch and Kadenach, 1985b; Zhang *et al.*, 1988; Zhang *et al.*,

1991). Most of these studies have confirmed the transmembrane predictions of the hydropathy plots and have indicated which side of the membrane exposes the N and C-termini (see Capaldi, 1990a for review). Near-neighbor relationships of the subunits have been studied by crosslinking. Since IV, Va, Vb, VIc are the four subunits common to all eukaryotes (except plants), it might be expected that they would interact with the mitochondrially-encoded subunits, which is confirmed by the cross-linking data (Briggs and Capaldi, 1977; Briggs and Capaldi, 1978; Jarausch and Kadenbach, 1985a). Unfortunately, interpretation of the results of these studies is very difficult because of the different gel systems used in the studies, some of which were unable to resolve all 13 subunits.

5.3.3 Prosthetic groups

Cytochrome oxidase contains two irons and at least two coppers. The irons are both inserted in the porphyrin ring of a heme A, which is distinguished from other heme types by its formyl in position 8 and an isoprenoid chain in position 2. The hemes have been designated hemes *a* and a_3 (hence cytochrome *c* oxidase is often referred to as cytpchrome aa_3), the latter binding O_2 and poisonous ligands such as HCN, CO, and HN_3 (Keilin and Hartree, 1939). These hemes produce cytochrome oxidase's characteristic visible absorption spectra: in the Soret region of the beef heart cytochrome oxidase spectrum, a broad peak centered at 421 nm in the oxidized state and a narrower peak at 443 nm in the reduced state results from the equal, additive contributions from heme *a* (λ_{max}=426/444 nm, ox/red) and a_3 (λ_{max}=414/442.5 nm, ox/red) whereas heme *a* contributes 80% to the α band at 605 nm in the reduced enzyme (Vanneste, 1966). One of the copper ions is designated Cu_A; when oxidized it produces a broad visible

absorption centered at 830 nm (Wharton and Tzagoloff, 1964; Beinert *et al.*, 1980) and a characteristic electron paramagnetic resonance (EPR) signal at g=2.03 (Beinert *et al.*, 1962; Aasa *et al.*, 1976) which distinguishes cytochrome oxidase from many other copper-containing proteins. Another copper is designated Cu_B and is EPR-undetectable. The heme *a* and Cu_A have a role in accepting electrons from cytochrome *c* and have been shown to be located near the cytoplasmic membrane face (Rich *et al.*, 1988; Ohnishi *et al.*, 1985). Heme a_3 and Cu_B are magnetically coupled and form a "binuclear center" possibly located more deeply in the membrane (Ohnishi *et al.*, 1985). Recent structural models, however, place the two hemes at similar depths in the membrane (Shapleigh *et al.*, 1992).

In addition to the two coppers and two irons thought to be catalytically active, one zinc and one magnesium per monomer, and one additional copper per *dimer* was found in the bovine heart oxidase with inductively-coupled (Einarsdóttir and Caughey, 1984, 1985) or direct current (Pan *et al.*, 1991a) plasma atomic emission spectroscopy (AES). Other groups calculated that cytochrome oxidase contained one additional copper per *monomer*, not dimer, in bovine heart and *Paracoccus denitrificans* enzyme by using inductively-coupled plasma AES (Steffens and Buse, 1987) as well as in different tissues of several mammals by proton-induced X-ray emission (Bombelka *et al.*, 1986), and in bovine heart with energy dispersive X-ray fluorescence (Öblad *et al.*, 1989). This copper has been designated "Cu_X". It has been suggested that this copper is "adventitious" since fully functional oxidase can be prepared lacking it (Y. Li *et al.*, 1987); however, some other attempts to remove the copper by dialysis against chelating agents failed (Yewey and Caughey, 1988). In contrast to earlier reports that subunit III could be removed with no change of metal content (Yewey and

Caughey, 1987), Pan *et al.* (1991a) report that Cu_X is removed by monomerization of the enzyme or subunit III removal, suggesting that Cu_X associates with subunit III and may play a role in dimerization.

The roles of the zinc and magnesium remained undefined. Zinc could not be removed by dialysis against EDTA (Naqui *et al.*, 1988; Yewey and Caughey, 1988), but could be partially removed by various chemical treatments with little or no loss in activity, suggesting a structural role (Naqui *et al.*, 1988; Moubarek *et al.*, 1987). A role for zinc in dioxygen reduction and proton translocation has been suggested (Einarsdóttir *et al.*, 1988; Yoshikawa and Caughey, 1990) although there is no evidence for this. Magnesium is proposed to associate with an ATP which binds to the complex (Yewey and Caughey, 1987).

5.3.3.1 Cu_A site

Homology between a region of subunit II and blue copper proteins such as azurin and plastocyanin was first noticed by Steffens and Buse (1979) who therefore suggested that subunit II might contain the Cu_A site. Blue copper proteins contain a cysteine followed a few amino acids later by a histidine with a methionine a few residues beyond. A histidine earlier in the sequence is often used in the blue copper site. In cytochrome oxidase, Cys-196 and 200 are conserved, as well as His-204 and Met-207, in analogy to the blue copper proteins. In addition, His-162 is also invariant, perhaps providing another ligand for Cu_A. None of these residues is conserved in non-Cu_A-containing oxidases such as cytochome *bo* in *E. coli.* and the aa_3-type quinol oxidases in *B. subtilis* (Lauraeus *et al.*, 1991).

The unique EPR spectrum of cytochrome oxidase attributable to Cu_A (Beinert *et al.*, 1962) is dissimilar to that of the blue copper proteins. Therefore, although only one cysteine ligand is proposed for blue copper

proteins, two were proposed for cytochrome oxidase based on chemical modification studies (Mann and Auer, 1980; Darley-Usmar *et al.*, 1981; Verheul *et al.*, 1982). A number of spectroscopic studies support these predictions. Changes in electron-nuclear double resonance (ENDOR) spectra using yeast oxidase with incorporated ^{15}N-His and ^{2}H-Cys indicated that at least one histidine and one cysteine were ligands of Cu_A (Stevens *et al.*, 1982). Using ^{13}C-Cys, Martin *et al.* (1988) concluded that two symmetric cysteines could explain the ENDOR data. Extended X-ray absorption fine structure (EXAFS) confirmed previous hypotheses, demonstrating two sulfur and five or six nitrogen or oxygen ligands for both coppers together (Scott *et al.*, 1986). The contributions of the two coppers were deconvoluted by removing the Cu_A by chemical modification, leaving Cu_B (P.M. Li *et al.*, 1987). Thus Cu_A was shown to have two sulfur and two nitrogen or oxygen ligands. However, a ba_3 oxidase (catalyzing cytochrome *c* oxidation) purified from *Thermus thermophilus* as a single polypeptide was found to have only one cysteine but a normal Cu_A (EPR) spectrum (Zimmerman *et al.*, 1988). More recent evidence indicates that the oxidase is coded for by genes for subunits homologous to I, II, and III of the eukaryotic oxidase with a conserved Cu_A site in subunit II (Keightley *et al.*, 1992). Therefore, most models of the Cu_A site are constructed using two cysteines and two histidines (Holm *et al.*, 1987) or two cysteines, one histidine and one methionine (Covello and Gray, 1990).

The Cu_A site of cytochrome oxidase has always been thought to be "unique" based on its spectroscopy and liganding. Recently though, nitrous oxide reductase, the terminal oxidase in the denitrification pathway of bacteria, has been cloned and purified from *Pseudomonas stutzeri* and is found to have sequence homology to the proposed Cu_A site of cytochrome

oxidase (Viebrock and Zumft, 1988). Moreover, EPR, magnetic circular dichroism (MCD), and EXAFS spectra were all similar (Scott *et al.*, 1989), suggesting a similar Cu_A environment between the two enzymes. Kroneck *et al.* (1988, 1990) interpret low frequency EPR data to indicate a Cu-Cu interaction in both enzymes, which cannot be ruled out in view of the evidence for Cu_X in cytochrome oxidase.

The proposed Cu_A site has been shown to be on the cytoplasmic side of the membrane near several acidic residues predicted to be involved in cytochrome *c* binding (Millett *et al.*, 1983). Rich *et al.* (1988) have supported this peripheral location by their finding that Cu_A is only 10% or less into the membrane dielectric.

5.3.3.2 Heme a and binuclear center

Evidence concerning the sites of heme *a* and the binuclear center (heme a_3 and Cu_B) has been derived from two main sources. The identity of the residues ligating these centers has been determined by various spectroscopies, while the location of these residues has been deduced from site-directed mutagenesis experiments and analysis of conserved residues. The heme *a* iron is low spin and six-coordinated: four nitrogenous ligands from the heme ring, and two histidine ligands from the protein, as shown by Raman (Babcock *et al.*, 1981), EXAFS (Powers *et al.*, 1981); MCD (Carter and Palmer, 1982), and ENDOR (Martin *et al.*, 1985) spectroscopy. Heme a_3 iron is high spin and six-coordinate when oxidized and five-coordinate when reduced (Babcock *et al.*, 1981). The coordination position not attributable to the heme ring, the fifth, or "axial" position, is ligated by histidine, as determined by EPR (Blokzil-Homan and vanGelder, 1971; Stevens and Chan, 1981), Raman (Babcock *et al.*, 1981), and EXAFS (Powers *et al.*, 1981) spectroscopies. The sixth position, a bridging ligand between

heme a_3 and Cu_B remains unidentified, but it has been proposed to be sulfur or chloride (Powers *et al.*, 1982; P.M. Li *et al.*, 1987; Scott *et al.*, 1988) in the oxidized state or H_2O or OH^- under turnover conditions (Cline *et al.*, 1983).

Cu_B is EPR-invisible in the oxidized state, but signals from the partially reduced center allowed three nitrogenous ligands to be identified using ENDOR spectroscopy (Cline *et al.*, 1983). EXAFS spectra on Cu_A-depleted enzyme verify this result (P.M. Li *et al.*, 1987), although preliminary EXAFS results from cytochrome *bo*, which lacks the potentially interfering Cu_A, suggest two nitrogens and a sulfur as Cu_B ligands (Ingledew and Bacon, 1991), a possibility also raised by Scott *et al.* (1986). The sulfur could perhaps correspond to the bridging ligand mentioned above; however, there are no conserved cysteines in subunit I, the presumed binding site (see below), and methionine has therefore been suggested (Haltia and Wikström, 1992).

The locations of these liganding residues was limited to subunit I and II by the purification of active two-subunit oxidase from *Paracoccus denitrificans* (Ludwig and Schatz, 1980) which contained all four metals. Winter *et al.* (1980) initially placed heme *a* in subunit II with Cu_A based on analysis of electrophoretically-purified subunits; however, it is now generally accepted that both hemes and Cu_B reside in subunit I, while only Cu_A residues in subunit II (Saraste, 1990; Shapleigh *et al.*, 1992; Haltia and Wikström, 1992) since there are insufficient conserved histidines in subunit II to ligate both heme *a* and Cu_A. As oxidases from many sources were sequenced, predictions of transmembrane helices (by hydropathy plot) along with a shortening list of conserved residues provided tools to model builders. Wikström *et al.* (1985) observed a weak homology between regions

of subunit I and several hemoglobins and based a model of heme a_3 and Cu_B liganding on it. Holm *et al.* (1987) attempted to fit the ligands onto three helices (VI, VII, and X) with two conserved histidines each. However, this model needed to be updated because one heme *a* ligand was no longer found to be conserved. The Saraste (1990) model counted on only 7 invariant histidines and added helix II to replace the lost heme *a* ligand. Additionally, other ligands were exchanged to increase the Fe-Fe distance.

None of these models were based on experimental evidence, and shifting of residues in and out of predicted transmembrane helices made comparisons difficult. The recent work of Shapleigh *et al.* (1992) [see also Minagawa *et al.* (1992), and Lemieux *et al.* (1992)] predicts a model based on changes in spectroscopic properties of site-directed mutants of cytochrome aa_3-type oxidase from *Rhodobacter sphaeroides*, which is highly homologous to the mammalian enzyme (see 5.3.1.1 and Hosler *et al.*, 1992). The authors mutated each of the conserved histidines and purified the mutant oxidases for visible and Raman spectroscopic studies. All of the mutants except the His-411 (in the *R. sphaeroides* numbering system), which is out of the membrane in this model, were inactive, suggesting that His-411 is not a metal ligand. The visible 605 nm peak attributable to heme *a* was disrupted in His-102 (helix II, BH61) and His-421 (helix X, BH378) mutants, but not in the others, indicating that the former are the heme *a* ligands. The remaining ligands were assigned based on the features of the resonance Raman spectra and structural analysis. According to the model, heme a_3 is liganded by His-284 (helixVI, BH240), while the three Cu_B ligands are His-333, His-334 (helix VII, BH 290, 291) and His-419 (helix X, BH 376).

Haltia and Wikström (1992) use the same set of ligands as Shapleigh *et al.* (1992) but shift the positions of the helices slightly. The planes of the two hemes are perpendicular in this model, as opposed to being nearly coplanar in other models, accounting for the relatively slow electron transfer between them measured by Moody *et al.* (1991). Helix VIII has been added to their model (facing the Cu_B site) because it contains a large number of conserved residues that have hydrogen-bonding capacity and could be involved in protonation of O_2 reduction intermediates or proton pumping.

5.4 Function

5.4.1 Interaction with cytochrome *c*

The mobile one-electron carrier cytochrome *c* is reduced by cytochrome *c* reductase (bc_1) and then donates its electron to cytochrome oxidase. A region of lysines on one face of the globular cytochrome *c* molecule has been shown to interact with cytochrome oxidase (Ferguson-Miller *et al.*, 1976, 1978; Osheroff *et al.*, 1980) in an ionic-strength dependent manner (Ferguson-Miller *et al.*, 1976). The area of interaction has been localized to several carboxyls on the C-terminal domain of subunit II.

5.4.1.1 Cytochrome c binding site

When a cytochrome *c*–cytochrome oxidase complex formed at low ionic strength was covalently crosslinked, adducts of subunit II with cytochrome *c* were identified (Briggs and Capaldi, 1978). Subunit II was also implicated in cytochrome *c* binding by crosslinking with the photoaffinity label arylazido cytochrome *c* (Bisson *et al.*, 1978). Crosslinking of the oxidase with cytochrome *c* via arylazido-modified Lys-22 (not on the

front face) did not inhibit the oxidase, and cytochrome *c* was found to be associated only with cardiolipin (Bisson *et al.*, 1980). However, when cytochrome *c* was modified at Lys-13, on the front face of the molecule in the vicinity of the heme edge, it was crosslinked to subunit II and inhibited the activity of the oxidase at an apparent stoichiometry of one cytochrome *c* per oxidase dimer. Tryptic mapping localized the labelling site to near Asp-158, Val-159, and His-161 (Bisson *et al.*, 1982). These are conserved residues in the C-terminal domain of subunit II, which is thought to extend into the intermembrane space (see above). Asp-158 on subunit II can thus be inferred to be a natural site of interaction with Lys-13 of cytochrome *c*.

Cytochrome *c* modified at Cys-107, on the back face of the molecule, was crosslinked to cytchrome oxidase and specifically labelled a residue in subunit III of yeast (Birchmeier *et al.*, 1976) and bovine (Fuller *et al.*, 1981) oxidase, later identified as Cys-115 (Malatesta and Capaldi, 1982). Fuller *et al.* (1981) found that exogenous cytochrome *c* could not stimulate activity of this cross-linked form, but ascorbate and TMPD alone could, presumably through reduction of the cross-linked cytochrome *c*. Darley-Usmar *et al.* (1984) found that although cytochrome *c* was crosslinked to both monomers and dimers, only the dimers were inhibited, suggesting that when cytochrome *c* is bound correctly to the subunit II of one monomer in a dimer, its back face is in close proximity to subunit III of the other monomer in the dimer. In this position it would block access to additional cytochrome *c* binding, giving rise to an inactive species.

A different approach to localizing the cytochrome *c* binding site was taken by Millett *et al.* (1983). Modification of the oxidase carboxyls with the carbodiimide ETC inhibited the activity of the oxidase; however, the presence of cytochrome *c* protected Asp-112, Glu-114, and Glu-198. Glu-198,

along with Glu-109, Asp-158, and Asp-173 are in fact strictly conserved in all subunit II sequences except those of cytochrome *bo*, which does not bind cytochrome *c*. Moreover, Glu-198 falls between putative Cu_A ligands Cys-196 and Cys-200, which provides the close association of cytochrome *c* and Cu_A necessary for electron transfer. This data, together with the arylazido–*c* labelling near Asp-158 (Bisson *et al.*, 1982) provides a structural model for the binding of cytochrome *c* to the conserved (and some species-specific) carboxyl residues in the C-terminal domain of subunit II.

5.4.1.2 Cytochrome c kinetics

Cytochrome oxidase can be assayed spectrophotometrically by following the oxidation of added cytochrome *c* (Smith and Conrad, 1956) or by measuring oxygen consumption polarographically in the presence of cytochrome *c* and the artificial reductants ascorbate and N,N,N',N'-tetramethyl *p*-phenylene diamine (TMPD, Nicholls, 1965; Ferguson-Miller *et al.*, 1976). At low ionic strength and low concentrations of cytochrome *c*, maximal rates measured by the spectrophotometric method are lower than the polarographic method because cytochrome *c* must dissociate from the oxidase and is rate limiting (Ferguson-Miller *et al.*, 1978; Wilms *et al.*, 1981; Sinjorgo *et al.*, 1984). In contrast, TMPD can reduce bound cytochrome *c*, so this step could become rate limiting in the polarographic assay (Ferguson-Miller *et al.*, 1978). Therefore it has been argued that the spectrophotometric assay is more physiological (Hüther and Kadenbach, 1987; Sinjorgo *et al.*, 1987; however see Cooper, 1990).

The kinetics of the reaction have been studied using both assay methods; however, some unusual results occur whichever method is used. First, when measured spectrophotometrically the reaction is first order under most conditions, and the first order rate constant changes with the

concentration of cytochrome *c* (Smith and Conrad, 1956). Second, when measured by either method the reaction does not follow Michaelis-Menten kinetics. When the data are plotted on an Eadie-Hofstee plot, two phases are observed: a high affinity, low turnover phase, and a low affinity, high turnover phase (Ferguson-Miller *et al.*, 1976). It has been a major focus of study to explain these unusual results in terms of a mechanism. As yet, no hypothesis has satisfactorily explained all the data and gained universal acceptance.

The simplest explanation for the biphasic behavior is that there are two binding sites for cytochrome *c*. Based on gel filtration-determination of K_D values which matched K_m values, Ferguson-Miller *et al.* (1976) proposed two binding sites which can transfer electrons independently. In agreement with this is the experiment of Veerman *et al.* (1980), which showed that binding of porphyrin (no iron) cytochrome *c* to the high affinity site did not inhibit the oxidase from accepting electrons from normal cytochrome *c* (which presumably bound at a second site). Further evidence for a two independent-site model is the kinetic behavior of the *caa*$_3$-oxidases of several species of thermophilic bacteria. TMPD alone catalyzes high turnover, but added soluble cytochrome *c* produces monophasic, low affinity kinetics (Nicholls and Sone, 1984; Yoshida and Fee, 1984).

The finding of two binding sites does not, however, imply that they act independently in electron transfer. Cytochrome *c* at the second binding site could merely influence the activity of the first binding site (Errede *et al.*, 1976; Errede and Kamen, 1978). Arguing against two independent sites are the experiments of Bisson *et al.* (1980) and Fuller *et al.* (1981). Covalent crosslinking of cytochrome *c* to the high affinity site rendered the oxidase inactive in the presence of additional cytochrome *c* which was free to bind at

a second site and donate electrons. Furthermore, while ascorbate/TMPD was able to stimulate some activity of the crosslinked complex, only after ascorbate/TMPD was added *and* cytochrome *c* allowed to bind to the second site was the activity fully restored. This could suggest that binding of the second cytochrome *c* increases electron transfer activity at the first site by an allosteric mechanism.

The "controlled binding" hypothesis of Speck *et al.* (1984) also proposes that the second cytochrome *c* site is regulatory, causing the catalytic site to increase activity by increasing the dissociation constant. This suggestion is supported by the work of Wilms *et al.* (1981), who showed that the "off" rate of the high affinity site is increased by binding of polylysine, which has a high concentration of positive charges simulating cytochrome *c*, at the low affinity site.

Since only one binding site was found on the oxidase by crosslinking studies (see above), and since the cytochrome *c* molecule is so highly positively charged, it was postulated that the low affinity or second site is comprised of negatively charged phospholipid associated with the protein. Bisson *et al.* (1980) showed that arylazidocytochrome *c* modified on a side or rear face did crosslink to phospholipid and did not inhibit activity. Vik *et al.* (1981) also showed that lipid-depleted enzyme exhibited monophasic kinetics; however, the highly lipid-depleted oxidase preparation of Thompson *et al.* (1983) displayed normal biphasic kinetics. Moreover, when Marsh and Powell (1988) substituted neutral phospholipids for the normal charged ones, biphasic kinetics were still observed.

Many other theories with only one binding site have been proposed. By using the small perturbation in the spectrum of cytochrome *c* upon binding to the oxidase, Michel and Bosshard (1989) concluded that there

was only one binding site; however, their methods have been criticized (Cooper, 1990). Because Nalecz *et al.* (1983) observed that dimers but not monomers exhibited biphasic kinetics, they proposed that the cooperation of two dimers each with a bound cytochrome *c* was required. However, others (Suarez *et al.*, 1984; Robinson and Talbert, 1986; Hakvoort *et al.*, 1987; Michel and Bosshard, 1989) have shown monomers to have biphasic kinetics .

The "sequential binding" hypothesis of Antalis and Palmer (1982) explained biphasic kinetics by a one-site model in which two cytochrome *c* molecules bind sequentially to the oxidase at the same binding site with identical association and dissociation constants. The second molecule of cytochrome *c* cannot react until the first dissociates, leading to rate equations containing higher order terms. Simulations using these equations yielded results matching some of the experimental data. However such simulations are problematic since, as pointed out by Myers and Palmer (1988), a rate equation taking into account all relevant intermediate species would contain more than 21,000 terms!

Two one-site models for cytochrome *c* binding involve conformational changes to account for the kinetic behavior. Brzezinski and Malmström (1986) propose that there are two conformational forms of the oxidized oxidase which are linked to proton pumping: the "input" form picks up protons, and the "output" form releases protons. The different interaction of cytochrome *c* with these two forms would result in biphasic kinetics. Alternatively, the hysteresis model of Garber *et al.* (1990) suggests that a substrate-induced conformational change primes the enzyme for faster turnover. The enzyme relaxes back to the slow form after electron transfer, but at high concentrations of cytochrome *c*, more substrate can bind before

the relaxation is complete, thus the substrate reacts immediately with the high turnover form of the oxidase, leading to biphasic kinetics.

5.4.2 Electron transfer pathway in cytochrome oxidase

As described above, studies on the cytochrome *c* binding site of the oxidase placed the interacting acidic residues very close to the histidines and cysteines which make up the Cu_A binding site. Holm's model (1987) emphasized the spatial closeness of the two sites and predicted that Cu_A must therefore be the initial electron acceptor from cytochrome *c*, despite studies suggesting that cytochrome *a* was reduced first (Gibson *et al.*, 1965; Andréasson *et al.*, 1972; Halaka *et al.*, 1984; Blair *et al.*, 1985). Further supporting the idea that Cu_A is the site of electron entry into the oxidase is the observation that cytochrome *bo* uses ubiquinone rather than cytochrome *c* as an electron donor and has lost Cu_A while still retaining the other redox centers (Dueweke and Gennis, 1991).

Recently Holm's prediction has been proven correct by a number of methods: pulse radiolysis with artificial electron donors (Kobayashi *et al.*, 1989) and flash photolysis of heme a_3-CO (Hill, 1991) on the purified enzyme, and steady-state spectroscopic measurements of reduction levels of Cu_A and heme *a* on intact mitochondria (Morgan and Wikström, 1991). However, even though Cu_A may be the first electron acceptor, it may not be strictly required for electron transfer as shown by Cu_A depletion studies (Gelles and Chan, 1985; Pan *et al.*, 1991b). Electrons rapidly equilibrate between Cu_A and heme *a* (Morgan *et al.*, 1989), and are passed onto the binuclear center from heme *a* (Hill, 1991; Oliveburg and Malmström, 1991). Babcock and Wikström (1992) argue that Cu_B is most likely the initial acceptor in the binuclear center.

It has been suggested that internal electron transfer (from heme *a* to the binuclear center) is the rate-limiting step of the catalytic cycle under high substrate conditions (Wilson *et al.*, 1981), and in support of this idea, Sarti *et al.* (1988) have shown that the rate of heme a_3 reduction is comparable to the turnover number. The rate of internal electron transfer is proposed to be increased by a conformational change corresponding to the "closed" to "open" transition during the catalytic cycle (Brzezinski and Malmström, 1987; Thörnström *et al.*, 1988). Reduction of Cu_A (Copeland *et al.*, 1987), heme *a* (Wilson *et al.*, 1988), or both (Scholes and Malmström, 1986) induces the "closed" to "open" transition, which is marked by a dramatic increase in the rate of cyanide binding (Jones *et al.*, 1984; Jensen *et al.*, 1984). This proposed conformational change has been suggested to have a role in proton pumping (Scholes and Malmström, 1986; Brzezinski and Malmström, 1987; Copeland *et al.*, 1987; Thörnström *et al.*, 1988); however, a conformational change has not been directly measured (Wilson *et al.*, 1988; Ferreira-Rajabi and Hill, 1989) and has been questioned (Moody *et al.*, 1991).

5.4.3 O_2 reduction

The mechanism of O_2 reduction by the binuclear center has been elucidated by time-resolved spectroscopy of photolyzed reduced Fe–CO oxidase as well as partial reversal of the reaction cycle in mitochondria under high protonmotive force such that reaction intermediates accumulate. Current ideas regarding reaction intermediates are reviewed in Babcock and Wikström (1992), and a proposed reaction sequence is illustrated in Figure 5.2. The oxidized (**O**) form of the binuclear center (Fe^{3+} Cu^{2+}) is reduced to the **R** form (Fe^{2+} Cu^{+}) in two steps in the "electron

filling" phase. Molecular oxygen binds weakly to Fe^{2+} to form an oxy-adduct (**A**) and is "trapped" by electron transfer from both metals to produce a peroxy intermediate (**P**). In the "power stroke" phase, the addition of an electron and two protons cleaves the O–O bond yielding the ferryl (**F**) form with H_2O coordinated to Cu^{2+}. The last electron leaves both metals with bound hydroxide as shown by the pH-dependence of this step (Wikström, 1988). Two more protons fully reduce the oxygen atoms to water, regenerating the oxidized form.

Since the rate of O_2 reduction by the **R** form is much faster (100 μs) than the maximal turnover rate under steady-state conditions with artificial electron donors (500–1000 s^{-1}), the **O** state is most commonly observed in spectroscopic studies. Physiological rates are even lower, only about 50 s^{-1}, since substrate is often limiting. Haltia and Wikström (1992) point out that these observations indicate that the "electron filling" phase, not O_2 reduction, is the rate-limiting step of the catalytic cycle.

5.4.4 Proton pumping

In Mitchell's chemiosmostic theory (1966), mitochondrial e^- transfer was proposed to be linked to proton translocation for the purpose of energy conservation. Wikström first observed proton pumping by cytochrome oxidase in 1977 although acceptance of the observation was slow (Moyle and Mitchell, 1978; Mitchell *et al.*, 1985). Despite some suggestions of a redox loop mechanism similar to the Q cycle in cytochrome *c* reductase (Mitchell, 1987), most researchers now agree that generation of an electrochemical gradient is the sum of the electrogenic transfer of electrons from

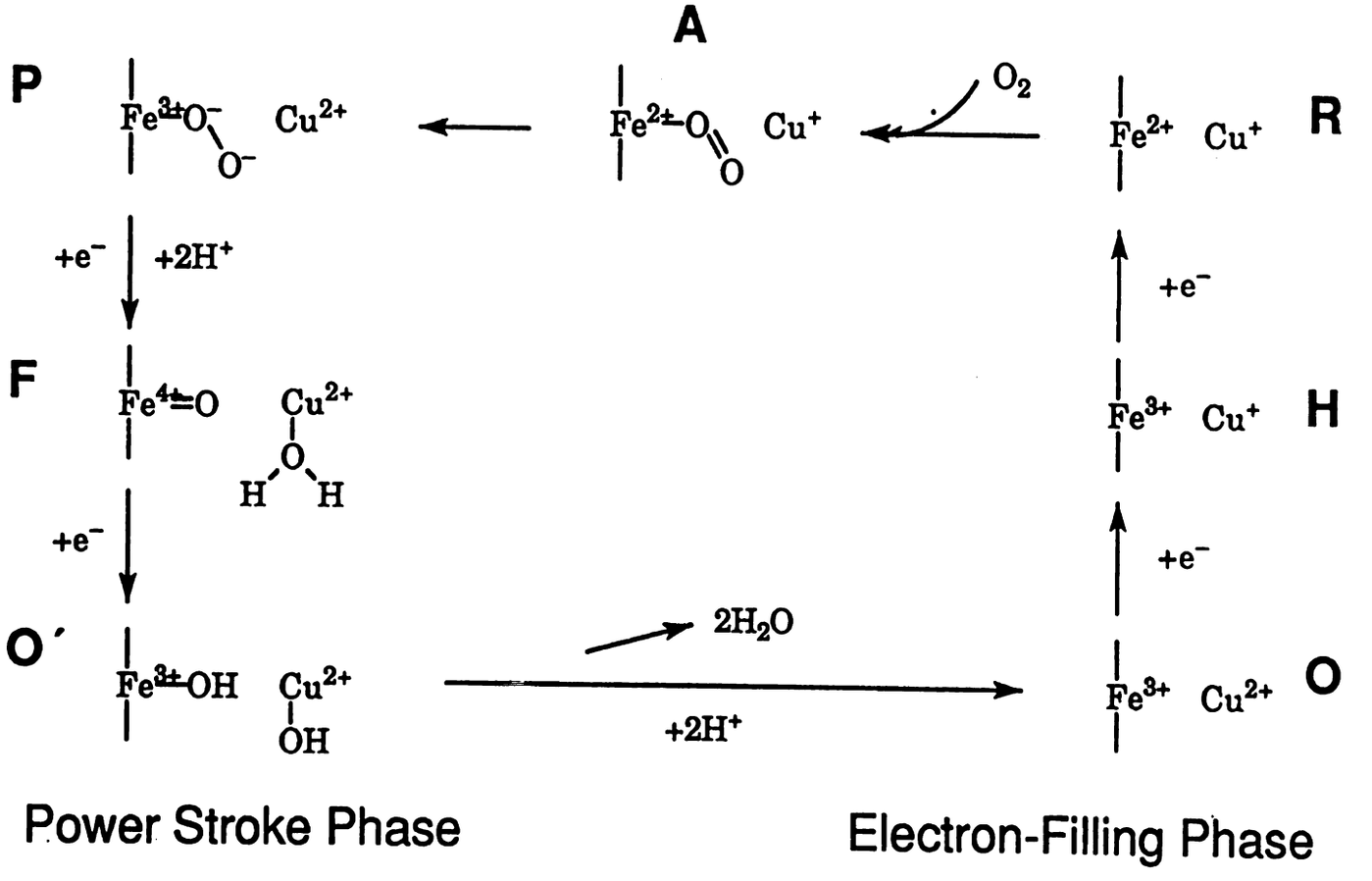


Figure 5.2: Proposed reaction sequence of O_2 reduction by the binuclear center of cytochrome *c* oxidase.

cytochrome *c* on the cytoplasmic side to the binuclear center inside the membrane, scalar consumption of protons taken up from the matrix for O_2 reduction (Wikström, 1988), and vectorial translocation of protons across the membrane (proton pumping). The coupling of proton pumping to electron transfer may be direct or indirect. In a direct mechanism, a redox-dependent protonation/deprotonation of the metal ligands themselves would occur (proton pumping by bacteriorhodopsin may be categorized as direct), while the coupling is mediated by long-range conformational changes in the indirect mechanism. (The Bohr effect of hemoglobin is an indirect mechanism.) Several models for a direct mechanism have been proposed.

Babcock and Callahan (1983) suggested that heme *a* provides a link between electron transfer and proton pumping. By comparing the spectral properties of heme model compounds to cytochrome *a* of the oxidase, they demonstrated that the formyl group of heme *a* probably hydrogen bonds to an –OH group. Resonance Raman spectroscopy showed that the strength of this bond is redox-dependent, providing a possible source of energy for coupling proton pumping to electron transfer.

Cu_A was suggested to be the coupling site by Gelles *et al.*(1986) based on experiments showing that modification of the Cu_A site by treatment with *p*-(hydroxymercuri)benzoate (Nilsson, *et al.*, 1988) and heat (P.M. Li *et al.*, 1988) eliminated proton pumping. They proposed that geometrical strain would cause a cysteine sulfur to exchange for a tyrosine during the redox cycle, with H^+ moving from one residue to the other in a vectorial fashion. Electron and proton "gating" (prevention of "slippage" or "short-circuiting") is imposed by conformational constraints.

Recently, Wikström (1989) showed that the the four electrons which reduce O_2 are not equally coupled to proton pumping. The **P→F** and **F→O** steps (Fig. 5.2) are each coupled to translocation of 2 H^+/e^-. This data has forced reconsideration of the above theories which have assumed that each electron is equally coupled to a proton. In order for heme *a* or Cu_A to be the coupling site, the assumption must be made that the path of electron transfer is different for the "electron filling" and "power stroke" electrons. Chan and Li (1990) suggest that the first two electrons are transferred from heme *a* to the binuclear center (without passing through Cu_A) and the second two are transferred through Cu_A, which is linked to the proton pump. If this mechanism is correct, both heme *a* and Cu_A should always be less than 50% reduced under steady state conditions, which has been shown (Moroney *et al.*, 1984; Gregory and Ferguson-Miller, 1989). Alternatively, Wikström (1989) points out that negative redox cooperativity between heme *a* and the binuclear center (Goodman, 1984; Blair *et al.*, 1986) could alter the configuration around heme *a* when O_2 is bound, and allow protons to be pumped only then.

Because the cytochrome *bo* complex, which has been shown to pump protons (Puustinen *et al.*, 1989), contains no Cu_A site (Chepuri and Gennis, 1990; Puustinen *et al.*, 1991), and neither of its hemes (B and O-types) have a formyl on the porphyrin ring (Puustinen and Wikström, 1991), it seems unlikely that the coupling site is related to these two centers. Babcock and Wikström (1992) conclude that Cu_B must therefore serve as the coupling site, eliminating heme *a* or the O_2 chemistry itself because the O_2 intermediates are so different between the **P** (peroxy) and **F** (ferryl) states. Since all four electrons must still go through the binuclear center, Haltia and Wikström (1992) suggest that conformational changes in the binuclear

center induced by reduction (Scott, 1989) allow only the O_2-bound form to pump protons, or that Cu_B only accepts e^- from heme *a* in the O_2-bound form. They propose that redox-linked geometrical changes in Cu_B allow two of the histidine ligands to protonate and deprotonate, and that a couple of conserved threonines (more than one must be involved since two protons are pumped in one step) in helix VIII form transient hydrogen bonds with the histidines as the first step of a proton ladder. Current site-directed mutagenesis work on subunit I residues should prove valuable in evaluating these theories.

5.4.5 The role of the nuclear-encoded subunits

Since the catalytic core is agreed to be in the mitochondrially-encoded subunits, the function of the nuclear-encoded subunits has been open to debate. Regulation of enzyme activity and assembly of the complex are the two most likely possibilities.

5.4.5.1 Regulation

As pointed out by Capaldi (1990b), regulation of the oxidase could occur at many steps: cytochrome *c* binding, O_2 binding, internal electron transfer, and proton pumping. The activity of the oxidase could be modulated at these steps by the presence of the nuclear-encoded subunits which do not exist in bacteria. Long-term control can be provided by subunits which assist in binding substrate or cofactors, or by the substitution of different subunits in different situations (isozymes). Short-term control can be exerted by allosteric effectors.

Binding of effectors. A number of effectors have been shown to alter the kinetic properties of the oxidase, including fatty acids (Thiel and Kadenbach, 1989), nucleotides (Ferguson-Miller *et al.*, 1976; Huther and

Kadenbach, 1986), and anions such as carbonate (Osheroff et al, 1980), citrate (Ferguson-Miller *et al.*, 1978), and phosphate (Ferguson-Miller *et al.*, 1978; Buge and Kadenbach, 1986; Malatesta *et al.*, 1987).

ATP has been shown to affect the kinetic properties of the enzyme (Ferguson-Miller *et al.*, 1976), abolishing the high affinity K_m and linearizing Eadie-Hofstee plots. The bacterial enzyme, however, is insensitive to intraliposomal nucleotides (Hüther and Kadenbach, 1988), suggesting that the effect may also be mediated by nuclear-encoded subunits. In fact, photoaffinity labelling studies with 8-arylazido ATP showed that the nuclear-encoded subunits IV and VIII bound ATP (Montecucco *et al.*, 1986; Bisson *et al.*, 1986), although Reimann *et al.* (1988) were unable to reproduce this result.

Binding of substrate and metals. Some studies suggest that the nuclear subunits form part of the binding site for cytochrome *c* along with subunit II and perhaps subunit III. When water-soluble carbodiimides were reacted with the oxidase, cytochrome *c* protected several of the nuclear-encoded subunits (Bisson and Montecucco, 1982; Kadenbach and Stroh, 1984; Taha and Ferguson-Miller, 1992). Since these subunits do not exist in the bacterial enzymes, it may be hypothesized that they are not strictly necessary for cytochrome *c* binding and may merely modulate the binding characteristics. However, this interpretation has been called into question (Capaldi, 1990b) since ATP protects the same set of subunits from carbodiimide labelling as does cytochrome *c* (Bisson *et al.*, 1987), suggesting that the protection is due to cytochrome *c*- and ATP-induced conformational changes. As mentioned above (section 5.3.1.2), a conserved cysteine on subunit VIa has been suggested to bind the zinc found in metal analyses (Naqui *et al.*, 1988). However, no role for this metal has been demonstrated.

Isozymes. The existence of isozymes of cytochrome oxidase strongly suggests that the nuclear-encoded subunits have a regulatory role. In mammals, subunits VIa, VIIa, and VIII have been shown to have tissue-specific forms based on differences in polyacrylamide gels (Merle and Kadenbach, 1980), immunological properties (Jarausch and Kadenbach, 1982; Kuhn-Nentwig and Kadenbach, 1985) and N-terminal amino acid sequences (Kadenbach *et al.*, 1982; Yanamura *et al.*, 1988; Kadenbach *et al.*, 1990). In different tissues of bovine oxidase, subunit VI can appear in either the liver form (l) or the heart form (h). In rat oxidase, however, heart contains both VI-h and VI-l forms, while the liver containes only VI-l (Schlerf *et al.*, 1988; Kadenbach *et al.*, 1990), demonstrating that the expression of isozymes varies between species. In fact, N-terminal protein sequences reveal that the rat brown fat oxidase contains the liver form of subunit VIa but the heart form of subunit VIII (Kadenbach *et al.*, 1990), suggesting that the oxidase from different tissues can "mix and match" isosubunits to suit their particular needs. A similar situation occurs in mouse, where the thymus oxidase contains liver-type VIa but heart-type VIII. In this case, the two forms of VIa are distinguished by their different staining intensities and not different electrophoretic mobilities (Van den Bogert *et al.*, 1992). A tissue-specific form of the oxidase in plants is suggested by the different reactivitites of oxidases from mung bean roots and hypocotyls to a subunit-specific antibody (Nakagawa *et al.*, 1988).

The sequence conservation of isozymes between different species is very strong. In fact, the homology is often greater between isozyme sequences from the same tissue of different species than between isozymes from different tissues in the same species. For instance, there is 50% homology between rat heart and liver subunit VIa isozymes, but 74%

homology between rat and bovine heart VIa isozymes (Schlerf *et al.*, 1988). The liver form contains five more negative charges than the heart form, perhaps modulating the electrostatic interactions with cytochrome *c*. This may explain the different kinetics observed for the two isozymes asssayed in intact or deoxycholate-dissolved membranes (Merle and Kadenbach, 1982) or reconstituted oxidase (Büge and Kadenbach, 1986) using the polarographic assay, although Sinjorgo *et al.* (1987) found no differences using the spectral assay. The effects of ATP and ADP on the kinetics of these two oxidases were also found to be different (Kadenbach *et al.*, 1991).

Environmental conditions also affect expression of the nuclear subunits. In yeast, subunit Va is expressed at high levels but Vb at low levels under normal aerobic conditions. A null mutant of Va results in a functional oxidase, but at only 10% of the normal levels due to lower expression relative to the normal Va (Poyton *et al.*, 1988). When O_2 or heme becomes limiting, Vb is expressed at higher levels while Va decreases. These two genes share a 66% homology (Cumsky *et al.*, 1987).

The slime mold *Dictyostelium discoideum* produces subunit VIIe during the exponential phase of growth and the unrelated VIIs during stationary phase (Bisson and Schiavo, 1986), thus suggesting that subunit expression can be controlled developmentally. This differential expression, like the yeast subunit V, is apparently controlled by O_2 levels (Bisson and Schiavo, 1988; Schiavo and Bisson, 1989).

5.4.5.2 Assembly of the complex

Null mutants of each of the nuclear subunits of yeast were created to study their effect on the assembly and function of the oxidase complex. Mutants in all subunits except VIII resulted in no detectable cytochromes aa_3 and no oxidase activity. The null mutant in subunit VIII reduced aa_3

and respiration to 80% of wild type (Patterson *et al.*, 1986), indicating that it is not absolutely necessary for assembly of the enzyme and may be the last subunit added. It is not clear what role the nuclear subunits have in proper assembly of the oxidase since bacterial oxidases assemble without them. The nuclear-encoded subunits may provide a "scaffolding" for assembly, assist in the folding of subunits, or regulate synthesis of the larger mitochondrial subunits.

5.5 Plant cytochrome oxidase

Interest in the cytochrome oxidase from plants increased after cytoplasmic male sterility (CMS, see chapter 2) was suggested to involve the enzyme. The subunit I gene from a CMS line of sorghum was shown to possess a unique C-terminal extension and different genomic location relative to a fertile line of sorghum (Bailey-Serres *et al.*, 1986) as a result of mitochondrial DNA rearrangements. Rearrangement of COX II in petunia (Young and Hanson, 1987) also correlates with CMS. Moreover, partially purified fractions of cytochrome oxidase from maize (R. Ingle, personal communication) as well as immunoprecipitates of the oxidase (C. Leaver, personal communication) appeared to contain the novel polypeptides produced as a result of these DNA rearrangements.

Further interest has been sparked by the identification of wheat as the only organism whose DNA does not encode a cysteine at position 196 (beef heart numbering), the putitive Cu_A ligand (Bonen *et al.*, 1984). RNA sequencing later showed that C→U editing of the message occurred, restoring the conserved cysteine (Covello and Gray, 1989). However, although extensive RNA editing appears to take place in the mitochondria

of plants (see section 1.4.4), only one amino acid sequence has been obtained from a plant protein that is produced from an edited message, that of the ATPase subunit 9 (Graves *et al.*, 1990).

Despite these motivations, cytochrome oxidase from plants, as well as many other plant proteins, have not been as well studied as those from mammalian, yeast, or bacteria for many reasons. A major problem is that the plant cytoplasm contains chloroplasts, and standard methods for preparing mitochondria from other sources fail to separate the two organelles because of their similar densities. To overcome this problem tissues containing undeveloped chloroplasts are often used, for example etiolated shoots (most plants, but especially grasses such as maize and wheat), roots or tubers (potato), fruit (tomato), seed ears (maize), or cell suspension culture. A second problem is that these sources yield low amounts of mitochondria. A third problem is that the presence of phenolic compounds used for cell wall synthesis tend to polymerize and bind to proteins, interfering with purification.

5.5.1 Purification and characterization

The first attempts to purify cytochrome oxidase from plant sources occurred in the 1940's. Goddard (1944) isolated a fraction by acetone-benzol extraction of wheat germ; however, many of the modern tools for analysis of purity and integrity were unavailable then. The activity, as measured by O_2 consumption, was low, and no UV-vis spectra were presented. Spectral properties of the oxidase and other plant cytochromes were later investigated in intact mitochondria, showing the plant oxidase to have unique spectral properties relative to other eukaryotes (Chance *et al.*, 1968). Specifically, the α band in the optical spectra of the reduced enzyme is blue-

shifted from 605 to 602 nm, a result confirmed later by several researchers on their purified fractions.

More recently, cytochrome oxidase has been purified from a number of plant sources. However, the various methods employed result in oxidases having widely ranging catalytic activities, and apparent subunit compositions different from other eukaryotic oxidases as well as from each other. The oxidase purified from sweet potato root tissue (Maeshima and Asahi, 1978) and etiolated pea seedling (Matsuoka *et al.*, 1981) by a selective solubilization of mitochondrial membranes with deoxycholate followed by DEAE chromatography in Triton X-100 and ammonium sulfate precipitation appears to be extremely labile; the purified enzyme had minimal catalytic activity. SDS-polyacrylamide gels suggest that plant oxidases have a simpler and somewhat different subunit composition than oxidases from mammals or yeast. Low-resolution gels identified only five subunits with molecular weights: I- 39, II- 33.5, III- 26, IV- 20, V-5.7 kDa (Maeshima and Asahi, 1978). Subunit V was later resolved into Va, b, and c with molecular weights 7.4, 6.8, and 6.2 kDa by using a higher resolution gel system (Nakagawa *et al.*, 1987). N-terminal amino acid sequencing of Vc revealed no homology to subunits in yeast or mammals. Polyclonal antibodies prepared against the purified enzyme immunoprecipitated additional polypeptides of 13 and 10 kDa (Matsuoka *et al.*, 1981).

In contrast, maize oxidase retains high catalytic activity when it is purified in lauryl maltoside by hyroxyapatite (Dutch *et al.*, 1987) or by anion-exchange FPLC followed by gel filtration (Hawkesford *et al.*, 1989). The subunit composition of the maize oxidase purified by FPLC was similar to the sweet potato oxidase except that the predicted molecular weights were different as a result of a different gel system. The largest four bands

assigned to be subunits approximately comigrated with those of the yeast oxidase; however, the additional gel bands in the maize preparation (including one near 10 kDa which was not a component of subunit V of Nakagawa *et al.* (1987)) were dissimilar to those of the yeast. Subunit-specific antibodies to yeast subunits I and II but not IV cross-reacted, suggesting that the plant and yeast nuclear-encoded subunits might not be related.

5.5.2 Proton pumping by cytochrome oxidase

Intact plant mitochondria are capable of ADP/O ratios near 3.0 (Dutch *et al.*, 1987), similar to mammalian values, suggesting that complexes I, III, and IV are all energy-conserving sites. Many studies on proton pumping using the O_2 pulse technique have analyzed large spans of the respiratory chain at once, often succinate to O_2 (deVirille and Moreau, 1984; Moreau and deVirville, 1985), confirming that the cytochrome pathway but not the alternative pathway pumps protons (Ishida and Palmer, 1988). deVrille and Moreau (1990) used ascorbate and TMPD as electron donors in O_2 pulse assays of antimycin-inhibited potato mitochondria to examine cytochrome oxidase directly, demonstrating H^+/e^- ratios of 1.0. No measurements of proton pumping by the purified cytochrome oxidase reconstituted into lipid vesicles has yet been reported.

5.5.3 Genes

Gene sequences have verified the similarity of the mitochondrially-encoded subunits in plants with those of yeast and mammals. The predicted amino acid sequence homologies between maize and yeast are 58% for subunit I (Isaac *et al.*, 1985) and 47% for subunit II (Fox and

Leaver, 1981), while the nucleotide sequence homology is 58% between maize and bovine subunit III genes (McCarty *et al.*, 1988). Among plant species, nucleotide sequence homologies are very high: subunit II genes are 99% homologous within monocots and 89% within dicots (Turano *et al.*, 1987). These values do not take into account RNA editing, which changes up to 5% of the amino acid residues in some genes (Covello and Gray, 1990). The subunit II gene of wheat was predicted to code for several radical amino acid substitutions, including an arginine for the conserved cysteine at position 228 (beef heart 196), which has been proposed to be a ligand of Cu_A (Bonen *et al.*, 1984). However, RNA editing "corrects" this anomaly, allowing, as in many other cases, the conserved residue to be appear in the sequence (Covello and Gray, 1989; Covello and Gray, 1990).

No genes for nuclear-encoded subunits of cytochrome oxidase have been isolated from plants. However, Nakagawa *et al.* (1990) have cloned a cDNA for subunit Vc from the sweet potato oxidase. This sequence shows regions of homology with beef heart oxidase subunit VIII and yeast oxidase subunit VIIa. Interestingly, the beef heart oxidase subunit VIII is expressed in a tissue-specific manner, while the plant Vc has been suggested to be involved in tissue-specific changes in the immunoreactivity of the plant oxidase (Nakagawa *et al.*, 1990). In the absence of further sequence information, it is not possible to say whether other nuclear subunits from plants share homology with their mammalian or yeast counterparts, but it seems likely. The homology of Vc to subunits from other oxidases stresses the importance of the nuclear subunits in oxidase function. The study of plant oxidase, in addition to answering basic questions on structure-function relationships, should provide a unique

opportunity to investigate the role of the non-catalytic subunits of the complex.

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ASSESSMENT OF THE ROLE OF PLANT MITOCHONDRIAL RESPIRATORY COMPLEXES IN CYTOPLASMIC MALE STERILITY AND STRUCTURAL ANALYSIS OF PLANT CYTOCHROME *C* OXIDASE

Volume II

By

Wendy Eileen Peiffer

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1992

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Chapter 6 – Purification and characterization of cytochrome *c* oxidase from wheat

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6.1 Introduction

The possibility that cytochrome oxidase plays a role in the cytoplasmic male sterility trait (see chapter 2) has generated increased interest in the enzyme from plants. We chose to purify the oxidase from wheat in particular because the DNA sequence of wheat subunit II predicts that an arginine replaces a conserved cysteine (228) at the putitive Cu_A site (Bonen *et al.*, 1984), implying that the liganding and functional properties of Cu_A might be altered in wheat cytochrome oxidase. Recently, however, RNA sequencing has shown that editing of the mRNA restores the cysteine (Covello and Gray, 1989). This has yet to be confirmed by direct protein sequencing. Studies on the plant oxidases may also provide new insight into the roles of the nuclear-encoded subunits, which in plants appear to differ from those of eukaryotic oxidases.

The plant cytochrome *c* oxidases studied thus far, from sweet potato (Maeshima and Asahi, 1978; Nakagawa *et al.*, 1987), pea (Matsuoka *et al.*, 1981), and maize (Dutch *et al.*, 1987; Hawkesford *et al.*, 1989), have an apparent subunit composition not only different from other eukaryotic oxidases, but different from each other. Moreover, electron transfer activity differs significantly between preparations. The preparations of Dutch *et al.* (1987) and Hawkesford *et al.* (1989) retained high activity by using the nonionic detergent lauryl maltoside, which has been shown to stimulate the activity of other eukaryotic oxidases (Rosevear *et al.*, 1980). The method of purification is well known to affect both the apparent subunit composition and activity. With multisubunit complexes, there is always a concern that

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preparations subjected to stringent purification conditions may lose both activity and subunits, while less rigorous purification may result in higher activity but contaminating proteins. Therefore, a goal of our preparation is to retain activity while achieving high enough purity to establish subunit composition.

The functional significance of the unusual subunit and spectral characteristics of plant cytochrome oxidases has not been established, in part due to the difficulty in obtaining enough plant mitochondria to purify a reasonably large quantity of the oxidase. Etiolated seedlings are frequently used as a starting material to reduce contamination of the mitochondrial fraction with chloroplasts, but this approach is time-consuming and low-yield. The use of wheat germ as an alternative source of plant material provides a substantial advantage in time and yield. Although it has been reported that unimbibed plant embryos do not contain recoverable intact mitochondria and have low amounts of mitochondrial protein (Sato and Asahi, 1975), we have isolated a membrane fraction from commercial wheat germ that has a protein complement and cytochrome oxidase specific activity similar to etiolated wheat seedling mitochondria. Using this convenient and rich source, we have succeeded in purifying by anion-exchange chromatography in lauryl maltoside a highly active cytochrome oxidase which is well suited for structural and functional analysis.

6.2 Materials and methods

6.2.1 Materials

Wheat germ was purchased from Star of the West Milling Company in Frankenmuth, Michigan, and King Milling Company in Lowell,

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Michigan. Wheat seed (*Triticum aestivus* var. Titan Red) was obtained from Michigan Foundation Seed Company. Maize seed (*Zea mays* variety GL466) was purchased from Great Lakes Hybrids, Inc. Lauryl ß-D-maltoside was synthesized according to Rosevear *et al.* (1980). Cytochrome *c* Sigma Type VI was further purified according to Thompson and Ferguson-Miller (1983). Asolectin vesicles were prepared by sonicating 40 mg/mL asolectin (Associated Concentrates, Long Island, NY) for 1 min/mL in 2% cholate and 75 m*M* K-HEPES (4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid), pH 7.4. Other chemicals were obtained from the sources indicated: BCA Protein Assay Reagent (Pierce Chemical Company), bovine serum albumin (BSA, Sigma fraction V), ultra-pure sucrose (ICN), E-64 (Boehringer-Mannheim), acrylamide and N,N'-methylene bisacrylamide (Serva), insoluble polyvinylpyrrolidone (PVP, Serva Polyclar AT), carboxymethylcellulose resin (Whatman), and goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate (Western blotting grade affinity purified, Bio-Rad Laboratories).

6.2.2 Preparation of wheat and maize seedling mitochondria

All operations were carried out at 0–4°C using pre-chilled detergent-free glassware. Intact wheat mitochondria for use in respiratory control studies were prepared by the method of Day and Hanson (1977) from etiolated seedlings grown in vermiculite for five days at 25°C and 80% relative humidity.

To prepare mitochondrial membranes for cytochrome oxidase isolation, wheat seeds were soaked in aerated water overnight, spread between two sheets of stiff plastic mesh, and suspended over distilled, aerated water. Soaked maize seeds were planted in vermiculite. Seedlings

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were grown in the dark at 25°C and 80% relative humidity for five days and were harvested using electric grass shears. The cut seedlings were soaked in cold (4°C) distilled water for an hour then drained before grinding 10 seconds at the highest speed in a commercial Waring blender (Model 34BL22) with 0.4 *M* mannitol, 50 m*M* KH_2PO_4, 5 m*M* EGTA, 0.2% BSA, pH 7.7, and PVP (0.1 g/g seedlings) at approximately 1 mL buffer per gram seedlings. The homogenate was filtered through four layers of cheesecloth and centrifuged at 500*g* for 15 minutes. The supernatant was recentrifuged at 27000*g* for 20 minutes to pellet the mitochondria, which were resuspended using a Potter-Elvehjem type tissue grinder (clearance 0.2 mm) in 0.4 *M* mannitol, 5 mM MOPS (3-[N-morpholino]propanesulfonic acid), 0.1% BSA, pH 7.2 and centrifuged at 40000*g* for 20 minutes. The pellet, resuspended in a small volume of 0.35 *M* mannitol, 5 m*M* MOPS , 50 m*M* KCl, and 1 m*M* EGTA, pH 7.2, was layered onto a 0.6 *M* sucrose cushion and centrifuged at 40000*g* for 20 minutes. In this step the mitochondria are pelleted, while the fluffy yellow contaminants remain in suspension and are easily poured off. The mitochondria were next depleted of cytochrome *c* by resuspending the pellet in a ten-fold volume of distilled water for one minute (to burst the outer membrane) before adding KCl to a final concentration of 0.15 *M*. This mixture was centrifuged at 40000*g* for 20 minutes and the mitochondrial pellet resuspended in the previous mannitol buffer and stored at -80°C. Cytochrome *c* could be recovered from the diluted supernatant by incubation with carboxymethylcellulose resin.

6.2.3 Preparation of wheat germ mitochondrial membranes

Wheat germ (unimbibed) was homogenized for fifteen seconds in a chilled (4°C or less) buffer (0.4 *M* sucrose or mannitol, 50 m*M* KH_2PO_4,

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5 m*M* EGTA, 0.2% BSA, pH 7.0 ± 2 m*M* phenylmethylsulfonyl fluoride (PMSF), 1 m*M* EDTA, and 10 μ*M* E64) using a food processor (Black and Decker model FP1D) at a ratio of 150 g wheat germ and 10 g PVP per liter buffer. The homogenate was centrifuged at 13000*g* for 15 minutes, and the supernatant filtered through 35 μm nylon mesh to remove congealed fat. The filtrate was then centrifuged at 28000*g* for 40 minutes. The supernatant contained the cytochrome *c*, which could be recovered by incubation with carboxymethylcellulose resin and subsequent chromatography. The loose mitochondrial pellet was resuspended in buffer (0.4 *M* sucrose or mannitol, 5 m*M* TES, 0.1% BSA, pH 7.2, ± 2 m*M* PMSF and 1 m*M* EDTA) using a Potter-Elvehjem type tissue grinder (clearance 0.2 mm) and then centrifuged at 3000*g* for 15 minutes to remove large debris. The resulting supernatant was filtered through 35 μm nylon mesh to remove congealed fat before centrifuging at 48000*g* for 20 minutes. The final mitochondrial pellet was resuspended in buffer (0.35 *M* sucrose or mannitol, 50 m*M* KCl, 5 m*M* TES, 1 m*M* EGTA, pH 7.2) and stored at -80°C.

6.2.4 Isolation of cytochrome oxidase from mitochondrial membranes

Mitochondrial membranes were incubated on ice for 30 minutes with 2 mg lauryl maltoside per mg mitochondrial protein, 10 μg DNase per mg protein and 1 m*M* $MgCl_2$. In some preparations, 2 m*M* PMSF and 10 μ*M* E-64 were added to the final suspension buffer. (Proteolytic inhibitors were normally used throughout in the wheat germ but not in the wheat or maize seedling preparations.) The mixture was centrifuged at 40000*g* for 20 minutes. The supernatant was filtered through a 0.45 μm membrane and loaded onto a Pharmacia MonoQ 10/10 anion exchange column on the Pharmacia FPLC system at 0.15 *M* KCl. Typically 900 mg of starting

mitochondrial protein were loaded onto the 10/10 column. A discontinuous gradient (described in the legend of Figure 6.3) of 0 to 1.0 *M* salt in buffer (FPLC buffer: 5% sucrose, 20 m*M* KH_2PO_4, 4 m*M* lauryl maltoside, pH 7.5) was applied to the column at 1 mL/min. The salt concentration was held constant at 0.32 and 0.39 *M* KCl, just before and just after the oxidase elutes at 0.35 *M* KCl. This separates the oxidase from both the large preceding peak and the following peak, cytochrome *c* reductase, eluting at 0.42 *M* KCl. The oxidase was concentrated by reloading the enzyme diluted ten fold onto a MonoQ 5/5 column, washing at 0.3 *M* KCl to remove contaminants and inactive enzyme. The column was then inverted, and the oxidase eluted with 0.5 *M* KCl in FPLC buffer at a very slow flow rate. The enzyme could then be further purified by running concentrated oxidase on a Superose 6 gel filtration column (equilibrated to 0.1 *M* KCl in FPLC buffer) at a flow rate of 0.2 mL/min.

Cytochrome oxidase was purified from maize mitochondria by a similar procedure except that a continuous (0 to 1 *M* KCl in FPLC buffer) gradient was used in the elution, no proteolytic inhibitors were used, and the fractions were sometimes frozen and thawed between purification steps.

6.2.5 Assay methods

The electron transfer activity of purified cytochrome oxidase was measured polarographically with a Gilson model 5\6H oxygraph at 25°C as described by Ferguson-Miller (1975) except that asolectin vesicles (1.1 mg/mL) and 0.1% lauryl maltoside were added to each assay mixture. Assays for maximal activity were performed in 50 m*M* KH_2PO_4, pH 6.5. Turnover numbers were calculated as described in Thompson and

Ferguson-Miller (1983). Cytochrome *c* kinetics were assayed in either 25 m*M* Tris-cacodylate, pH 7.9 or 50 m*M* KH_2PO_4, pH 6.5. Kinetics of the oxidase in whole mitochondrial membranes were measured without lauryl maltoside, while kinetics of detergent-solubilized membranes and purified oxidase were assayed with added asolectin vesicles (1.1 mg/mL) and 0.1% lauryl maltoside. Mitochondrial membranes were solubilized with either lauryl maltoside at 2 mg detergent per mg protein as described in 6.2.4 or with 3.5 mg Triton X-100 per mg protein and centrifuged at 40,000*g* for 15 minutes. The kinetic analysis of the oxidase using wheat cytochrome *c* was performed with whole membranes but with 0.1% added lauryl maltoside. Protein concentrations were determined by the bicinchoninic acid (BCA) method (Smith *et al.*, 1985).

6.2.6 Spectral methods

UV and visible spectra were recorded on a Perkin-Elmer Lambda 4B spectrophotometer. The extinction coefficients for absolute spectra of cytochrome aa_3 (dithionite reduced or air oxidized) were taken from the spectra published by van Buuren *et al.* (1972): cytochrome aa_3 (reduced), (605-650 nm) = 40 $mM^{-1}cm^{-1}$ and (440-490 nm) = 204 $mM^{-1}cm^{-1}$; cytochrome aa_3 (oxidized), (420-490 nm) = 140 $mM^{-1}cm^{-1}$.

6.2.7 SDS-PAGE of purified cytochrome oxidase

Subunit composition of the oxidase fractions was evaluated by SDS-polyacrylamide gel electrophoresis (Bio-Rad Protean II, 200 V for 2 hours in chilled buffer) using the procedure of Kadenbach *et al.* (1983) except that 0.04% bromophenol blue was added to the sample buffer. Protein samples

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were precipitated with 10% trichloroacetic acid (final concentration 4.5%) before resuspension in the sample buffer, and sonicated for 30 minutes at 15–20°C. Protein bands were stained overnight with Coomassie brilliant blue.

6.3 Results

6.3.1 Mitochondria from etiolated wheat seedlings and wheat germ

Mitochondria prepared by the method of Day and Hanson (1977) from etiolated wheat seedlings had respiratory control ratios ranging from 5.0–7.0, similar to values obtained for maize mitochondria prepared by the same method (Figure 6.1). These values deteriorated after an hour, faster than maize mitochondria. ADP/O ratios were 2.7-2.9 using glutamate/malate as substrate, similar to those reported for maize mitochondria (Dutch *et al.*, 1987). In contrast, the respiration rates of mitochondrial membrane fractions from wheat germ were not stimulated by ADP, and exogenously supplied cytochrome *c* had complete access to cytochrome oxidase, indicating that the mitochondria were not intact.

Mitochondrial membrane fractions isolated from etiolated wheat seedlings and from wheat germ appear to be very similar with respect to cytochrome content. The difference spectra (reduced minus oxidized) in Figure 6.2 show that the two sources of mitochondria yield similar amounts and ratios of cytochromes (0.12 and 0.19 nmol aa_3 per mg mitochondrial protein for wheat germ and seedlings, respectively). The cytochrome oxidase specific activity of seedling mitochondria was usually higher (0.11 nmol O_2 consumed per second per mg protein), but only by about 10%. An extra re-pelleting step increased the specific activity to equal the

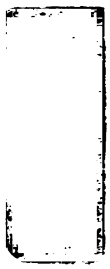
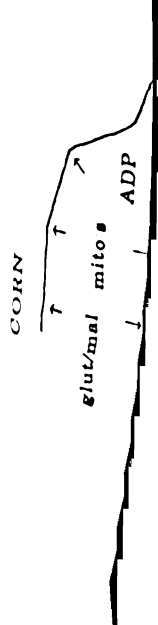


Figure 6.1: Polarographic measurement of respiratory control ratio of maize and wheat seedling mitochondria. Maize (upper) or wheat seedling (lower) mitochondria (0.3 mg protein) were assayed with glutamate/malate as substrate as described in the legend of Figure 3.1.



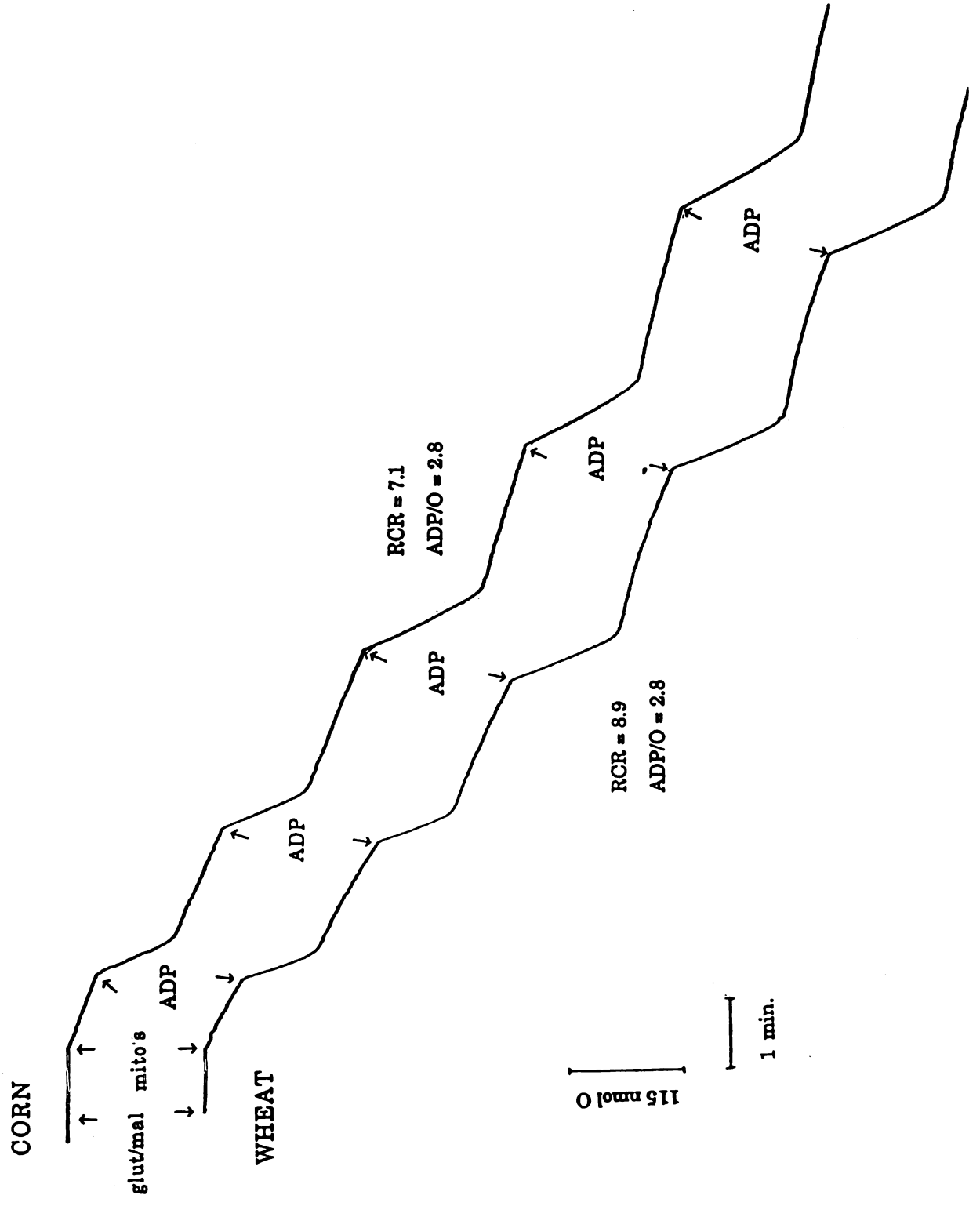

CORN
glut/mal mito's
ADP
WHEAT
ADP
ADP
RCR = 7.1
ADP/O = 2.8
ADP
RCR = 8.9
ADP/O = 2.8
ADP
115 nmol O
1 min.

Figure 6.1



Figure 6.2: Difference spectra (dithionite-reduced minus air-oxidized) of lauryl maltoside-solubilized (2 mg/mg protein) mitochondrial membranes from wheat germ (1.0 mg/mL, ----) and etiolated wheat seedlings (0.32 mg/mL, —).

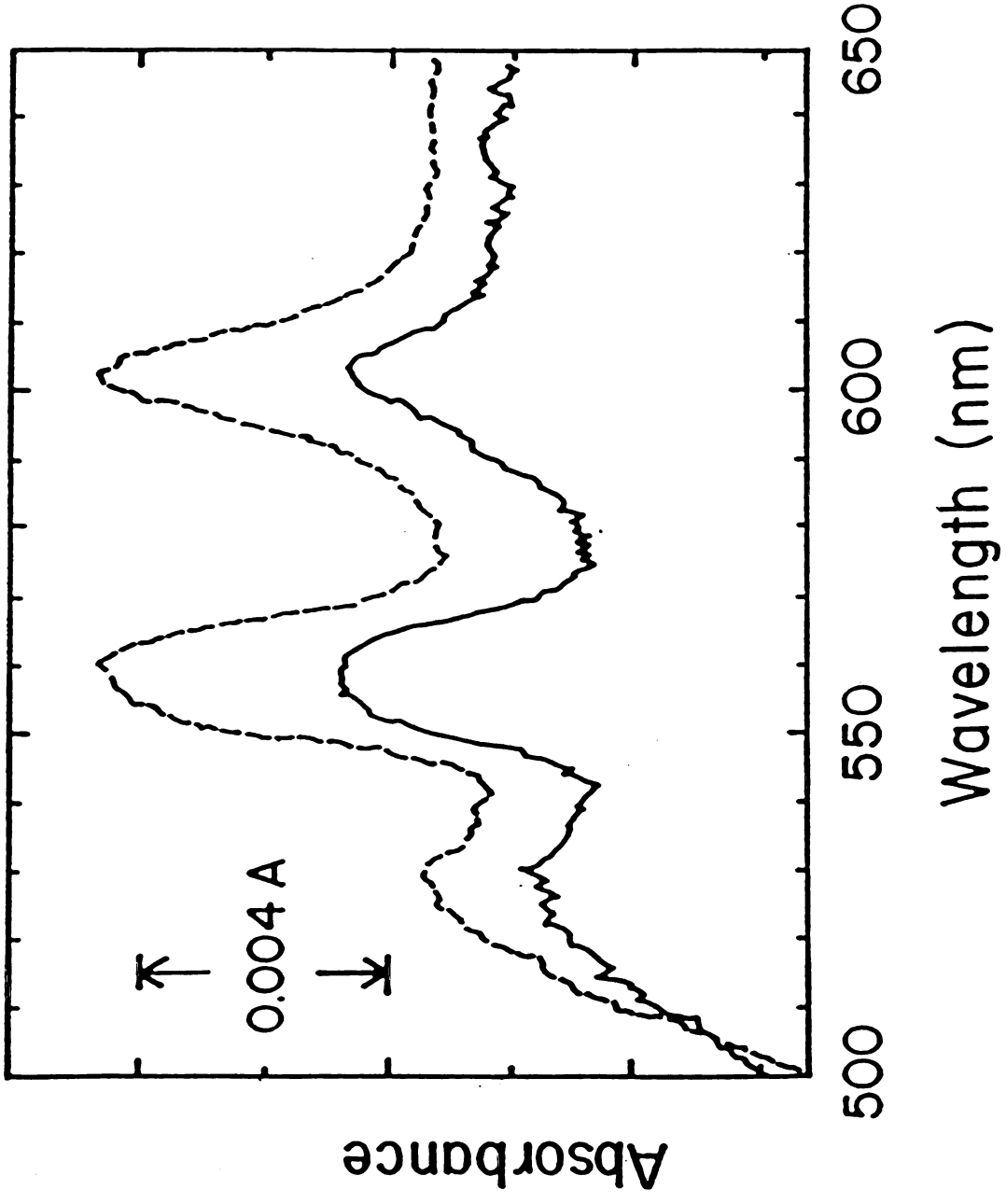


Figure 6.2

seedling mitochondria, although this step was not routinely done. FPLC elution profiles of solubilized mitochondrial membranes from both sources were nearly identical, suggesting a similar protein complement. Wheat germ obtained from two different milling companies and at various times during the year yielded the same results.

6.3.2 Purification of cytochrome oxidase by FPLC

When mitochondrial membranes solubilized in lauryl maltoside were applied to a Pharmacia MonoQ column, oxidase eluted at 0.35-0.37 *M* KCl, allowing separation from the bc_1 complex, which elutes slightly later at 0.42-0.45 *M* KCl. An elution profile of solubilized wheat germ mitochondrial membranes is shown in Figure 6.3, and is nearly identical to that obtained from wheat seedling mitochondria. Concentration of the oxidase fractions by the inverted column method described in section 6.2.4 can yield a highly concentrated enzyme; for example 0.5–1.0 mL of 50 μ*M* oxidase can be obtained from 900 mg mitochondrial protein (in addition to several more dilute fractions).

The visible spectra obtained from the final fraction are shown in Figure 6.4. Peaks at 441 nm and 602 nm in the reduced spectrum are blue-shifted relative to beef heart enzyme, whose reduced peaks are at 443 and 604 nm. A protein-to-heme ratio (A_{280nm} to A_{420nm}) of less than 2.2 is obtained after gel filtration. This final fraction has a turnover number of 500-800 s^{-1}, while fractions from earlier stages of purification have turnover numbers up to 1000 s^{-1}.

The visible spectrum from the bc_1 complex (Figure 6.5) is comparable to those from other organisms, with Soret peaks at 414 nm (oxidized) and 426 nm (reduced) and α peaks at 554–560 nm (reduced). A protein-to-heme

Figure 6.3: FPLC elution profile of lauryl maltoside-solubilized wheat germ mitochondrial membranes. Membranes made 0.2 *M* KCl were loaded onto a MonoQ 10/10 column and washed with FPLC buffer (5% sucrose, 20 m*M* KH_2PO_4, 4 m*M* lauryl maltoside, pH 7.5) to remove unbound protein. Bound protein was eluted at 1.0 mL/min with a discontinuous KCl gradient: 0–0.32 *M* in 15 mL, 0.32 *M* for 20 mL, 0.32–0.39 *M* in 7 mL, 0.39 *M* for 15 mL, 0.39–1.0 *M* in 16 mL. The solid line indicates the A_{280} and the dashed line indicates the KCl gradient.

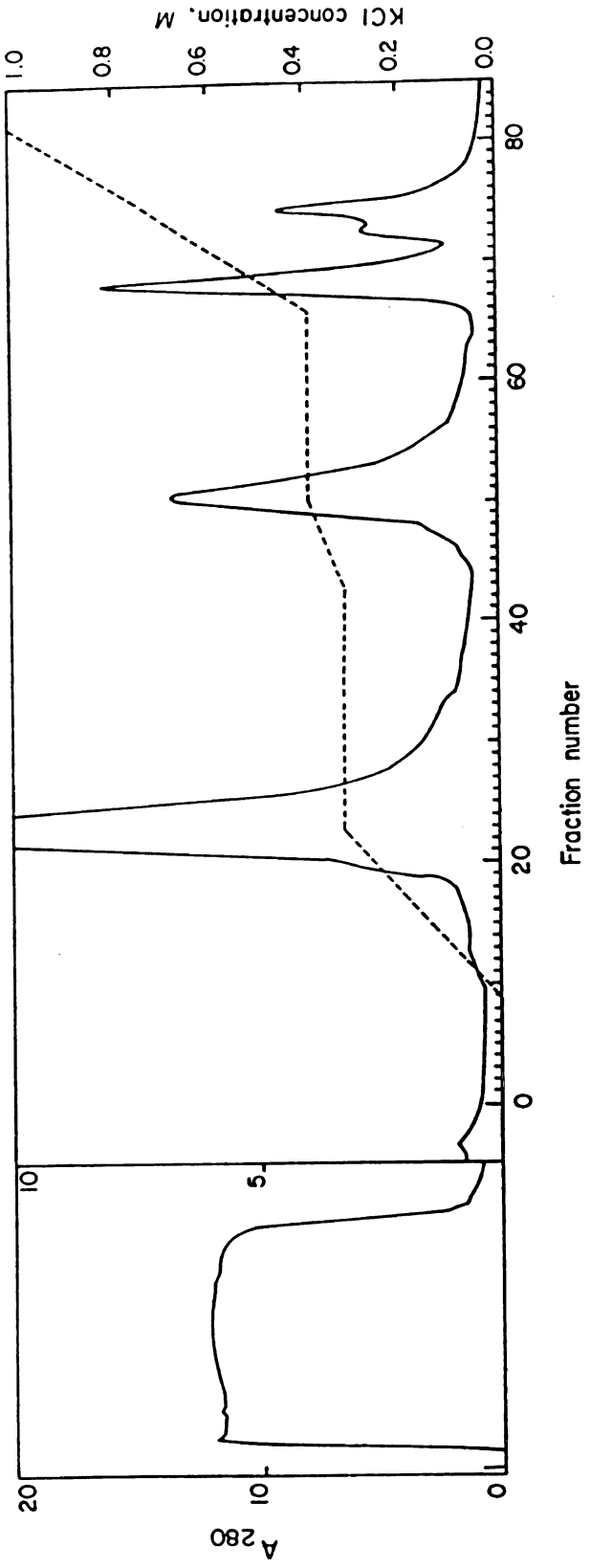

KCl concentration, M
1.0
0.8
0.6
0.4
0.2
0.0
80
60
40
20
0
Fraction number
A_{280}
20
10
0
10
5
0

Figure 6.3

Figure 6.4: Visible spectrum of purified wheat germ cytochrome *c* oxidase after gel filtration on Superose 6. Oxidase was 2.5 μ*M* in FPLC buffer (described in the legend of Figure 6.3). Air-oxidized (----); dithionite-reduced (—).

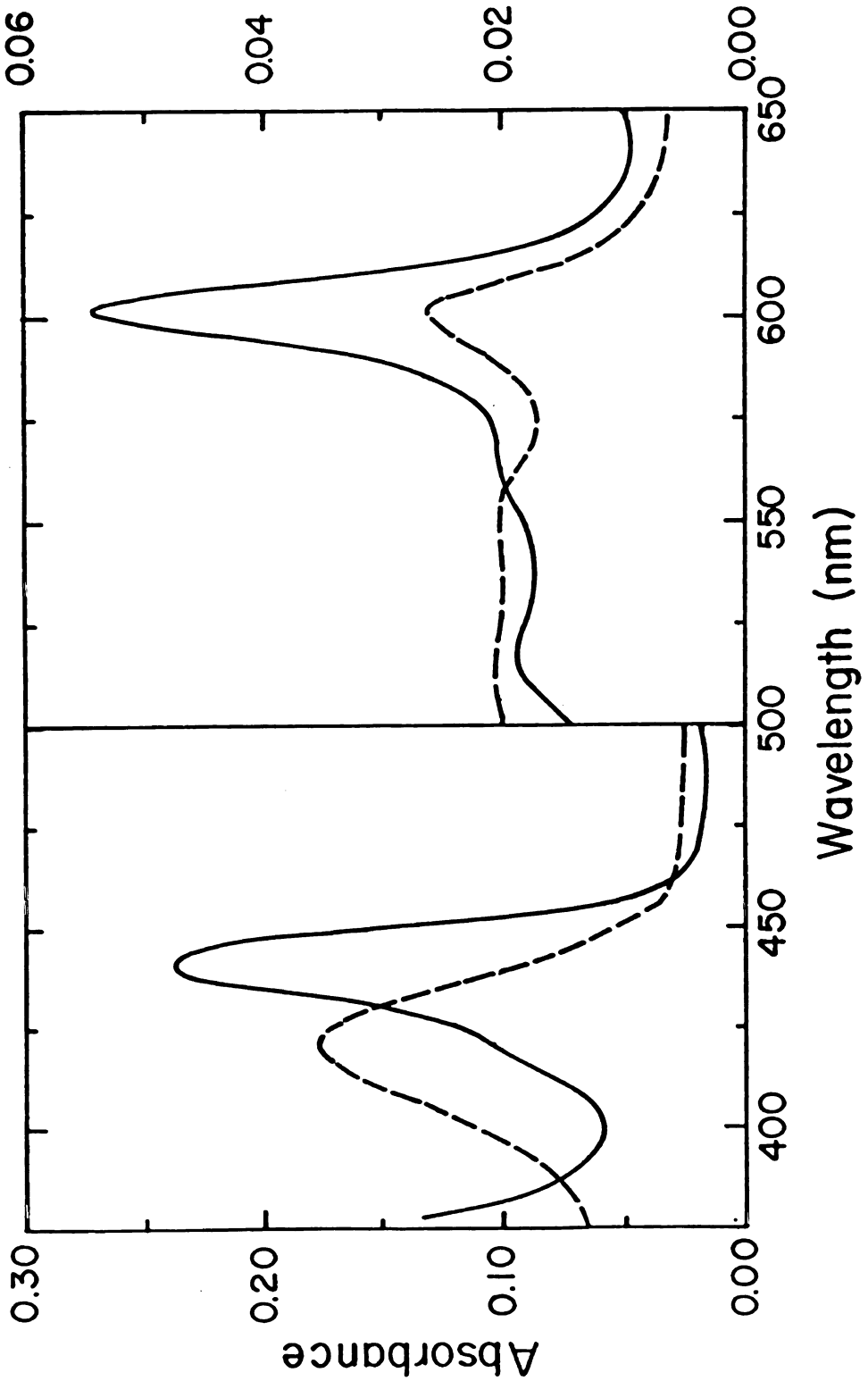

0.06
0.04
0.02
0.00
650
600
550
500
450
400
0.30
0.20
0.10
0.00
Absorbance
Wavelength (nm)

Figure 6.4

Figure 6.5: Visible spectrum of wheat germ bc_1 complex after one MonoQ fractionation. Air-oxidized (----); dithionite-reduced (—).

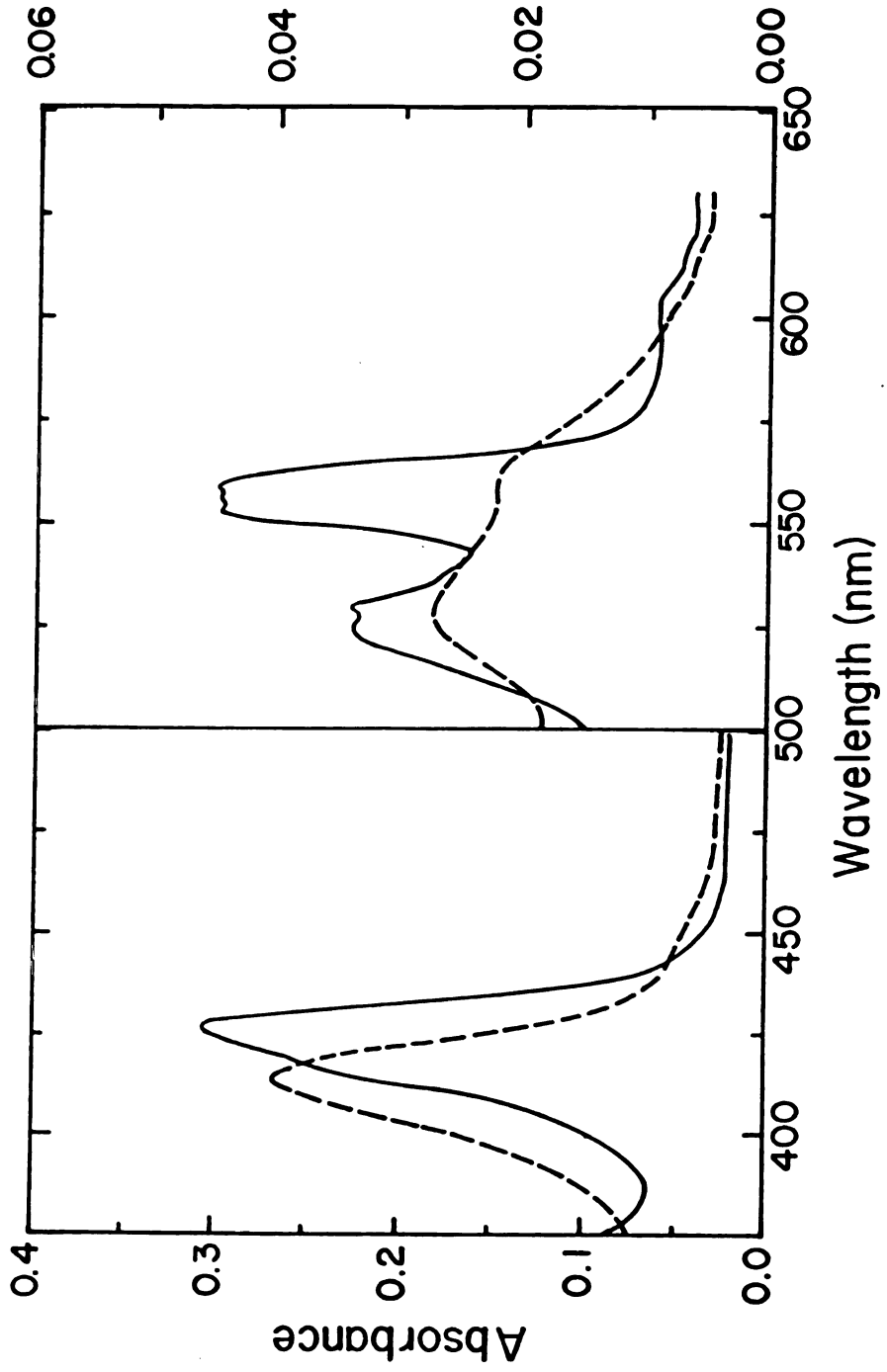

0.06
0.04
0.02
0.00
650
600
550
500
450
400
0.4
0.3
0.2
0.1
0.0
Absorbance
Wavelength (nm)

Figure 6.5

ratio (A_{280nm} to A_{414nm}) of 1.2 is typically obtained on peak fractions from the initial MonoQ separation. Further purification was not attempted. Yu *et al.* (personal communication) have found that our purified bc_1 has little associated phospholipid, relatively low turnover number, but show proton translocating activity.

We have been unable to demonstrate proton pumping by the purified oxidase reconstituted into phospholipid membranes using the method of Gregory and Ferguson-Miller (1989), although the vesicles show good respiratory control (ratio= 5–6). Upon addition of reduced cytochrome *c*, an alkalinization similar to reconstituted beef heart oxidase in magnitude and speed occurs outside the vesicles, but no initial acidification is seen. However, the normal ADP/O ratios achieved in wheat seedling mitochondria (Figure 6.1) suggests that the oxidase does pump protons. Future efforts should include assay of wheat seedling mitoplasts by the oxygen pulse method (Mitchell *et al.*, 1979) which does not require purification and reconstitution, as well as attempts to assay reconstituted oxidase purified from sodium bromide-washed or Triton X-100-purified mitochondrial membranes, which may have fewer contaminants (such as components of the ATP synthase complex) that might interfere with reconstitution or the assay.

6.3.3 Cytochrome *c* kinetics of wheat cytochrome oxidase

The kinetic properties of the plant cytochrome oxidase were measured under a variety of conditions to evaluate the similarity of the wheat germ and seedling oxidases as well as their similarity to other eukaryotic oxidases. Results are presented as an Eadie-Hofstee plot, in which the slope of the data is the negative inverse of the K_m. However, K_m

values calculated from these plots cannot be considered true K_m values because of the biphasic nature of the substrate dependence, and because substrate depletion occurs at low concentrations of cytochrome *c* (a problem in the high affinity phase) where substrate concentration approaches the enzyme concentration.

An Eadie–Hofstee plot of the kinetics of wheat germ and seedling oxidases in solubilized and whole mitochondrial membranes assayed in cacodylate is shown in Figure 6.6A and B respectively. Biphasic kinetics are clearly seen with both forms of the enzymes. The corresponding low and high affinity apparent K_m and V_{max} values are equal (average values for solubilized membranes: K_{m1}=0.06 μM, K_{m2}=4.0 μM; whole membranes: K_{m1}=0.02 μM, K_{m2}=0.6 μM), and compare favorably to previously published results for mammalian oxidases (Thompson and Ferguson-Miller, 1983). Furthermore, the kinetics of purified wheat germ oxidase are identical to the solubilized membranes used for fractionation (Figure 6.7), indicating that the sensitive kinetic properties of the enzyme have not been disrupted during the purification procedure. The higher turnover numbers in this plot result from the use of proteolytic inhibitors during the mitochondria preparation and subsequent purification steps.

Previous reports have shown that cytochrome oxidase activity is stimulated more in lauryl maltoside than in other detergents. However, wheat germ oxidase activity is stimulated almost equally by lauryl maltoside and Triton X-100. The cytochrome *c* kinetics of membranes solubilized by these two detergents were therefore measured and found to be identical (Figure 6.8). V_{max} values were equal using both detergents, in contrast to other oxidases, which exhibit much lower activity in Triton X-100 than in lauryl maltoside (Rosevear *et al.*, 1980; Sinjorgo *et al.*, 1987).

Figure 6.6: Eadie-Hofstee plot of the kinetics of reaction of solubilized (**A**) or whole (**B**) membranes from wheat germ (❑) and wheat seedling (◆) with horse heart cytochrome *c*. Assays were performed polarographically as described under section 6.2.5 in 25 m*M* Tris-cacodylate, pH 7.9. Oxidase concentration was 0.019 μ*M* for wheat seedling whole membranes and 0.017 μ*M* for solubilized membranes; cytochrome *c* concentration ranged from 0.01 to 28.4 μ*M*. Oxidase concentration was 0.029 μ*M* for wheat germ whole membranes and 0.026 μ*M* for solubilized membranes; cytochrome *c* concentration ranged from 0.025 to 25.5 μ*M*.

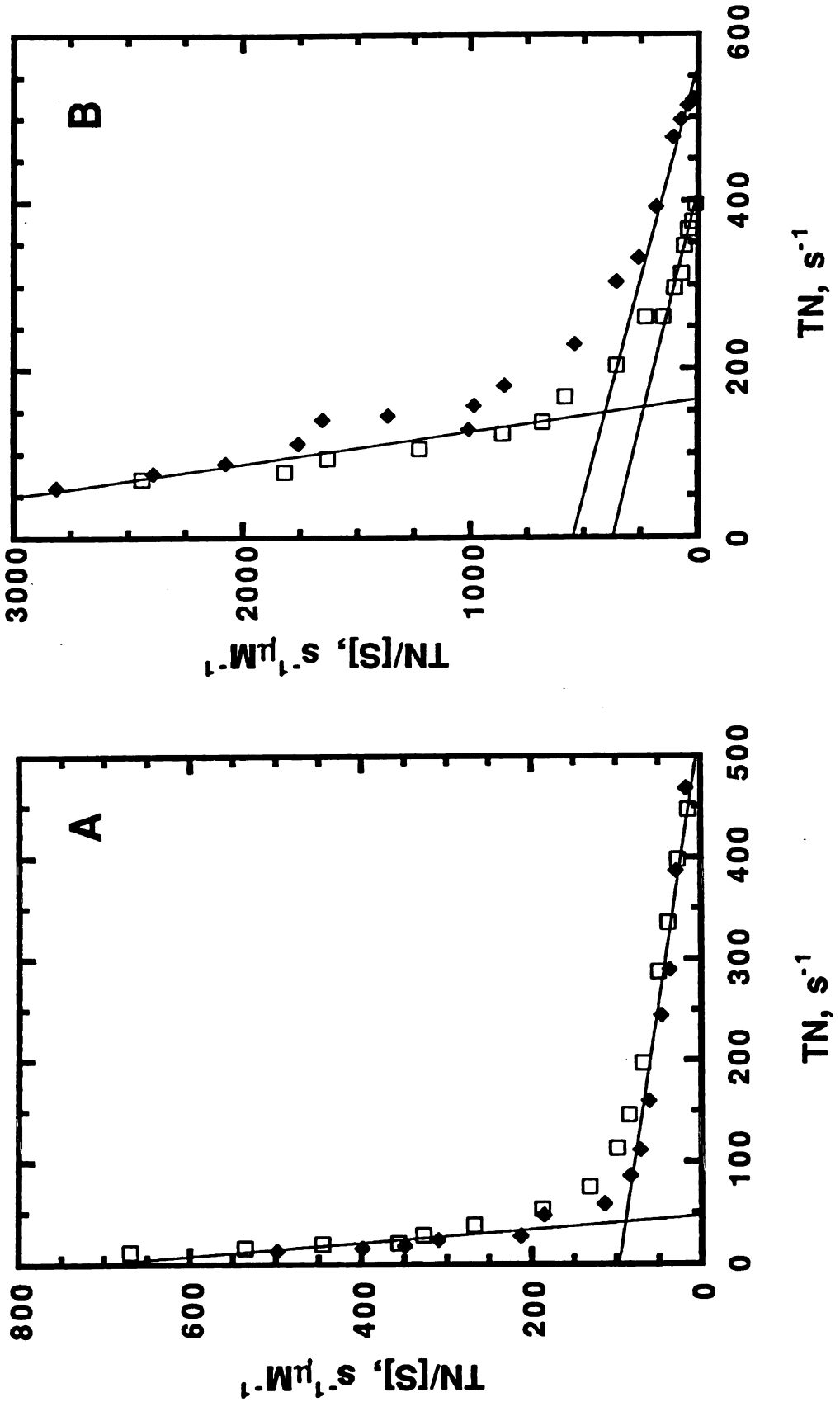


Figure 6.6

Figure 6.7:

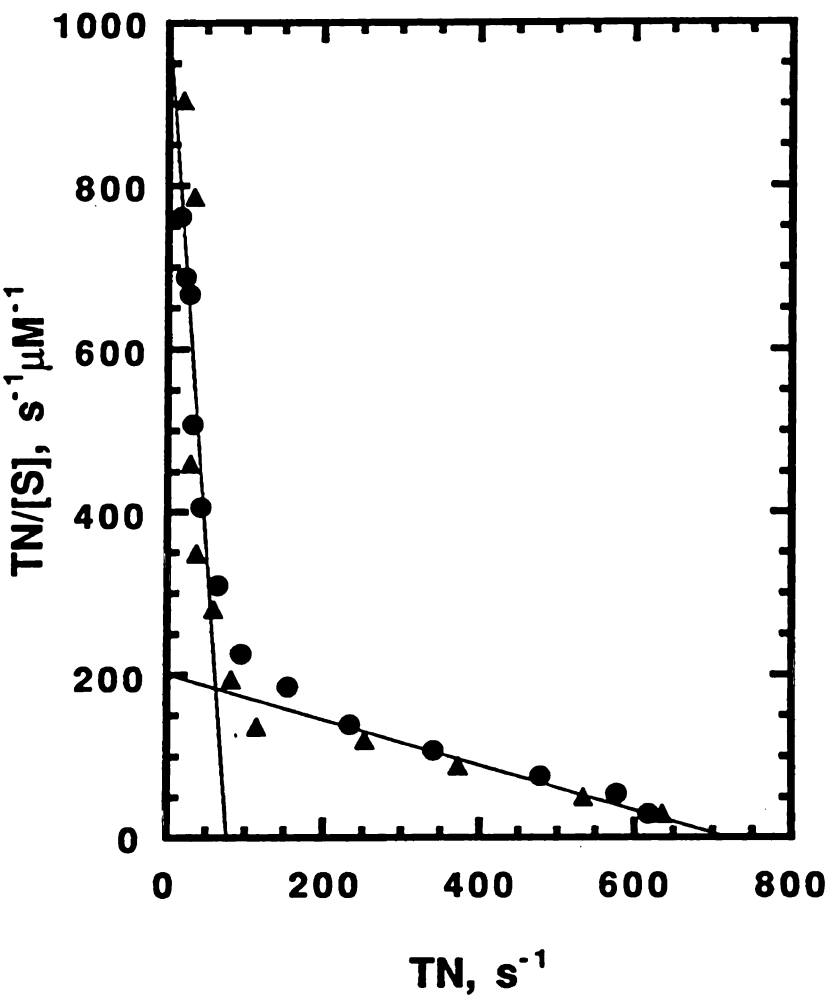


Figure 6.7: Eadie-Hofstee plot of the kinetics of reaction of solubilized mitochondrial membranes (●) and purified cytochrome *c* oxidase (▲) from wheat germ with horse heart cytochrome *c*. Assays were performed polarographically as described under section 6.2.5 in 25 m*M* Tris-cacodylate, pH 7.9. Oxidase concentration was 0.025 μ*M* for membranes and 0.021 μ*M* for purified oxidase; cytochrome *c* concentration ranged from 0.021 to 21.1 μ*M*.

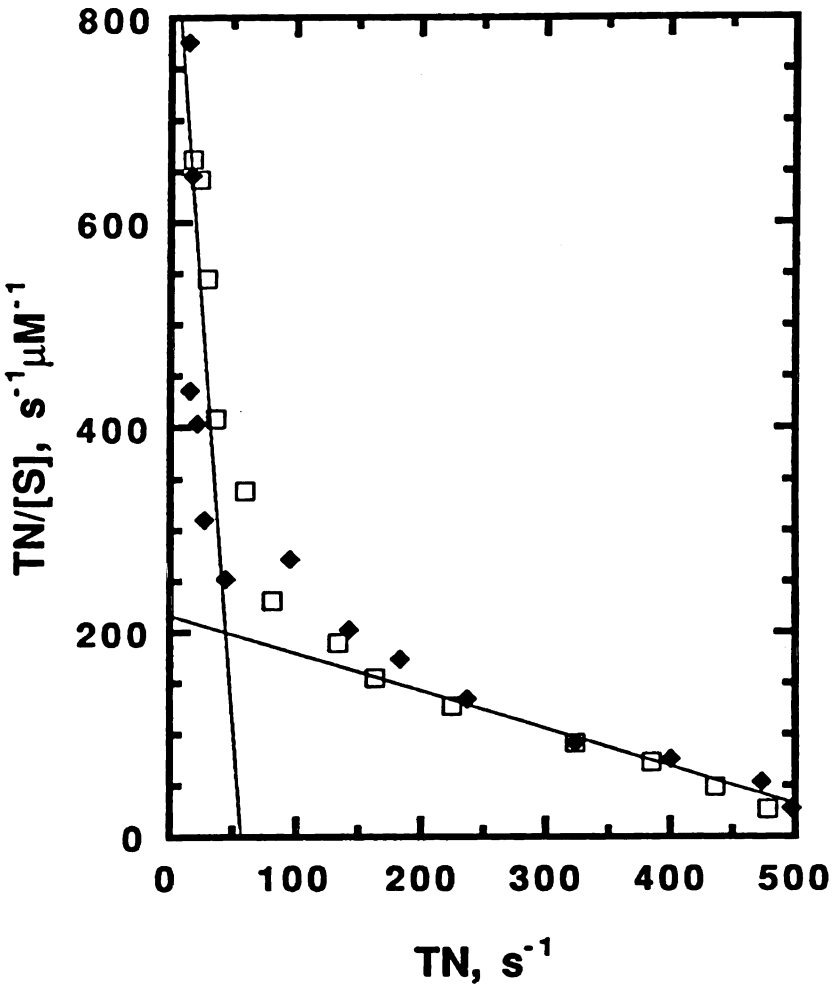


Figure 6.8: Eadie-Hofstee plot of the kinetics of reaction of mitochondrial membranes from wheat germ solubilized with lauryl maltoside (◆) and Triton X-100 (❑) with horse heart cytochrome *c*. Assays were performed polarographically as described under section 6.2.5 in 25 m*M* Tris-cacodylate, pH 7.9. Oxidase concentration was 0.022 μ*M*; cytochrome *c* concentration ranged from 0.026 to 17.6 μ*M*.

This unusual stability of the plant enzyme in Triton X-100 might allow purification of an intact, active enzyme in this inexpensive detergent not possible with other systems. This possibility is explored in chapter 7.

Cytochrome *c* from wheat seedling and wheat germ was purified in order to test the kinetic parameters with the natural substrate. Typically, cytochrome *c* from the same species as the oxidase exhibits lower K_m values because of optimized positioning of the positive and negative charges involved in the interaction of cytochrome *c* and cytochrome oxidase (Ferguson-Miller *et al.*, 1976). Preliminary results indicate that with wheat germ oxidase the K_m values obtained using cytochrome *c* from wheat germ are lower than those obtained using cytochrome *c* from wheat seedling, while both are lower than those obtained using horse heart cytochrome *c* (data not shown). This may reflect a real difference in interaction; however, because of the small amounts and lack of purity of the plant cytochromes *c*, further work is needed to verify this result.

6.3.4 Subunit analysis of wheat oxidase

SDS-urea polyacrylamide gels of wheat cytochrome oxidase, Figure 6.9, suggest a radically different subunit composition from the mammalian enzyme, but a similar composition to what has been reported for other plant oxidases. Subunit designations of the beef heart enzyme are shown next to it in lane (a). Lane (b) is wheat germ oxidase after MonoQ purification and concentration. The four largest bands have a similar migration behavior to the first four subunits of the beef heart enzyme in lane (a). Lane (e) is the bc_1 complex as it appears after elution from the first MonoQ column. Comparison shows that contamination of the oxidase from bc_1 does exist (especially in the region below subunit II), though it is not spectrally



Figure 6.9: Subunit composition analysis of wheat germ and seedling cytochrome oxidase by SDS-PAGE. Lane (a), beef heart oxidase, with subunit designations (I-IV) to the right; lane (b), wheat germ oxidase concentrated from the first MonoQ run, with subunit designations to the left; lane (c), wheat seedling oxidase concentrated from the first MonoQ run, (*) designates a possible alternative subunit as described in Results; lane (d), corn seedling oxidase concentrated from the first MonoQ run fractions; and lane (e), bc_1 complex from the first MonoQ run. The position of molecular weight standards (Amersham rainbow markers) in kDa are shown to the right of lane (e).

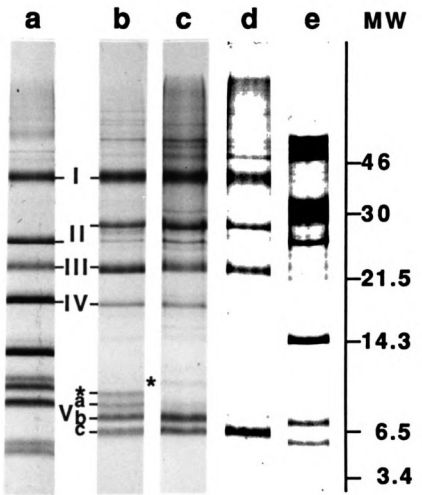


Figure 6.9

evident in the fractions. Oxidases purified from wheat seedling and maize seedling mitochondria are shown in lanes (c) and (d) respectively. The oxidase from wheat seedling has retained some subunit IV without the use of proteolytic inhibitors; however, corn seedling oxidase purified by a similar (but less rapid) procedure has little subunit IV except for some lower molecular weight bands that are perhaps degradation products.

Of particular interest is an additional band in the wheat germ cytochrome oxidase, labelled V*, that migrates near 9 kDa immediately above the three small subunits labelled Va, b, and c. It is reproducibly present and has a different mobility than faint bands in that region of wheat seedling (lane c) or maize (lane d) oxidase. This band has not been observed in previous reports of purified plant oxidase. In the case of the seedling oxidases, a band at 10 kDa is seen with variable intensity throughout the purification (lane d, *); this may represent an alternative subunit to V* which is developmentally regulated, similar to subunit VII in slime mold oxidase.

6.4 Discussion

Commercial wheat germ is inexpensive, readily available, and not highly variable. If stored cold (4°C) in closed containers, it can be used for at least six months. Although intact mitochondria were not obtained, commercial wheat germ yields a mitochondrial membrane fraction with a similar content of cytochromes bc_1 and aa_3 to that found in mitochondria isolated from etiolated seedlings. The relatively easy and rapid preparation we have developed offers the potential of isolating large quantities of cytochrome oxidase and many other plant mitochondrial proteins as well.

Since the germ is an embryonic tissue, developmental and tissue-specific changes in enzymes may also be studied.

Cytochrome *c* oxidase can be purified in relatively large amounts from these wheat germ mitochondrial membranes using anion exchange FPLC. Thus we can now do studies on plant oxidase that were formerly not possible due to lack of material, such as protein sequencing and Raman spectroscopy (see chapter 8). In addition to quantity, our enzyme is highly active, while previous studies on plant cytochrome oxidases show significant loss of activity during purification. These reports have also not provided any analysis of the enzyme's kinetic characteristics. The purified oxidase retains high and low affinity apparent K_m values identical to those of the solubilized membranes, indicating that the purification procedure has not altered the oxidase with respect to this sensitive measure of oxidase integrity. In contrast, the kinetic parameters of rat liver oxidase are different from those of the solubilized membranes, including a serious decrease in the V_{max} (Thompson and Ferguson-Miller, 1983). Maize oxidase purified by a similar but less rapid procedure exhibits a dramatic loss of affinity for cytochrome *c* in the initial kinetic phase, compared to the membrane-bound form (R. Ingle, personal communication). Concomittantly, the maize oxidase shows almost complete loss of subunit IV, which suggests a possible role for this subunit in determining the cytochrome *c* affinity of the initial kinetic phase, though other less obvious proteolytic damage could be involved. Subunit IV appears to be particularly labile to proteolytic degradation; even when proteolytic inhibitors are used in the purification of wheat germ oxidase, subunit IV is often present in less than stoichiometric amounts. The apparent content of subunit IV may also be affected by electrophoretic separation conditions such as extent

of solubilization in the loading buffer or diffusion from the gel during staining. In general, however, rapid purification appears to be essential for maintaining the native characteristics of plant cytochrome oxidase.

The UV-vis spectral properties of the wheat germ and seedling oxidases are very similar to those reported for maize and other plant oxidases. The blue shift of the reduced peaks indicates an altered heme *a* environment (Chance *et al.*, 1968), a conclusion that is supported by analysis of the plant oxidase by resonance Raman spectroscopy (Chapter 8).

Plant oxidases have previously been shown to have nuclear-encoded subunits that differ from animal or yeast oxidases. The larger subunits (I, II, III, IV) of the wheat germ oxidase appear to be similar in size to the beef heart enzyme, but the 3 or 4 smaller subunits below 10 kDa are similar only to other plant oxidases. A subunit of about 9 kDa is retained during purification of the wheat germ oxidase, but is not seen at any stage of purification of the wheat seedling enzyme, whereas the latter enzyme appears to have a larger (10 kDa) peptide that does not appear in the germ. This suggests a possible developmental form of the oxidase in wheat, similar to the developmental forms found in slime mold (Bisson and Schiavo, 1986). The subunit composition of the wheat oxidase is discussed further in chapter 7.



Chapter 7 – The number and nature of subunits in cytochrome *c* oxidase from wheat

7.1 Introduction

Chapter 6 describes a purification procedure yielding highly active cytochrome oxidase from wheat germ and wheat seedling. However, the apparent subunit composition on SDS-gels differs from other eukaryotic oxidases and even from several other plant oxidase preparations. Our preparation differs most notably from the other plant oxidases by the absence of bands in the 10–13 kDa molecular weight range on SDS-gels. However, a 9 kDa peptide (designated subunit V*) is retained during anion exchange and gel filtration chromatography of wheat germ but not seedling oxidase, while a band of variable intensity often appears at 10 kDa in the wheat seedling oxidase. We have suggested that this difference in apparent subunit composition between wheat germ and seedling oxidase may be developmentally controlled, as in slime mold oxidase, or tissue-specific, as in mammalian oxidase. Another issue relates to the identity of an intensely-staining band (designated "mystery band") that runs below subunit II on SDS-gels. This band seems to appear in other plant oxidase preparations as well (Nakagawa *et al.*, 1987; Hawkesford *et al.*, 1989). Therefore, it is critical that we establish the true subunit composition of the wheat oxidase, with particular reference to V* and the "mystery band".

The determination of subunit composition is a difficult problem with a multisubunit complex such as cytochrome oxidase, and indeed, there has been much disagreement over the years about the true number of subunits in the oxidases from mammals and yeast (see section 5.2). There are a

number
procedu
differen
required
activity
are miss
analysis
subunit
analyzin
overlap
anomolo
particula
have bee
(usually
subunits
relatively
enable u
characte

Th
wheat b
cysteine
likely th
1989), th
mitochon
sites in
understa
as the in

number of reasons for this disagreement. Different preparation procedures, such as the use of various detergents, result in oxidases with different apparent subunit compositions. Since not all subunits may be required for the aspects of activity that are readily measured, retention of activity may not be a good enough criterion to judge whether subunits that are missing are actually part of the true enzyme complex. The methods of analysis of preparations also contributed to the difficulty in agreeing upon a subunit composition. Researchers often employ different conditions in analyzing subunit composition by SDS-gel electrophoresis, so peptides may overlap on one, but separate on another gel system. Moreover, the anomolous migration behavior of some hydrophobic peptides (subunit III in particular) leads to confusion in assigning subunits. These difficulties have been largely overcome by the use of one highly resolving gel system (usually that of Kadenbach *et al.*, 1983), by analyzing immuno-reactivities of subunits from different oxidases, and by protein and gene sequencing. The relatively large amounts of plant oxidase which we can now purify should enable us to employ some of these methods in order to identify and characterize the subunits of the wheat oxidase.

The amino acid sequence of subunit II is of particular interest in wheat because of the possibility that an arginine replaces a conserved cysteine suggested to be a Cu_A ligand (Bonen *et al.*, 1984). While it is very likely that RNA editing restores the conserved cysteine (Covello and Gray, 1989), this has yet to be confirmed by protein sequencing. Only one plant mitochondrial protein has been sequenced; therefore, sequencing of editing sites in subunit II, the cysteine in particular, is important for understanding the process of plant mitochondrial protein synthesis, as well as the importance of certain residues of the oxidase in catalysis.

7.2 Materials and methods

7.2.1 Gel electrophoresis and immunoblotting

SDS-urea gel electrophoresis was performed by the method of Kadenbach *et al.* (1983) as described in chapter 6. Preparative gel electrophoresis used to purify oxidase subunits for subunit II digestion and antibody production were run by the same procedure; 3 nmol of oxidase was run per 0.75 mm Bio-Rad Mini-PROTEAN II gel. Gels were stained for 10 minutes in 50% methanol, 7.5% acetic acid, and 0.25% Coomassie blue, then destained for 10 minutes in 25% methanol, 10% acetic acid. Gels were soaked for an additional 5–10 minutes in water to remove SDS, urea, and destaining solution. Bands excised with a razor blade were neutralized with 2 *M* ammonium bicarbonate, and then minced and incubated with 0.2 *M* ammonium bicarbonate and 0.1% SDS for 18–24 hours at room temperature with agitation.

Gels used for Western blot analysis were blotted onto nitrocellulose (0.45 μm pore size, Bio-Rad Laboratories) in a buffer containing 12.5 m*M* Tris, 96 m*M* glycine, and 20% methanol (in which the gel was pre-equilibrated for 10 minutes) for 1 hour at 100 V. (The filter paper on the cathode side of the gel was soaked in this buffer with 0.5% SDS for ten minutes before blotting.) The blots were stained for five minutes with amido black (0.025% in 7.5% acetic acid and 22.5% ethanol) and destained with water. Blots were blocked for thirty minutes to overnight in a solution of 5% milk powder in 20 m*M* Tris, 0.5 *M* NaCl, and 0.05% Tween-20, pH 7.5. Yeast subunit IV antibodies were incubated for four hours, while nuclear subunit antibody was incubated overnight. Goat anti-rabbit IgG alkaline phosphatase conjugate was applied as the secondary antibody, with

5-bromo

immun

B

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7.2.2 Pr

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5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium used for immunodetection.

Blotting for amino acid sequencing was performed in 10 m*M* 3-cyclohexylamino-1-propane sulfonic acid (CAPS), pH 11.0, with 10% methanol (in which the gel was pre-equilibrated for 10 minutes) at 100V for 30 minutes onto ProBlot (Applied Biosystems). The blots were rinsed in 20 m*M* KH_2PO_4, 150 m*M* KCl, pH 7.2 followed by water to neutralize the high pH before staining with 1% acetic acid and 50% methanol in 0.05% Coomassie Brilliant Blue R for 30 seconds. Destaining was performed in staining solution without dye.

7.2.2 Preparation of antibodies

Monospecific polyclonal antibodies to the nuclear-encoded subunits of yeast cytochrome oxidase were kindly provided by Dr. Robert Poyton (University of Colorado, Boulder). The cell line producing a monoclonal antibody to yeast oxidase subunit II was the gift of Dr. Thomas Mason (University of Massachusetts, Amherst). Monoclonal and polyclonal antibodies against wheat oxidase peptides were prepared by Carrie Hiser and Roxy Nickels in Dr. Lee McIntosh's laboratory (Michigan State University) using the protocol described by Elthon *et al.* (1989b). Monoclonal antibodies to the "mystery band" were prepared by injecting FPLC-purified wheat germ oxidase into mice. A polyclonal antibody ascites fluid was generated by injecting pooled fractions of nuclear-encoded subunits (IV, V*, Va, Vb, and Vc) purified by high performance liquid chromatography (HPLC, see section 7.2.4), deliberately excluding the "mystery band" and other large subunits. Ascites fluid was removed from the abdomen of the mice.

7.2.3 Purification of wheat oxidase for subunit analysis

7.2.3.1 Sodium bromide wash of mitochondrial membranes

Solid sodium bromide was added to stirring mitochondrial membranes on ice to a final concentration of 2 *M*. After 15 minutes, the mitochondria were diluted five-fold in 0.35 *M* sucrose or mannitol, 50 m*M* KCl, 5 m*M* TES, 1 m*M* EGTA, pH 7.2 and centrifuged at 40000*g* for 30 minutes. The pellet was resuspended in this buffer, recentrifuged, and resuspended. These washed membranes were then subjected to lauryl maltoside solubilization and FPLC as described for unwashed membranes in chapter 6.

7.2.3.2 Solubilization of mitochondrial membranes with Triton X-100

Mitochondrial membranes were solubilized with 1.5 to 3.5 mg Triton X-100 per mg protein and 0.2 *M* KCl (final concentration). The mixture was agitated for 30 minutes and centrifuged at 40000*g* for 30 minutes. The supernatant was removed from beneath a layer of cloudy white material floating on top. If the pellet contained the oxidase, it was resuspended in buffer (0.35 *M* sucrose or mannitol, 50 m*M* KCl, 5 m*M* TES, 1 m*M* EGTA, pH 7.2) to which 2 mg lauryl maltoside per mg protein was added. After 30 minutes the mixture was centrifuged at 40000*g* for 30 minutes, and the greenish supernatant containing the oxidase used for FPLC fractionation.

7.2.4 HPLC of wheat oxidase

To purify the subunits of the wheat germ oxidase for antibody production and subunit II digestion and proteolytic cleavage, we attempted to use the method of Robinson *et al.* (1990). 200 µL of 20 µM oxidase was dissolved in 0.1% trifluoroacetic acid (TFA) and filtered before loading onto a Vydac C4 column pre-equilibrated with 0.1% TFA. A gradient from 0.1%

TFA in water to 0.1% TFA in 100% acetonitrile was performed by a Waters Prep LC 3000 at a flow rate of 1 mL/min in 45 minutes and the absorbance was monitored at 214 nm and was recorded by the Waters Data Module. When this procedure was applied to beef heart oxidase, the elution profile obtained was similar to that reported by Robinson *et al.* (1990). Since subunit II from wheat germ oxidase did not elute under these conditions, several variations on this method were tried, including substitution of isopropanol for acetonitrile, or a 55:45 (v/v) mixture of isopropanol and acetonitrile. Fractions were dried under vacuum before further treatment.

7.2.5 Proteolytic digestion of subunits

Protein prepared by either HPLC was dissolved in 0.2 *M* ammonium bicarbonate and 0.1% SDS; protein from preparative gel electrophoresis was already in this buffer. For trypsin digests, 0.2–1 μL of 1 mg/mL trypsin (Sigma) was added to 50–100 μL of subunit II and for V8 digestion, 1 μL of 1 mg/mL V8 (Boehringer Mannheim) was added per 300 μL. In both cases, approximately 3 μg subunit II was digested per tube. The digests were incubated for 3 hours at 37°C and dried under vacuum. Endoproteinases Lys-C and Arg-C (Boehringer-Mannheim) and Endo-peptidase Pro-C (Seikagaku Kogyo Co.) were also used under the conditions of V8 but at concentrations of up to 20 μL per 300 μL sample and digestions times up to 24 hours. SDS was removed by cold 80% acetone precipitation. The pellets were dried to remove acetone before adding gel loading buffer (as described in Kadenbach *et al.* (1983) with the addition of 0.04% bromophenol blue). Samples were sonicated at 15–20° C for 30 minutes and left overnight before running them on gels.

7.2.6 Sequencing of wheat oxidase peptides

Four to five bands were excised from ProBlot for each amino acid sequencing on an Applied Biosystems Model 477 pulsed-liquid peptide sequencer with an on-line Model 120 PTH-amino acid analyzer.

7.3 Results

7.3.1 Determination of the true subunit composition of wheat oxidase

Since the wheat germ oxidase purified by the FPLC methods of the last chapter contains high amounts of "mystery band" and apparently stoichiometric amounts of V*, the putitive "developmental" subunit, we have tried to better define the subunit composition of the wheat oxidase with particular reference to these two peptides. A first analysis can be made by examining the SDS-gels of oxidase from different points on the eluted peak from a typical mitochondrial fractionation on MonoQ. Figure 7.1 shows two fractions before the peak (lanes 1–2, corresponding to fraction 45–46 in Figure 6.3), the peak (lane 3), and one fraction after the peak (lane 4, corresponding to fraction 55 in Figure 6.3) which is contaminated with peptides from the bc_1 complex (lane 5). The "mystery band" (shown by an arrow) is not present in the earliest fraction, but begins to appear in lane 2. It is very strong in the peak fraction, and is reduced again in the later fractions. The early fractions which lack the "mystery band" are active (TN=450 s^{-1}), showing that its presence is not required for activity and suggesting that it is perhaps not a true subunit. The "developmental" subunit, V*, appears to be relatively stoichiometric throughout the elution profile, suggesting that it is a true subunit. Both the "mystery band" and V* are present in similar amounts after gel filtration (Pharmacia Superose 6)

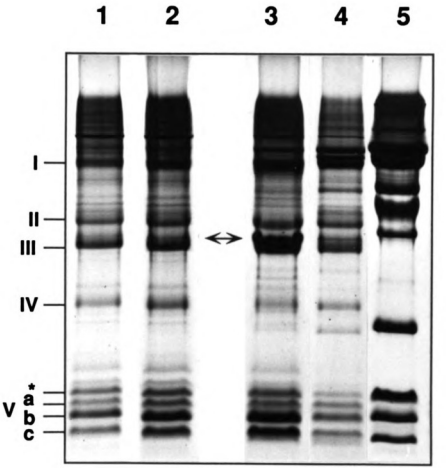


Figure 7.1: SDS-PAGE analysis of wheat germ cytochrome oxidase FPLC fractions. Lane 1 and 2 are fractions preceding the peak, lane 3 is the most concentrated fraction of the peak, lane 4 is a fraction after the peak, and lane 5 is the bc_1 complex in the peak following the oxidase. Designations of the subunits of the wheat germ oxidase are marked to the left of lane 1. The arrow points to the position of the "mystery band".

of MonoQ-purified oxidase. Further evidence, however, was needed to support these conclusions.

7.3.1.1 Native gel electrophoresis

The recent method of Schägger and vonJagow (1991) appeared very promising for application to our problem of determining subunit composition with minimal processing of the enzyme which could lead to alteration of the peptide content. This unique method utilizes the protein dye Coomassie Blue G in the sample and cathode buffer to induce "charge shift", similar to the action of SDS in denaturing gels. Therefore the complexes run more or less according to their molecular weights. The complexes are solubilized by lauryl maltoside and kept soluble in the gel by high concentrations of aminocaproic acid. Since some dye remains bound to the proteins, visualization is immediate. Bands can be excised from the gel and run under denaturing conditions to determine the subunit composition.

We have tried to reproduce the results of Schägger and vonJagow (1991) with only limited success. In our hands, lauryl maltoside-solubilized beef heart inner mitochondrial membrane particles, which should be analogous to the beef muscle mitochondria used in the above report, produced several wavy bands on top of a streaked background. Purified beef heart oxidase produced two major and several minor bands (the multiple bands probably arise from oligomeric states of the enzyme), as did purified wheat oxidases, although not reproducibly. However, when solubilized mitochondria from wheat seedling, wheat germ, and yeast mitochondria were run on the native gel system, intense smearing resulted throughout the entire lane, completely obscuring any bands which may have formed. Various modifications of the procedure with regard to protein

concentration and detergent concentration in the sample and gel gave no improvements. We have therefore abandoned this method as an approach to determining subunit composition of the wheat oxidase.

7.3.1.2 Immunological methods

Immunoblots. To determine whether the absence of the V* band in wheat seedling is the result of its loss during purification, we used polyclonal antibodies against HPLC-purified nuclear-encoded subunits from wheat germ oxidase to analyze solubilized mitochondria from both wheat seedling and germ. Figure 7.2 shows an immunoblot of purified wheat germ oxidase (lane 1), solubilized wheat germ mitochondria (lane 2), and solubilized wheat seedling mitochondria (lane 3). Reaction with V* is clear in the two wheat germ lanes; however, there is no reaction at that molecular weight in the wheat seedling lane. A very faint reaction occurs at a slightly higher molecular weight in wheat seedling mitochondria (marked by an arrow), at a position which may correspond to the 10 kDa putitive analog to V*. No reaction is seen at that position in either wheat germ lane. Therefore, the absence of V* at its "native" molecular weight is confirmed by this blot, but the possibility remains that an analog that is immunologically related exists in the seedling.

Immunoprecipitation. Antibodies against oxidase from several sources were tested for ability to immunoprecipitate, as measured by ability to remove activity from the protein-containing solution. A yeast oxidase subunit IV polyclonal antibody, a yeast oxidase subunit II monoclonal, the "mystery band" monoclonal, and the HPLC-purified nuclear-encoded wheat germ oxidase subunits mouse polyclonal all were strongly positive in an enzyme-linked immunosorbent assay (ELISA); however, none were able to

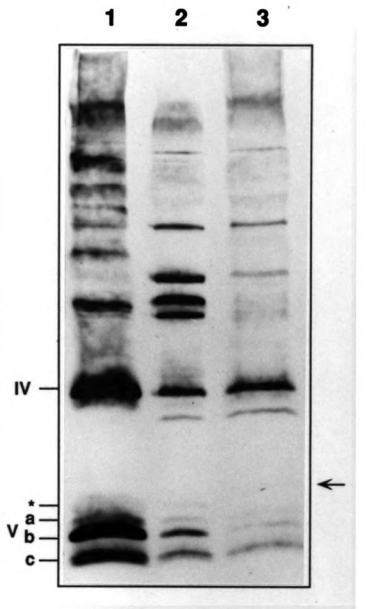


Figure 7.2: Immunoblot analysis of purified wheat germ cytochrome oxidase (lane 1), wheat germ mitochondrial membranes (lane 2), and wheat seedling mitochondrial membranes (lane 3). Membranes were solubilized with lauryl maltoside as described in section 6.2.4 and prepared for electrophoresis as described in section 6.2.7. The blot was incubated overnight with antibodies against HPLC-purified nuclear-encoded subunits (described in section 7.2.2).

immunoprecipitate oxidase activity from solubilized wheat germ mitochondria under a broad range of protein and detergent concentrations.

7.3.1.3 Other purification techniques

Sodium bromide wash. Since a number of unidentified peptides in the higher molecular weight range (> 50 kDa) appear in SDS gels of the wheat germ cytochrome oxidase preparation, in addition to the "mystery band" (which runs below subunit II on gels), as well as several faint bands above Va (including the putative developmental V*), we subjected our mitochondrial membranes to a wash with sodium bromide, which removes peripheral membrane proteins. Typically the ATPase α and β, and a number of the NADH dehydrogenase subunits are removed with this treatment in other mitochondrial and microbial membrane preparations.

The twice buffer-washed and resuspended pellet from sodium bromide-washed membranes retained only 40–50% of the original cytochrome oxidase activity. Another 20% was found in the supernatant of the wash. When the washed membranes were solubilized and subjected to anion-exchange FPLC, the elution profile was similar to those from unwashed membranes (see Figure 6.3), except that the off-scale peak prior to the elution of the oxidase was much lower relative to the height of the oxidase peak, as would be expected since the ATPase α and β subunits make up a large part of this peak. However, fractions from the tail of this off-scale peak were greenish, and contained high concentrations of oxidase which had little or no activity. The presence of many other components prevented precise identification of which subunits were present in this inactive complex. The oxidase peak eluting in the expected position had typical turnover numbers (> 500 s^{-1}) and spectral characteristics. However, SDS gels of this fraction show little if any improvement over concentrated

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fractions purified from unwashed membranes, and the "mystery band" and V* are both still present. This result suggests that the activity lost during the procedure was largely due to oxidase being inactivated by the sodium bromide, possibly due to denaturation of the enzyme.

Triton X-100 solubilization. Since the cytochrome oxidase of wheat germ mitochondria appears to be reasonably stable in Triton X-100 (see chapter 6), we have attempted to perform initial steps of our purification in this detergent. One immediate benefit is conservation of the expensive detergent lauryl maltoside which is used in large quantities for the initial mitochondrial solubilization. A second benefit is that Triton allows a "membrane splitting" similar to that achieved with deoxycholate in beef heart oxidase preparation, removing many potential contaminants early in the procedure.

At moderate concentrations of Triton X-100 (1.75 mg detergent per mg protein, or "mg/mg"), many mitochondrial proteins are selectively solubilized while others remain membrane-embedded and are easily pelleted by centrifugation. The bc_1 and ATPase complexes are solubilized, but the cytochrome oxidase is not. In fact, 90% of the total protein is solubilized, resulting in an immediate ten-fold purification of the oxidase. At higher concentrations (above 2 mg/mg) the oxidase is also solubilized. Difference spectra of mitochondria extracted in either Triton X-100 or lauryl maltoside are shown in Figure 7.3.

Solubilization of the Triton-insoluble pellet (from the 1.75 mg/mg solubilization) with 2 mg lauryl maltoside/mg protein allows recovery of an active cytochrome oxidase. However, this fraction is unable to pass through a 0.45 μm filter, a necessary step prior to loading onto the pressure-sensitive FPLC column. This may be due to large micelles of incompletely-

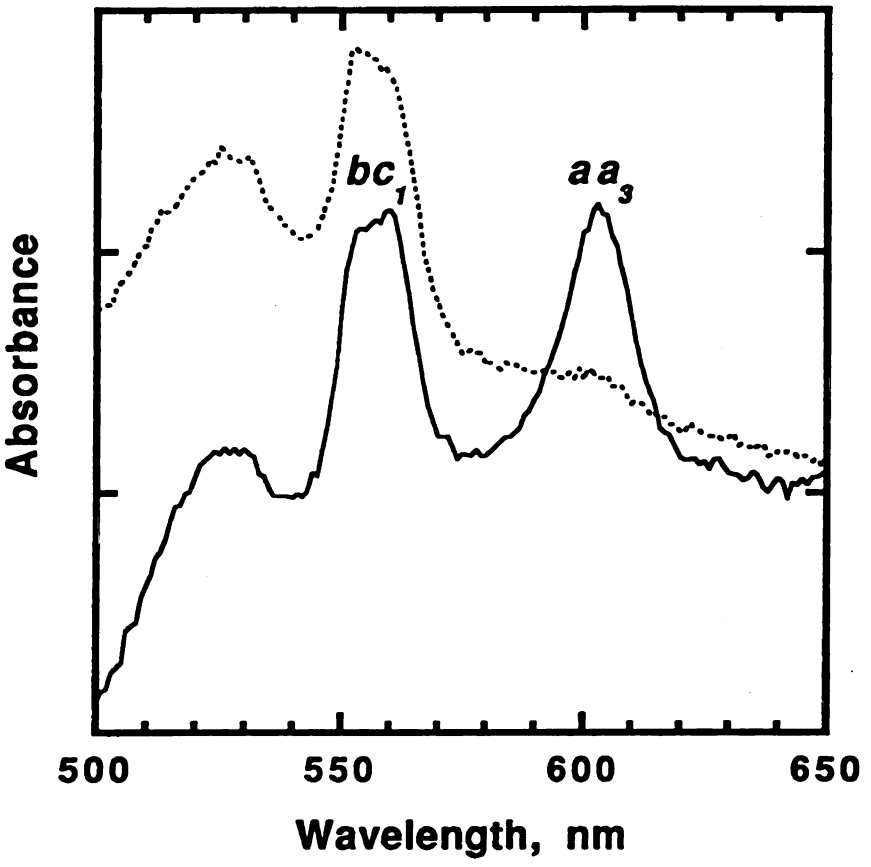


Figure 7.3: Difference spectra (dithionite-reduced minus air-oxidized) of wheat germ mitochondrial membranes extracted with lauryl maltoside or Triton X-100. Solubilization was performed as described in section 7.2.3.2 with lauryl maltoside (—) at 2 mg/mg protein or Triton X-100 (----) at 1.75 mg/mg protein. In the latter case, the oxidase is not significantly extracted, as indicated by the absence of a peak at 602 nm.

solubilized proteins and lipid formed at lower Triton concentrations. In fact, the supernatant of the 1.75 mg/mg solubilization can also not be filtered, but a 3.5 mg/mg supernatant (which contains the oxidase) can be filtered. One attempt at a MonoQ fractionation of the lauryl maltoside-solubilized pellet of the 1.75 mg/mg extraction made without filtering resulted in loss of a majority of the oxidase which presumably was retained by the column filter. Oxidase which did elute was active, but retained both the mystery band and V* (data not shown). More work is needed to alleviate the technical problems which currently render this promising procedure unusable.

7.3.2 Amino acid sequence of subunit II

The first step in sequencing subunit II RNA editing sites was to separate subunit II from the other subunits. Because none of the RNA editing sites occurred near the N-terminus of the protein, and in fact several important ones appear only near the C-terminus (*e.g.* Cys-228), it was necessary to digest the subunit into smaller pieces using proteolytic enzymes. The resulting peptides required separation and transfer to a matrix appropriate for amino acid sequencing.

7.3.2.1 Isolation of subunit II

HPLC. The technique of Robinson (1990) for large scale purification of subunit II from beef heart oxidase was tried for the wheat oxidase. The nuclear subunits and the mystery band elute as sharp peaks; however I, II, and III do not elute and presumably remain bound to the column (Figure 7.4). Peaks were identified by the mobilities of the fractions on SDS gels (Figure 7.5). Substituting the less polar isopropanol or isopropanol: acetonitrile (55:45, Figure 7.6) for acetonitrile and increasing the steepness

of the gradient in order to decrease residence time on the column elutes III, II, and I (only with pure isopropanol), in that order, although the three subunits tend to overlap, particularly when using isopropanol alone. Column life was short when the wheat germ oxidase was run; only 20 runs could be done with good recovery, even under optimal conditions with extensive washing between runs with methylene chloride.

Since the beef heart oxidase subunit II elutes from the column in a well-resolved peak under all the above conditions (in our hands), we tried to determine what differences might exist between the wheat germ and the beef heart enzyme which may account for this observed difference in elution. We suspected that our enzyme might contain more associated lipid than the beef heart enzyme due to its different methods of purification, and that this, combined with the natural hydrophobicity of I and III, was causing II to elute later than the beef heart II. We attempted to remove the lipid by precipitation with ammonium sulfate (the last step of beef heart oxidase purification) and trichloroacetic acid (TCA), and to remove I and/or III by heat and acetone precipitation, which aggregate the subunits. We also tried III-depleted (but still containing I) enzyme normally produced in the FPLC purification (by wash of the column-bound purified oxidase with 0.3 *M* KCl, see section 6.2.4). None of these techniques improved the elution of II.

Another difference between the wheat subunit II and the beef heart II sequences is that the wheat sequence contains five cysteines instead of two. When II is denatured, the cysteines may crosslink II to itself or to I and III, resulting in aberrant elution. However, neither addition of high concentrations of βME and dithiothreitol nor covalent modification of the reduced cysteines with 4-vinylpyridine altered the elution profiles.

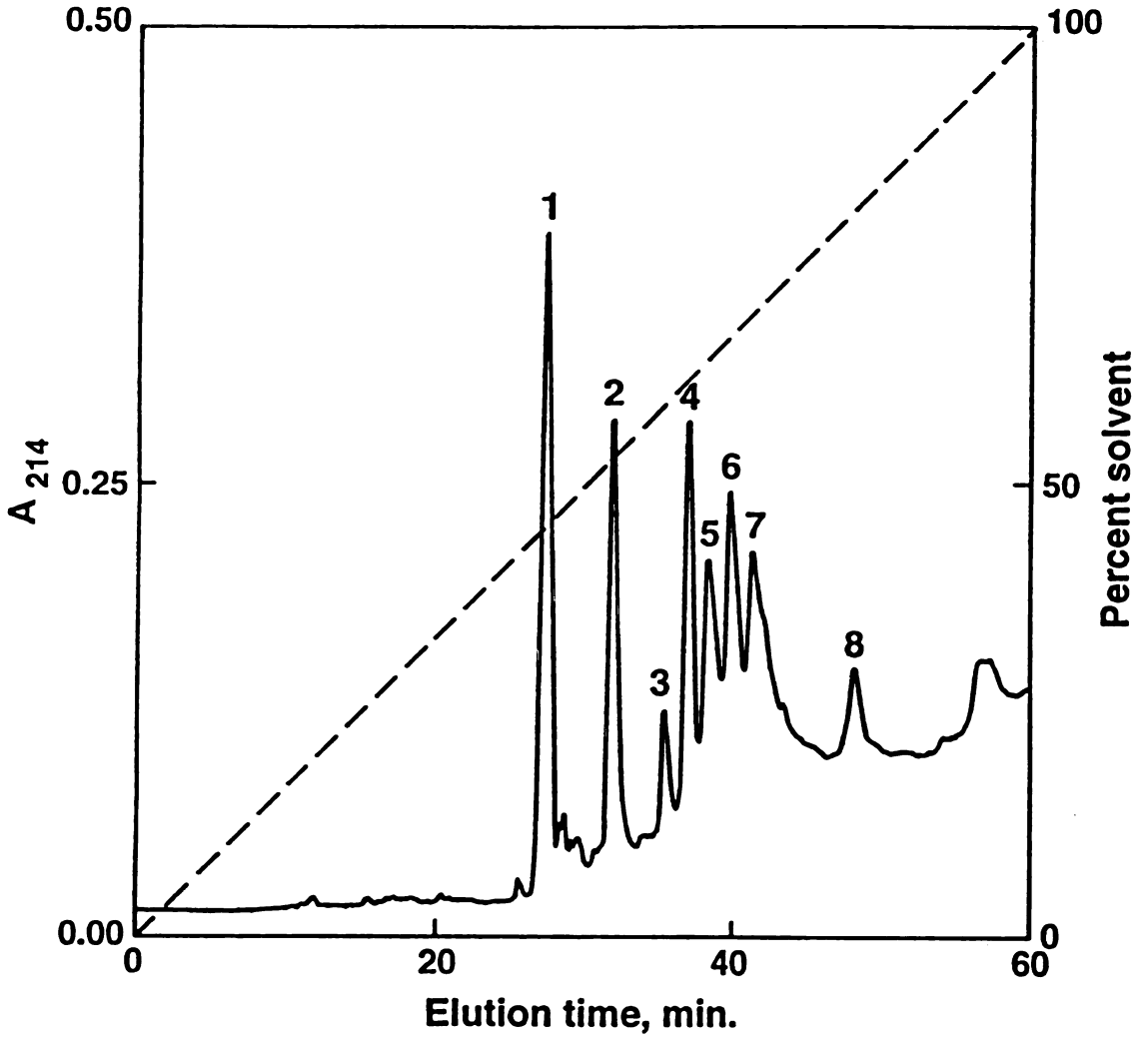


Figure 7.4: Separation of cytochrome oxidase subunits by reverse-phase HPLC. Oxidase was run on a C4 column from water/0.1% TFA to 100% acetonitrile/0.1% TFA in 60 minutes. Eluted protein was detected by absorbance at 214 nm.

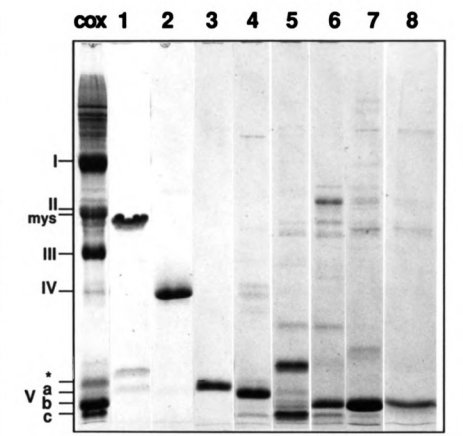


Figure 7.5: SDS-PAGE analysis of peaks from reverse-phase HPLC in Figure 7.4. HPLC fractions were dried under vacuum and incubated in sample buffer with 4% SDS (section 6.2.7) overnight prior to electrophoresis.



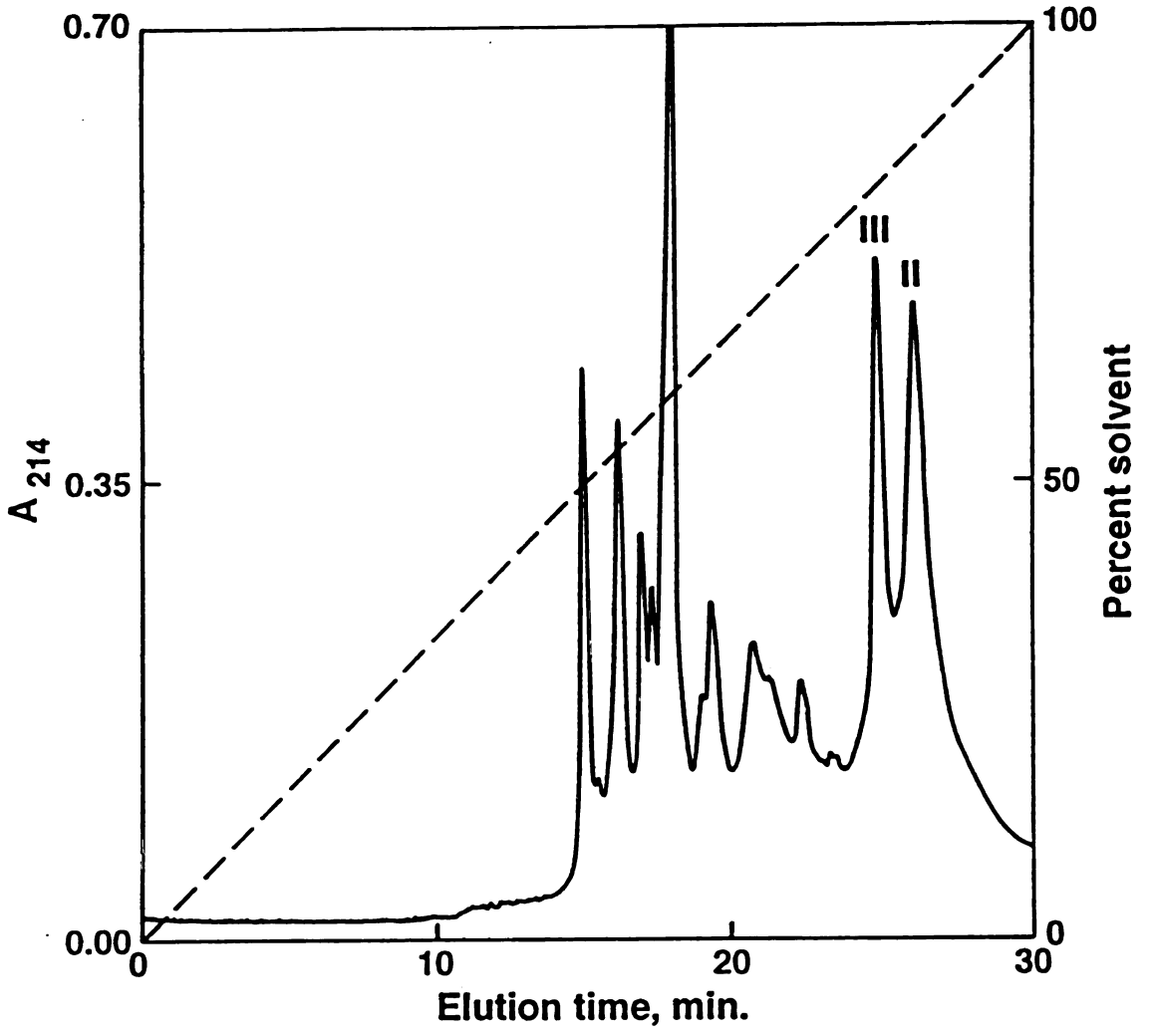


Figure 7.6: Purification of subunit II by reverse-phase HPLC. Cytochrome oxidase diluted in 0.1% TFA was loaded onto a C4 column and eluted by a gradient of H_2O/0.1% TFA to 55:45 (v/v) isopropanol: acetonitrile/0.1% TFA in 30 minutes. Protein was detected by absorbance at 214 nm.

Regardless of the elution protocol, HPLC-prepared subunit II was incompletely soluble in 0.1% SDS. SDS-urea gels of it and its proteolytic digests had a considerably higher Coomassie blue staining background than the same digest on beef heart II or wheat subunit II eluted from preparative gels (Figure 7.7, lanes 1 and 2). This background was accentuated upon transfer to nitrocellulose. Coomassie blue staining of the blot resulted in a blue smear down the lane to the position of the lowest band, with discrete bands barely discernible (not shown). This is most likely due to insufficient solubilization of the protein in the gel loading buffer (4% SDS) causing the protein to enter the gel at different times and creating a continuous stream down the lane. The appearance of the gel was not altered by the addition of 8% SDS or 6 *M* urea, acetone precipitation, and heating immediately before loading the gel. The protein has perhaps become too seriously denatured from the HPLC solvents or column to be resolubilized, and we therefore abandoned the HPLC method of purifying subunit II.

Preparative gel electrophoresis. Because HPLC purification proved unfeasible, we attempted to isolate subunit II by preparative gel electrophoresis. We have preliminarily determined that the recovery of subunit II (determined by protein assay) was 15 μg per gel when ~1.5 nmol oxidase was loaded (30–50%). In the future, electroelution may be attempted to increase the yield. When the dried acetone-extracted sample was solubilized in the standard sample buffer (containing 4% SDS) and re-run on a gel, subunit II ran cleanly with almost no background Figure 7.7, lane 3). Therefore, this method was used for all subsequent proteolytic digestion experiments.

Figure 7.7: SDS-PAGE analysis of subunit II and its proteolytic digestions purified by HPLC and preparative gel electrophoresis. Subunit II was purified by these method as described in sections 7.2.4 and 7.2.1 respectively; trypsin and V8 digests were performed and samples prepared for electrophoresis as described in section 7.2.5. Lane 1: subunit II purified by HPLC as in Figure 7.6; lane 2: trypsin digest of HPLC-purified subunit II; lane 3: subunit II purified by preparative gel electrophoresis; lane 4: trypsin digest of gel-purified subunit II; lane 5: V8 digest of gel-purified subunit II; lane 6: Coomassie-blue stained blot (CAPS-transferred) of V8 digest of gel-purified subunit II.

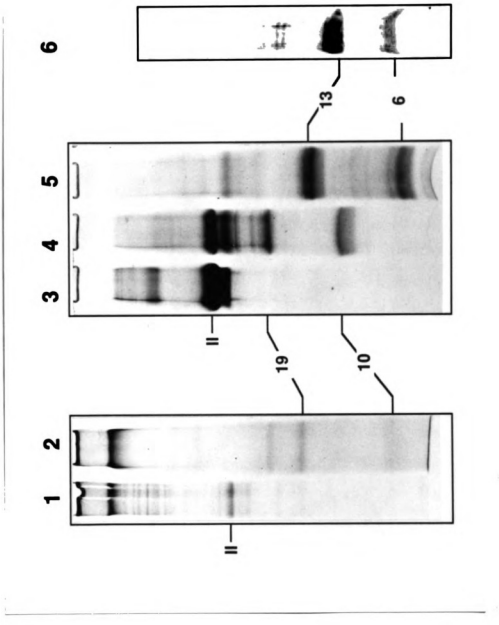


Figure 7.7

7.3.2.2 Proteolytic digestion of subunit II

Endoproteinase Lys-C, Arg-C, Pro-C, trypsin and V8 (Glu-C) were tested for their ability to digest subunit II into pieces appropriate for subsequent amino sequencing. The sequence of subunit II is shown in Figure 7.8, with editing sites double-underlined. Lys-C, Arg-C, and Pro-C were unable to cleave subunit II, most likely because of their lability in SDS, which is required to maintain solubility of subunit II. Trypsin and V8 are not as sensitive to SDS, and adequate cleavage was in fact achieved. Figure 7.7 shows the results of these digests from subunit II prepared by both HPLC and preparative gel electrophoresis. A significant amount of undigested subunit II remains after trypsin digest (lane 4); however, more complete digestion, achieved by adding more trypsin, does not alter the pattern of peptides (lane 2). The trypsin digest appears similar to that obtained with beef heart oxidase, and antibodies reacted with the lower molecular weight band in both cases, supporting the conclusion that the same cleavage site, Arg-162 (Taha and Ferguson-Miller, 1992), is involved. However, no RNA editing sites are found within 10 amino acids of this site, therefore trypsin cleavage was not used further.

Analysis of the sizes of the V8 cleavage products suggests that several pieces might contain RNA editing sites near the N-terminus. The band below the 14 kDa molecular weight marker could result from cleavage at Glu-132, Glu-141, Glu-154, Glu-158, or Glu-165 (indicated within the amino acid sequence of subunit II in Figure 7.8 by vertical arrows), which would result in pieces of 14.3, 13.2, 11.8, or 11.3 kDa respectively, with Glu-141 being the most likely cleavage site based on apparent size of the peptide on the gel. The lower molecular weight bands were more difficult to analyze because of the distortions of predicted molecular weight at that

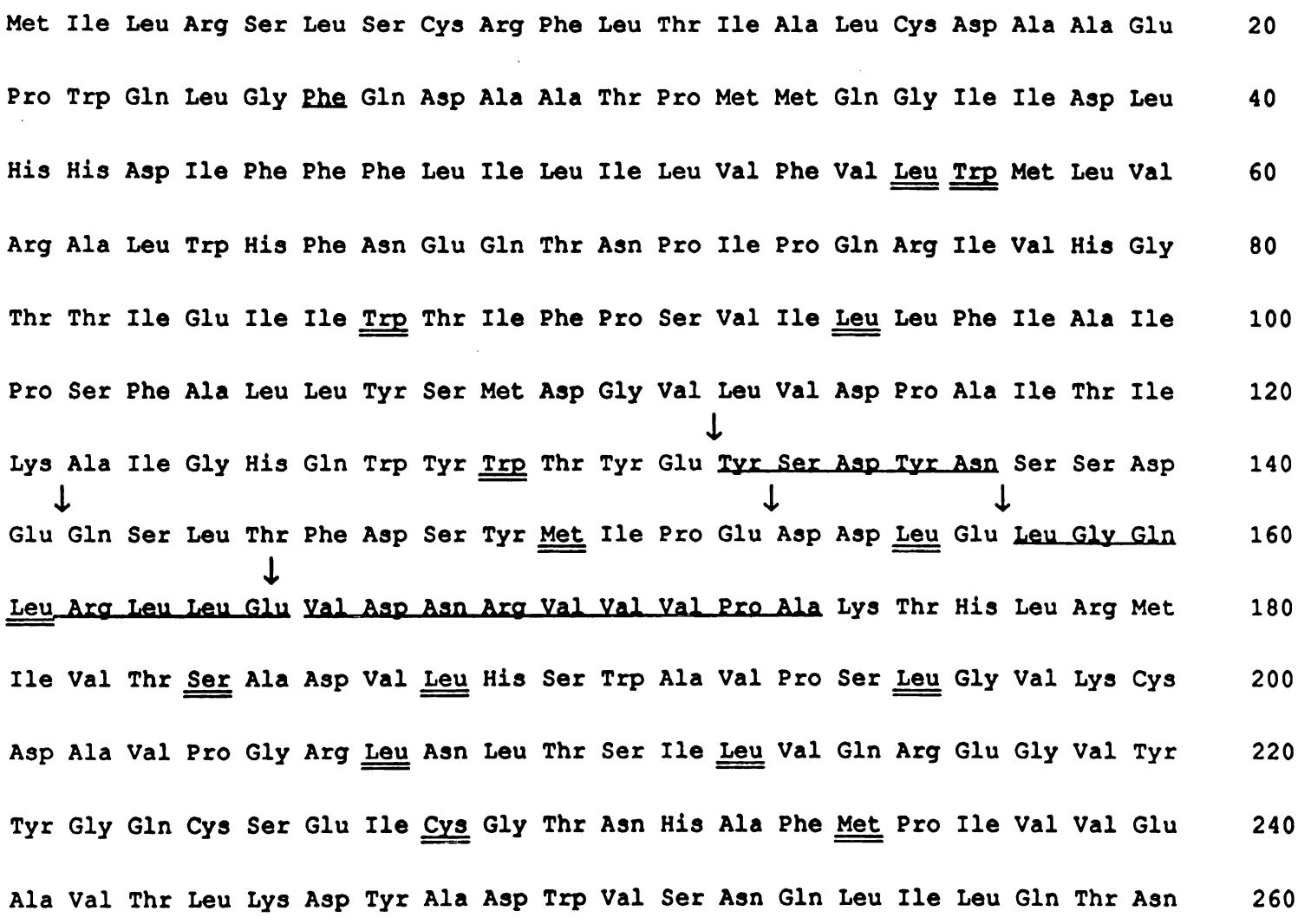


Figure 7.8: Predicted amino acid sequence of wheat cytochrome oxidase subunit II. Sites of RNA editing are double-underlined; sequenced peptides are underlined, and arrows point to predicted V8 cleavage sites.

position in the gel, and because many cleavages would result in pieces of that size. Assuming only one cut per molecule, however, the most likely cleavage sites producing these bands would be Glu-217 or Glu-226. The latter would be most ideal because sequence confirmation would be possible of both Cys-228 and Met-235, two highly conserved residues and possible Cu_A ligands.

7.3.2.3 Amino acid sequences of subunit II peptides

Based on the predicted cleavage sites, we sequenced both the 13 kDa peptide, as well as the lower band of the smaller peptides. The sequence of subunit II is shown in Figure 7.8, with sequenced peptides underlined, while edited residues are double-underlined. The first five amino acids of the 13 kDa peptide match exactly the sequence starting after Glu-132. Therefore, since the nearest editing site is Met-150, no information on the presence of RNA editing was possible from this peptide.

Sequence analysis of the second band proved more ambiguous. The residues produced in each sequence cycle are shown in Figure 7.9. Two sequences can be extracted from this complex mixture: one starting after Glu-157, and a second after Glu-165 (underlined on Figure 7.8). Many of the residues corresponding to a third sequence starting at Glu-153 appear to be in the sequencing cycles, but that sequence is inconclusive. The exact sizes of these pieces cannot be determined, since several sizes are possible for the first sequence; however, the piece starting at Glu-165 must continue to at least Glu-217, making it 5.7 kDa. (There would then be a 4.8 kDa or less piece starting at Gly-218, but we have not recovered it.) For the editing site contained within this sequences, Leu-161, there is no evidence of the DNA-predicted residue, Ser, which would be expected if any protein had been made from unedited mRNA. Therefore, it is most likely that the

	Cycle								
	1	2	3	4	5	6	7	8	9
Site of cleavage									
	T								
	S								
	D				E				
Glu-165:	V	D	N	R	V	V	V	P	A
Glu-157:	L	G	Q	L	R	L	L	E	

Figure 7.9: Amino acids obtained in each sequencing cycle of the smallest band on SDS-gels of a V8 digest of subunit II.

mature protein is synthesized only from the completely edited mRNA, as was found by Graves *et al.* (1990) for ATPase subunit 9.

7.3.3 Comparison of wheat oxidase subunits to other oxidases

7.3.3.1 Immunoblot comparisons

Comparison to plant oxidases. The mouse polyclonal antibodies against the HPLC-purified wheat germ nuclear-encoded subunits cross-react with other plant mitochondria tested, including maize, pea, tomato, and potato (data not shown). However, in each case, only subunit IV and Va, Vb, and Vc can be seen; V* is only seen in wheat germ. A monoclonal antibody against the "mystery band" reacts only with wheat germ and seedling mitochondria, not the closely related maize or any other plant, despite the fact that a similar-looking band at the same location is clearly evident in gels of the maize oxidase. Monoclonal antibodies against yeast oxidase subunit II strongly cross-react with wheat and beef heart oxidase as well as all plant mitochondria tested. These results suggest that although some mitochondrial proteins may not be not well-conserved among the plants (*e.g.* the "mystery band"), the subunits of cytochrome oxidase are in fact well-conserved even among plants which are not closely related. No other plant was found to possess V*, despite the variety of plants and different tissues tested.

Comparison to yeast oxidase. Since subunit IV was readily lost during purification, it was important to determine whether it was an authentic oxidase subunit. The availability of monospecific polyclonal antibodies to yeast oxidase subunit IV allowed us to test whether it was a related peptide. Nitrocellulose blots of yeast mitochondria (Y) and purified beef heart (B) and wheat germ (W) oxidases are shown in Figure 7.10.

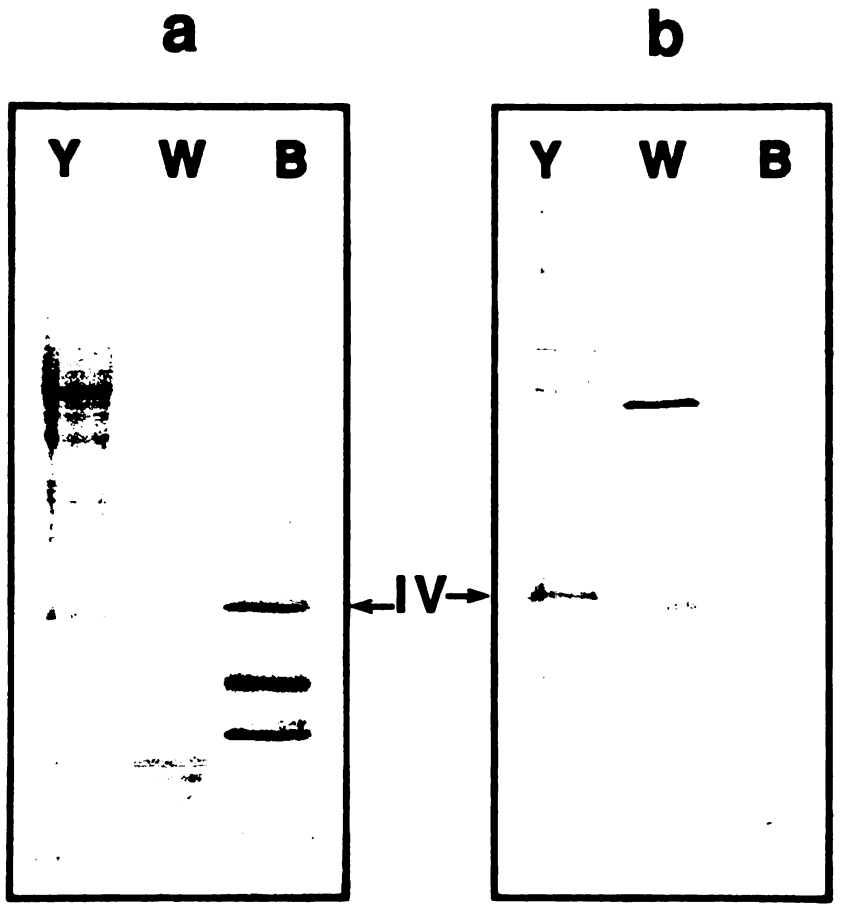


Figure 7.10: Western blot analysis of wheat germ cytochrome oxidase. Nitrocellulose blots in panels (**a**) and (**b**) contain lanes (Y), yeast mitochondria; lanes (W), wheat germ oxidase; and lanes (B), beef heart oxidase. Panel (**a**) is the amido black stain of the nitrocellulose blot, and panel (**b**) is the immunodetection of subunit IV on the blot using monospecific polyclonal antibodies to yeast oxidase subunit IV. The position of subunit IV is indicated by the arrows.

Panel (a) is the amido black stain of these transfers; most of the subunits of the oxidases are visible, but subunits I and III transfer less efficiently, leaving a "hole" in the transfer. Panel (b) shows the same samples as in (a) after incubation with monospecific polyclonal antibodies to yeast oxidase subunit IV, followed by secondary antibody labelling and alkaline phosphatase color development. Subunit IV of the yeast oxidase and wheat germ oxidase react with these antibodies, but the purified beef heart oxidase does not. The position of subunit IV, which is higher in the yeast than in the beef heart or wheat germ oxidases, is marked by the arrows. Some non-specific reaction with higher molecular weight species is also seen. Monospecific polyclonal antibodies against yeast subunits Va, VI, VII, and VIIa did not cross-react with the wheat oxidase by immunoblot or ELISA analysis.

7.3.3.2 N-terminal sequences

We have attempted to determine N-terminal sequences of several of the nuclear-encoded subunits. Subunit Va was dried and resuspended in water after HPLC purification, but did not yield a sequence. Nakagawa *et al.* (1987) also found this subunit to be blocked when they cut it out of polyacrylamide gels. Sequencing of several other subunits was attempted using protein blotted in CAPS onto Problot. Subunit Vc was cut from a blot of purified oxidase and successfully sequenced. This sequence compared favorably to that of the sweet potato by Nakagawa *et al.* (1987) with five or six identical residues out of ten (Table 7.1). The first uninterpretable residue (#4) could be a histidine as Nakagawa *et al.* (1987) found, but the seventh is not. The lack of a residue in the fourth position of our sequence could indicate the presence of a cysteine, since modification of this residue was not performed prior to sequencing. The sequence of subunit IV was also

Table 7.1: Sequence alignment of subunit Vc from wheat and sweet potato (Nakagawa *et al.*, 1987) cytochrome oxidase. Identical residues between the two sequences are underlined. No residue appeared in cycle four of the wheat sequence, and two residues (D/P) appeared in cycle seven.

WHEAT:	A G G V AD/PT Y
SWEET POTATO:	A G G H V A H L V Y K G P S V

Table 7.2: Sequence alignment of subunit IV from wheat, yeast, and *N. crassa*, and subunit Vb from bovine cytochrome oxidase. Sequences for comparison were taken from Power *et al.* (1984). Identical residues between two sequences are underlined. The residue from the wheat sequence in parentheses is less definite than the rest of the sequence.

WHEAT:	S A D K P A A(G)G T K V V M
yeast:	E Q K P V V K T A Q N L A E V N G P.E T L I G P G A K E
N. crassa:	E T K P V P H I K R L S E
Bovine Vb:	A S G

obtained from the same blots (Table 7.2). The homology between wheat IV and yeast IV appears to be limited, although there is a match of Lys(K)–Pro(P) across the three sequences at positions 4 and 5 of the wheat sequence. The next two amino acids are Val(V)–Val(V) in the yeast sequence, which is similar to Ala(A)–Ala(A) in the wheat sequence. Since antibodies to yeast subunit IV cross-react with wheat, it may be possible that later regions of the sequence share greater homolgy. However, the homologous beef heart Vb, which lacks a major N-terminal segment, does not react with the same antibody. HPLC-purified V* was run on gels and blotted, but yielded no sequence. V* was cut from the same blots which successfully yielded IV and Vc sequence, but again no sequence was obtained, suggesting natural blocking.

7.4 Discussion and future prospects

The apparent subunit composition of our wheat germ and seedling oxidases purified by anion-exchange FPLC exhibits some differences compared to oxidases purified from other plants. We obtain a strongly-staining band below subunit II on SDS-gels which we call the "mystery band" and believe to be a contaminant of the oxidase preparation. More interestingly, in wheat germ but not wheat seedling, we reproducibly obtain an additional band, called V*, which runs above Va, and is strongly retained throughout purification by anion-exchange and gel filtration. We have postulated that the expression of this peptide may be developmentally regulated. In this chapter we have attempted to provide evidence for our hypotheses that the "mystery band" is not, but that V* is, a true subunit of the oxidase.

We have been unable to use native gel electrophoresis or immunoprecipitation for the purpose of determining subunit composition. However, neither peptide is removed by gel filtration, sodium bromide wash, or Triton X-100 membrane-splitting. This is not necessarily surprising, since many peptides with hydrophobic properties are not easily separated once they have associated. However, the fact that the "mystery band" shows a different elution profile than oxidase suggests that it is not a true subunit.

The unusual behavior of the "mystery band" in gels suggests that it is the same peptide that Nakagawa *et al.* (1987) designates as subunit III. The mobility of this peptide is dependent on the acrylamide concentration: at 18% it runs below subunit II, at 14% it runs above (data not shown). This behavior was described by Nakagawa *et al.* (1987) for their subunit III. Moreover, they report that their subunit IV binds DCCD, while their subunits I and IV aggregate upon heating, properties of eukaryotic subunit III. Therefore, these results strongly suggest that Nakagawa *et al.* (1987) has incorrectly assigned subunit IV as III, and it is likely that their subunit III is the "mystery band". If so, they do not retain a subunit IV; however, we have also observed its lability in the case of the maize oxidase purification. Because our monoclonal antibody against the "mystery band" does not cross-react with any other species, including maize, we have not been able to use immunological techniques to identify it. This lack of cross-reactivity may indicate that even an N-terminal sequence would not necessarily be useful in identifying it. Our only clue at this time is its visual similarity to the 30 kDa β-hydroxybutyrate dehydrogenase: this enzyme forms a band in gels that is unusually "wavy" (Nelson *et al.*, 1984) just like we see for the "mystery band". Furthermore, β-hydroxybutyrate

dehydrogenase has both hydrophobic and hydrophilic behavior; it requires detergent to be solubilized from membranes but not for further purification. Similarly, the "mystery band" is initially solubilized by detergent, but thereafter has more hydrophilic behavior, such as ease of transfer to blots and early elution in reverse phase HPLC.

Immunoblot analysis using antibodies against the wheat germ oxidase small peptides shows that the absence of V* from wheat seedling oxidase is not the result of loss during purification. We must still consider the possibility that its presence in wheat germ may be an artifact of the differences in mitochondrial preparation. The wheat germ mitochondrial membranes require much higher speeds to pellet, presumably because they are membrane vesicles not whole mitochondria. Therefore, this preparation may contain more microsomal contamination, introducing co-eluting peptides not present in the wheat seedling mitochondria.

In the immunoblot, the faint reaction in wheat seedling mitochondria of a peptide at a slightly higher molecular weight than the wheat germ V* suggests that an alternative subunit may in fact be present. Frequently, bands in this region are seen in wheat seedling oxidase; however, these could be contaminants from the NADH dehydrogenase which elutes just prior to the oxidase. These contaminants could be more concentrated in wheat seedling oxidase because it is less pure. It needs to be pointed out that the HPLC-purified subunits used to generate antibodies are not free of all contaminants; NADH dehyrogenase peptides could be present (likely what is seen above subunit V* in lanes 1–3 of Figure 7.1) and react (faintly) only with wheat seedling because of a slight concentration difference. In any case, our inability to obtain N-terminal sequence of V*

hinders our ability to determine homology to existing proteins, which could clarify its identity.

We are currently attempting to produce polyclonal antibodies to V* which has been purified by preparative gel electrophoresis. The ability of these antibodies to immunoprecipitate the entire complex from a solution of solubilized membranes would provide stronger evidence of V* being a subunit. Moreover, we would be able to explore whether V* is developmentally expressed, by testing reactivity at different developmental stages. The antibodies would provide a tool for cloning; the developmental regulation of its expression could then be studied.

At least some of the nuclear-encoded subunits in plant oxidases are related to those in other eukaryotic oxidases. Immunoblot analysis and N-terminal sequencing shows that subunit IV from wheat oxidase is related to that from yeast oxidase, while the cDNA sequence of sweet potato Vc exhibits sequence similarity to beef heart oxidase subunit VIII. Therefore, it seems likely that as other plant subunits are sequenced, more similarities will be found. Homologies in the nuclear-encoded subunits between plants and other eukaryotes would seem to support the idea that only one endosymbiotic event took place, giving rise to the mitochondria of both plants and other eukaryotes. This is in contrast to the theory of Gray, which suggests a separate endosymbiotic event leading to plant mitochondria, based on similarities of ribosomal protein sequence. However, if bacterial enzymes started out more complex, and evolved to be simpler as they are today, then the theory of Gray might still apply, despite this evidence.

We have been unsuccessful in isolating the peptides necessary to confirm the presence of cysteine at residue 228; however, much evidence

indicates that it is indeed present. Graves *et al.* (1990) have shown that the amino acid sequence of ATPase subunit 9 reflects complete editing of its RNA template. While we have not unequivocally shown that the wheat subunit II amino acid sequence also is the result of completely edited RNA, the absence of the residue predicted from the unedited sequence suggests that, in the cases of Leu-161 at least, that protein is synthesized only from completely edited RNA. On this basis, and on the basis of spectral evidence presented in the next chapter supporting the presence of a normal Cu_A site, we conclude that residue 228 is indeed a cysteine in wheat oxidase.

Chapter 8 - Spectral characterization of cytochrome *c* oxidase from wheat

8.1 Introduction

Several studies in whole mitochondria have suggested that the plant oxidase has unique spectral properties. The α band of plant oxidases is blue-shifted from 604 nm in beef heart to 602 nm (Lance and Bonner, 1968). Furthermore, low temperature difference spectra of heme *a* show a split peak, with a maximum at 438 nm and a shoulder at 445 nm (Bendall and Bonner, 1966; Lance and Bonner, 1968). The Soret absorbance of heme A groups in general is expected to have some splitting due to the innate asymmetry of the heme substituents (formyl and vinyl on opposite corners), but usually these two contributions are so close together that they are not easily resolved, especially at room temperature. The accentuation of this split suggests differences in the heme *a* environment of plant oxidase relative to mammalian oxidase.

These previous studies of the spectral characteristics of plant cytochrome oxidases were hampered by the presence of other cytochromes in whole mitochondria. Because of the relatively large amounts of purified wheat oxidase now available, we have been able to examine its spectral properties without this difficulty. Additionally, we have extended our investigation of the spectral properties of the wheat oxidase to include Cu_A because of the possibility that the Cu_A site is disrupted by an amino acid substitution at one of the copper ligands (Bonen *et al.*, 1984).

If there are changes in the structure or environment of the heme *a* or Cu_A sites of plant oxidases, the function of the oxidase might be affected. In fact, some models for coupling of proton pumping to electron transfer are based on the premise that proton pumping activity is related to specific structural features of Cu_A or heme *a*. Gelles *et al.* (1986) have described a mechanism whereby shuttling of protons is promoted by redox-linked changes in the coordination of Cu_A. In support of this model, Nilsson *et al.* (1988) have observed that perturbation of the Cu_A site leads to a marked decrease in the proton-pumping activity of cytochrome oxidase. Another model is based on spectroscopic observations that indicate that the formyl group of heme *a* is hydrogen-bonded to a protein residue (Babcock and Callahan, 1983). The observed energy differential between the hydrogen bond to the formyl group in the oxidized and reduced forms of heme *a* is postulated to be a source of energy for pumping protons across the membrane.

A more recent model suggests that the heme a_3–Cu_B site provides the mechanistic coupling between electron transfer and proton translocation in cytochrome oxidase (Wikström, 1989). This model gains support from the report that the bacterial cytochrome *bo* acts as a proton pump (Puustinen *et al.*, 1989), despite the absence of Cu_A (Chepuri and Gennis, 1990). Also, the *b* and *o* hemes do not have the peripheral formyl group that is thought to be the critical feature of a heme *a*-mediated proton pump (Puustinen and Wikström, 1991). However, it can be argued that the proton pumping mechanism need not be the same in the two enzymes since *bo* is accepting electrons from ubiquinol rather than cytochrome *c*.

A comparative approach has been used to link structure to function in cytochrome oxidase. We have isolated cytochrome oxidase from maize

and wheat germ and determined the spectroscopic properties of the Cu_A and heme *a* sites. By comparing our results with those for the beef heart enzyme, and more recently from the simpler enzyme from the bacteria *Rhodobacter sphaeroides*, it is possible to pinpoint those structural characteristics that are common to the Cu_A and heme *a* sites of all cytochrome oxidases. These comparisons, in turn, may help us determine if these sites correlate with ability to pump protons.

The first target of our investigation was Cu_A. A broad absorption band at 830 nm and a characteristic g=2 signal in the EPR spectrum are normal features of oxidized Cu_A in beef heart oxidase. If these spectral properties are unchanged in wheat oxidase, it would indicate that the protein ligands of Cu_A are the same as in other oxidases, instead of altered as suggested by DNA sequence information (Bonen *et al.*, 1984).

The environments of hemes *a* and a_3 can be investigated by both visible and resonance Raman (RR) spectroscopies. The similarities and differences between the plant and mammalian systems are discussed in light of the possible involvement of heme *a* in proton pumping.

8.2 Materials and methods

8.2.1 Preparation of cytochrome oxidases

Beef heart cytochrome oxidase was isolated by using a modified Hartzell-Beinert protocol (Centeno, 1987). Wheat germ cytochrome oxidase was prepared according to the procedure outlined in chapter 6.

8.2.2 Spectral methods

EPR spectra of air-oxidized oxidase were recorded at X-band with a Bruker ER200D with 1 mW microwave power and 10G modulation amplitude. Samples were kept at 10 K by using an Oxford ESR-9 liquid helium cryostat.

Visible absorption spectra were obtained at room temperature with a Perkin-Elmer Lambda 6 spectrophotometer. Difference and second derivative spectra were calculated by the Perkin-Elmer UV Data Manager computer software. Samples of cytochrome oxidase were air-oxidized unless complete oxidation by potassium ferricyanide is indicated. Fully oxidized enzyme was separated from ferricyanide on a G-50 column equilibrated in 5% sucrose, 20 m*M* KH_2PO_4, pH 7.5, and 0.5 % lauryl maltoside. For cyanide binding, 5 m*M* KCN was incubated with the fully oxidized enzyme on ice for 6-12 hours. The mixed valence form (reduced heme *a*, oxidized heme a_3) was generated by reduction of the CN-bound enzyme with ascorbate/TMPD (5 m*M*/1 m*M*). The fully reduced form was generated from the air-oxidized enzyme with sodium dithionite. To bind CO, pure CO gas (1 mL) was slowly bubbled through dithionite-reduced oxidase over 5 minutes and the spectra taken immediately.

Unless otherwise noted, samples for resonance Raman spectroscopy were typically 25-70 μ*M* enzyme in a buffer system that consists of 100 m*M* KH_2PO_4 (pH 7.5), 0.5 *M* KCl, 5% sucrose and 4 m*M* lauryl maltoside. Reduction of cytochrome oxidase was achieved by addition of excess sodium dithionite under anaerobic conditions.

Excitation into the Soret bands of hemes *a* and a_3 was achieved by using either a Coherent INNOVA-90K Kr^+ laser (406.7 nm) or a Liconix 4240N He-Cd laser (441.6 nm). Visible band excitation at 602 nm was

obtained with a Spectra Physics Model 375 dye laser, with Rhodamine R6G as the dye, pumped by a Spectra Physics Model 164 Ar^+ laser. The fully oxidized samples were contained in a quartz spinning cell that was open to the atmosphere. These conditions, combined with low laser powers (5-10 mW) prevented the photoreduction of the enzyme. The fully reduced and mixed-valence samples were contained in 0.2 cm quartz cuvettes adapted for anaerobic work. Raman scattering was collected in a 90° geometry and dispersed in a Spex 1401 spectrometer. The detector was a thermoelectrically-cooled photomultiplier tube (RCA 34031A) operated in the photon counting mode. Data acquisition and signal averaging were accomplished with a DEC LSI11 computer. Spectral resolution was 5-6 cm^{-1}. The temperature of the samples was kept between –5°C and 4°C by flowing cold nitrogen gas around the sample cell. O_2 consumption measurements showed that prolonged exposure to the laser beam decreased the activity of wheat germ cytochrome oxidase only by 10–25%. No changes in the absorption spectrum of the enzyme were detected after the Raman experiment.

8.3 Results

8.3.1 EPR spectrum of wheat cytochrome oxidase

The EPR spectra of the purified wheat germ and maize oxidases are shown in Figure 8.1. The g=2 signals due to Cu_A are very similar and show the small copper nuclear hyperfine coupling typical of other cytochrome oxidases. The g_z signal at 3 and the g_y at 2.29 are normal for the low-spin iron of cytochrome a. The weak signal at g=4.3 indicates very low levels of contaminating metals, such as non-integral iron, and since there are no

Figure 8.1: EPR spectra of purified maize (60 μM, upper trace) and wheat germ (64 μM, lower trace) cytochrome *c* oxidase in FPLC buffer (described in the legend of Fig. 6.3) with 0.5 *M* KCl. Spectra were run at 10 K as described in the section 8.2.2.

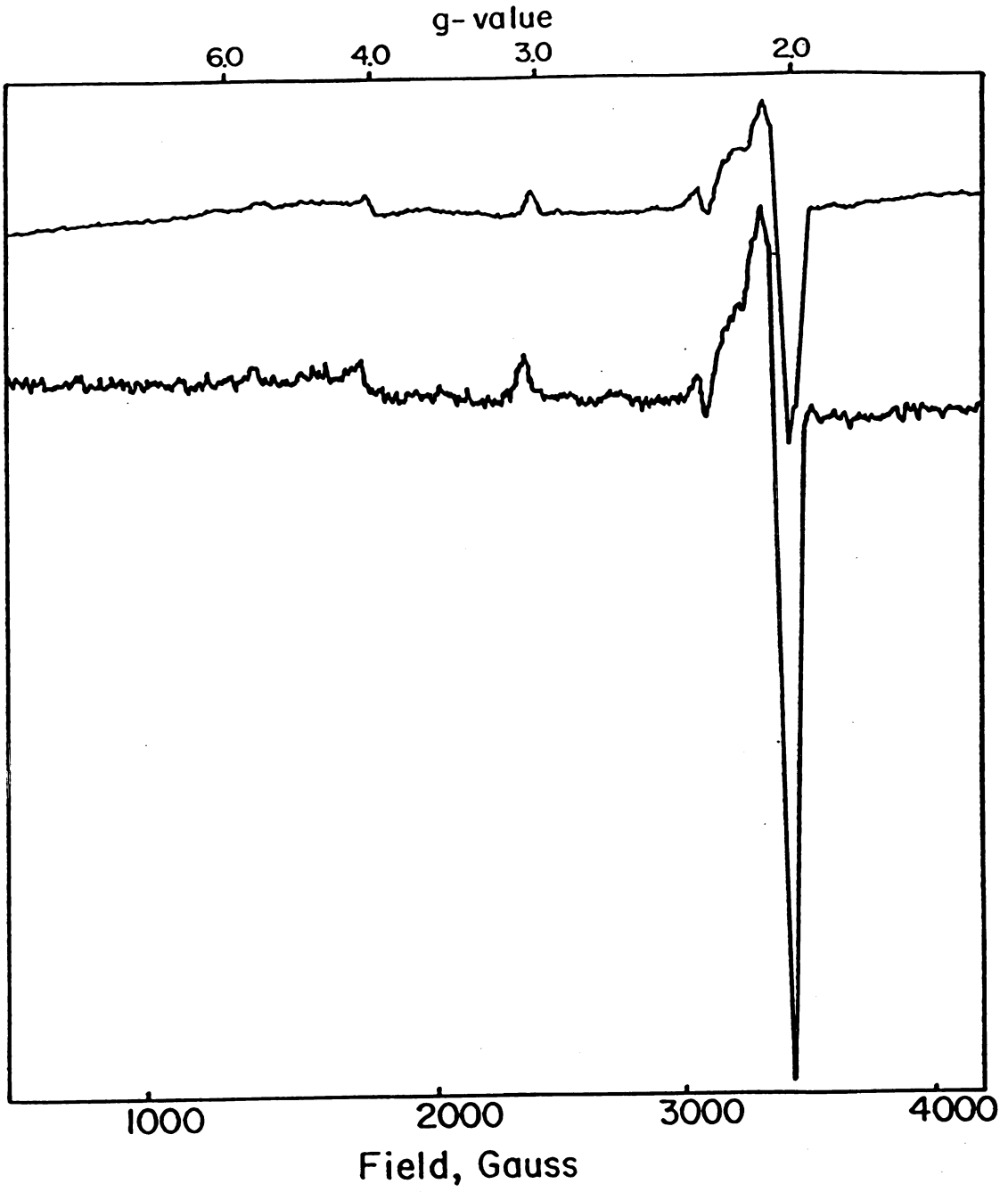


Figure 8.1

further major signals, cytochromes a_3 and Cu_B are EPR-silent in the resting enzyme. Thus, the EPR spectra demonstrate both the similarity of the wheat germ oxidase to other oxidases as well as the purity and integrity of the enzyme.

8.3.2 Visible absorption spectra

The broad absorption band at 830 nm due to oxidized Cu_A is shown in Figure 8.2. This band appears similar to the Cu_A signal in beef heart oxidase (not shown), further supporting the EPR evidence that the environment of Cu_A is not altered in wheat oxidase.

The complete visible spectra of air-oxidized and dithionite-reduced wheat oxidase were presented in chapter 6 (Figure 6.4) and are similar to those of purified maize oxidase (dePaula *et al.*, 1990). The spectra show that the Soret and α bands of the reduced enzymes, which occur at 441–442 and 602 nm, respectively, are blue-shifted relative to the equivalent bands in the beef heart enzyme, which occur at 443 and 604 nm, respectively. The visible absorption bands of the reduced plant enzymes are still, however, considerably red-shifted from the absorption bands of six-coordinate heme a^{2+} model compounds in protic solvents (Babcock and Callahan, 1983). An important factor in producing this shift is proposed to be the existence of a hydrogen bond between the protein and the heme *a* formyl. The greater shift in beef heart oxidase suggests a stronger hydrogen bond in beef heart than in wheat oxidase; however, other changes in the immediate protein environment may be involved.

The plant oxidase is mostly oxidized after purification; however, while the α band of oxidized beef heart oxidase is at 598 nm, this peak appears at 601-602 nm in "air-oxidized" wheat oxidase (Figure 8.3). The



Figure 8.2: 830 nm absorption band of oxidized (upper) and reduced (lower) wheat cytochrome oxidase. Oxidase was 70 μM in FPLC buffer (described in the legend of Figure 6.3).

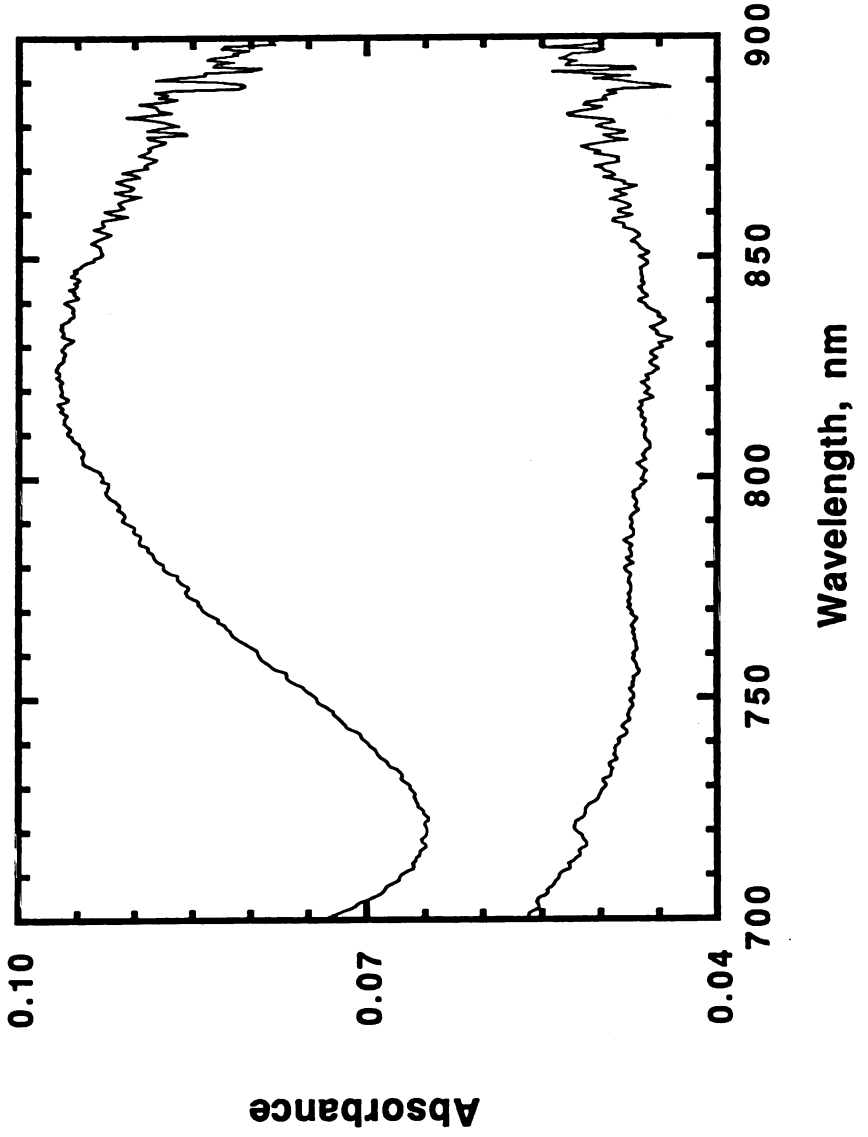


Figure 8.2

Figure 8.3: Comparison of α band absorbance in air-oxidized (---) and ferricyanide-oxidized (—) wheat germ oxidase. The vertical line is centered on the absorption maximum of the ferricyanide reduced at 598 nm, blue-shifted from the absorption maximum of the air-oxidized at 602 nm, indicating partial reduction of the air-oxidized form. Oxidase was 10.8 μM in FPLC buffer (described in the legend of Figure 6.3).

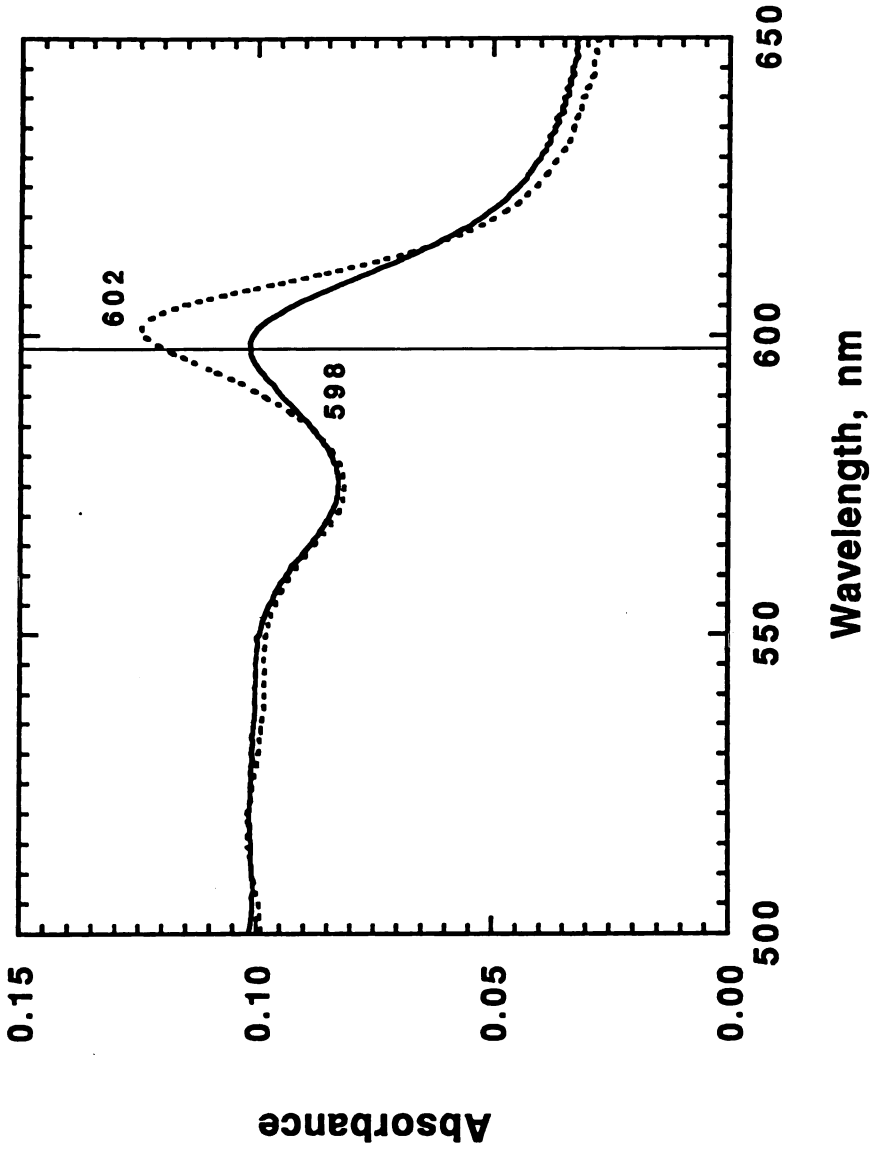

602
598
650
600
550
500
Wavelength, nm
0.15
0.10
0.05
0.00
Absorbance

Figure 8.3

intensity and position of the 602 nm peak (which is due mostly to heme *a*) suggests partial reduction (~20%) of heme *a* in the purified enzyme. Complete oxidation with potassium ferricyanide shifts the maximum to 598-599 nm (Figure 8.3). The intensity of the 830 nm Cu_A band, however, does not increase upon ferricyanide oxidation, indicating that it is fully oxidized even though heme *a* is partly reduced. Heme *a* does not become re-reduced after the ferricyanide is removed by gel filtration.

While the 602 nm α peak of the reduced enzyme is 80% due to heme *a*, the 442 nm Soret peak is composed of roughly equal contributions of *a* and a_3. The spectrum of heme *a* alone can be obtained by subtracting the spectrum of CN-bound fully oxidized oxidase from the spectrum of the the CN-bound sample reduced with ascorbate/TMPD. Since cyanide remains bound to oxidized a_3 under both these conditions, the contribution of a_3 cancels out upon subtraction, leaving a reduced minus oxidized spectrum of heme *a* alone. When this procedure is done with beef heart oxidase, we observe the heme *a* peak at 446 nm (Figure 8.4A), in general agreement with Vanneste (1966).

In the purified wheat oxidase, heme *a* is partially reduced and becomes more reduced during incubation with cyanide. Thus, subtracting its spectrum from a fully reduced oxidase spectrum yields only a portion of the total heme *a* content. In an attempt to overcome this problem, the enzyme was oxidized with ferricyanide and then separated from it, yielding a stable fully oxidized enzyme. However, when this fully oxidized enzyme is incubated with cyanide, heme *a* is again reduced. The 598 nm peak shifts to 602 nm and increases dramatically in intensity, indicating a heme *a* reduction level of near 70% (Figure 8.5). Thus, only about 30% of the heme *a* is observed in the difference spectrum. Under these conditions, we can

Figure 8.4: Difference spectrum of heme *a* in beef heart (**A**) and wheat germ (**B**) oxidases. Spectra were obtained by the cyanide binding procedure described in section 8.3.2 using 5.6 μM beef heart oxidase and 7.0 μM wheat germ oxidase in FPLC buffer (described in the legend of Figure 6.3). The Soret band in the wheat enzyme is split, with maxima at 440 and 444 nm, while the single beef heart oxidase maximum occurs at 446 nm.

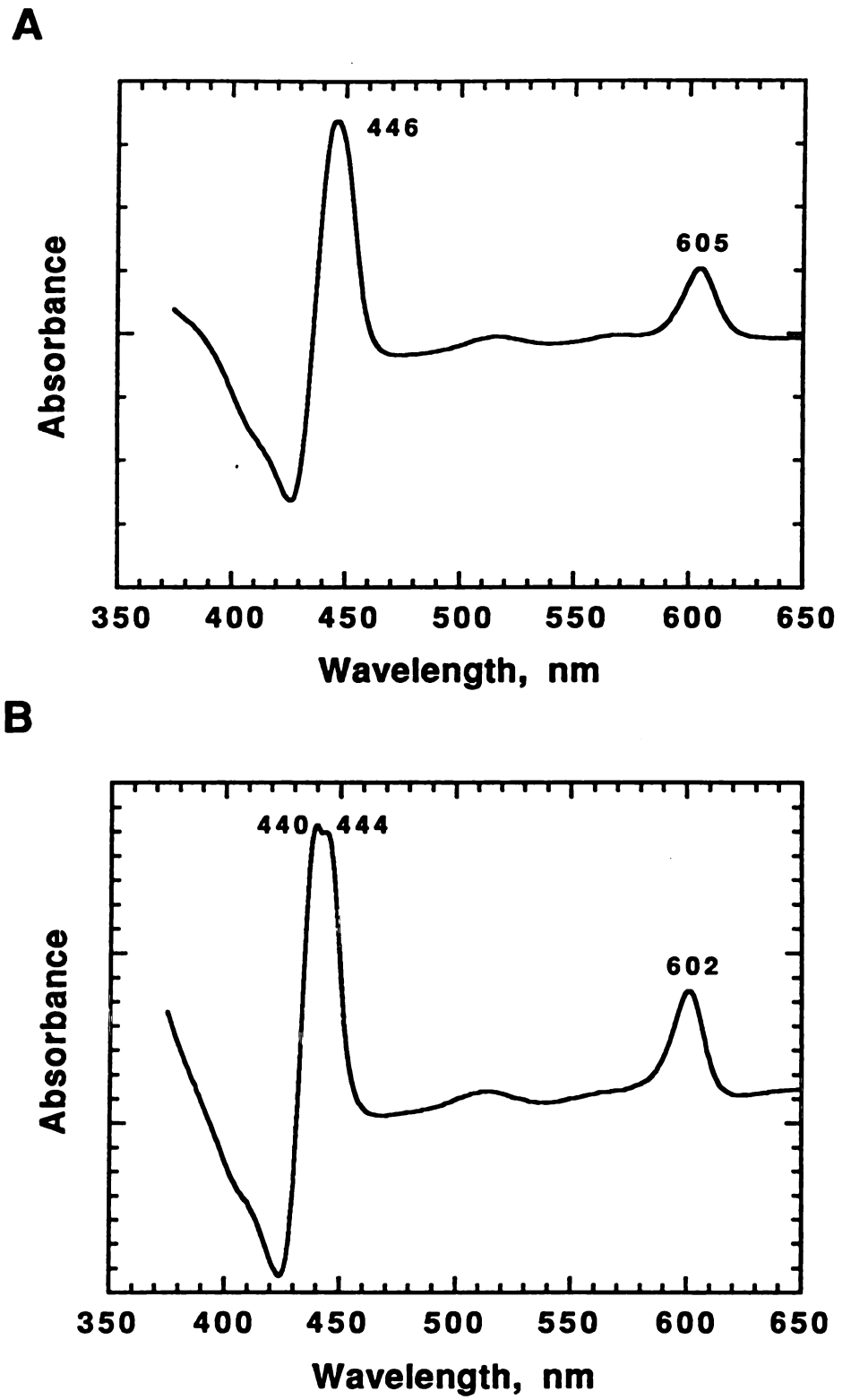

A
446
605
Absorbance
350
400
450
500
550
600
650
Wavelength, nm
B
440
444
602
Absorbance
350
400
450
500
550
600
650
Wavelength, nm

Figure 8.4

Figure 8.5: Effect of cyanide binding on the absorption spectrum of wheat cytochrome oxidase. The absorption maxima of fully oxidized oxidase (prepared by ferricyanide oxidation and removal as described in section 8.2.2) was at 420 nm (——), while the CN-bound oxidized absorbed at 432 nm (——) and the ascorbate TMPD-reduced CN-bound absorbed at 437 nm (----). Oxidase was 7.0 μM in FPLC buffer (described in the legend of Figure 6.3).

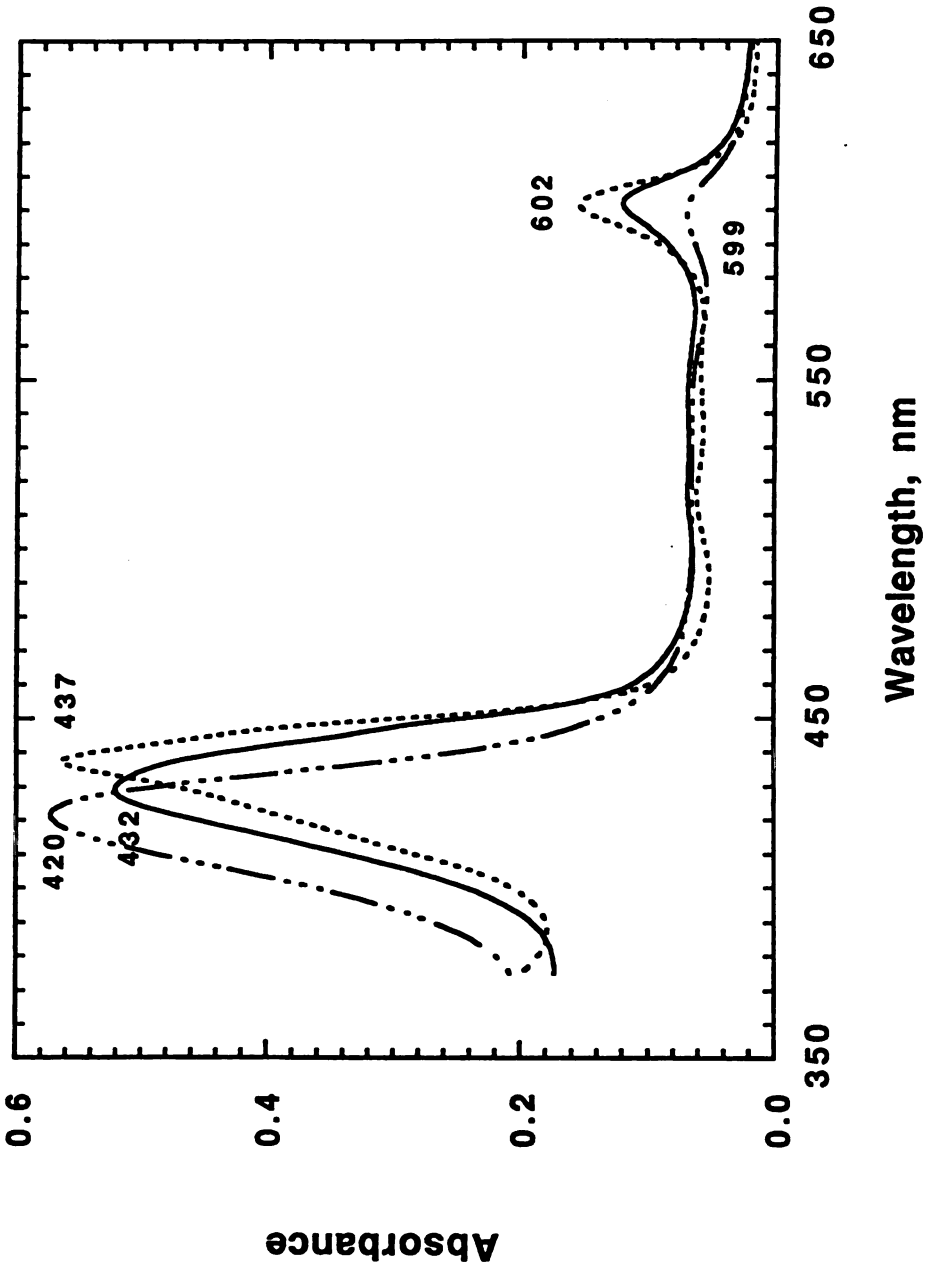

Absorbance
0.0
0.2
0.4
0.6
350
450
550
650
Wavelength, nm
420
432
437
599
602

Figure 8.5

observe a partial splitting of heme *a*, with maxima at 440 and 444 nm (Figure 8.4B).

Spectral peaks due to partially overlapping contributions can sometimes be deconvoluted by second derivative analysis. Sherman *et al.* (1991) have recently used this technique to resolve the heme contributions to the spectrum of CN or CO-bound cytochrome oxidase. The second derivative of the wheat heme *a* difference spectrum in the presence of CN reveals two components with maxima at 438 and 446 nm (Figure 8.6B), similar to values obtained at low temperature in plant mitochondria. In contrast, the second derivative of the beef heart oxidase heme *a* difference spectrum under the same conditions shows maxima at 443 and 451 (Figure 8.6B), considerably shifted from the wheat values. This strongly suggests a difference in heme *a* between wheat and beef heart oxidase even though no quantitative conclusions can be drawn with regard to the relative amounts of the absorbing species.

Analysis of the fully reduced CO-bound enzyme also provides additional evidence for the spectral shift of heme *a* and possibly a_3. Binding of CO shifts the Soret peak from 442 to 434 nm (Figure 8.7), compared to 432 in beef heart. Since heme *a* is reduced both before and after CO binding, the difference in the wavelength maxima of the CO bound form may be due to a difference in a_3. However, the second derivative of the CO-bound spectra indicates that the CO-a_3 complex actually absorbs at 429 nm in both enzymes (Figure 8.8A). Peaks at components at 429 nm due to CO-bound a_3, and at 438 and 448 nm are due to reduced heme *a* in the wheat oxidase, while these heme *a* components occur at 442 and 451 nm in beef heart oxidase (Sherman *et al.*, 1991, Figure 8.8B). These compnents are similar to those seen for heme *a* in the CN-spectra, strongly suggesting a change in

Figure 8.6: Second derivative analysis of heme *a* difference spectra in beef heart (**A**) and wheat germ (**B**) oxidase. The maxima at 438 and 446 nm in wheat germ oxidase and at 443 and 451 in beef heart oxidase represent the splitting of the heme *a* absorption due to the asymmetry of the heme ring substituents. Oxidases were as described in the legends of Figures 8.4.

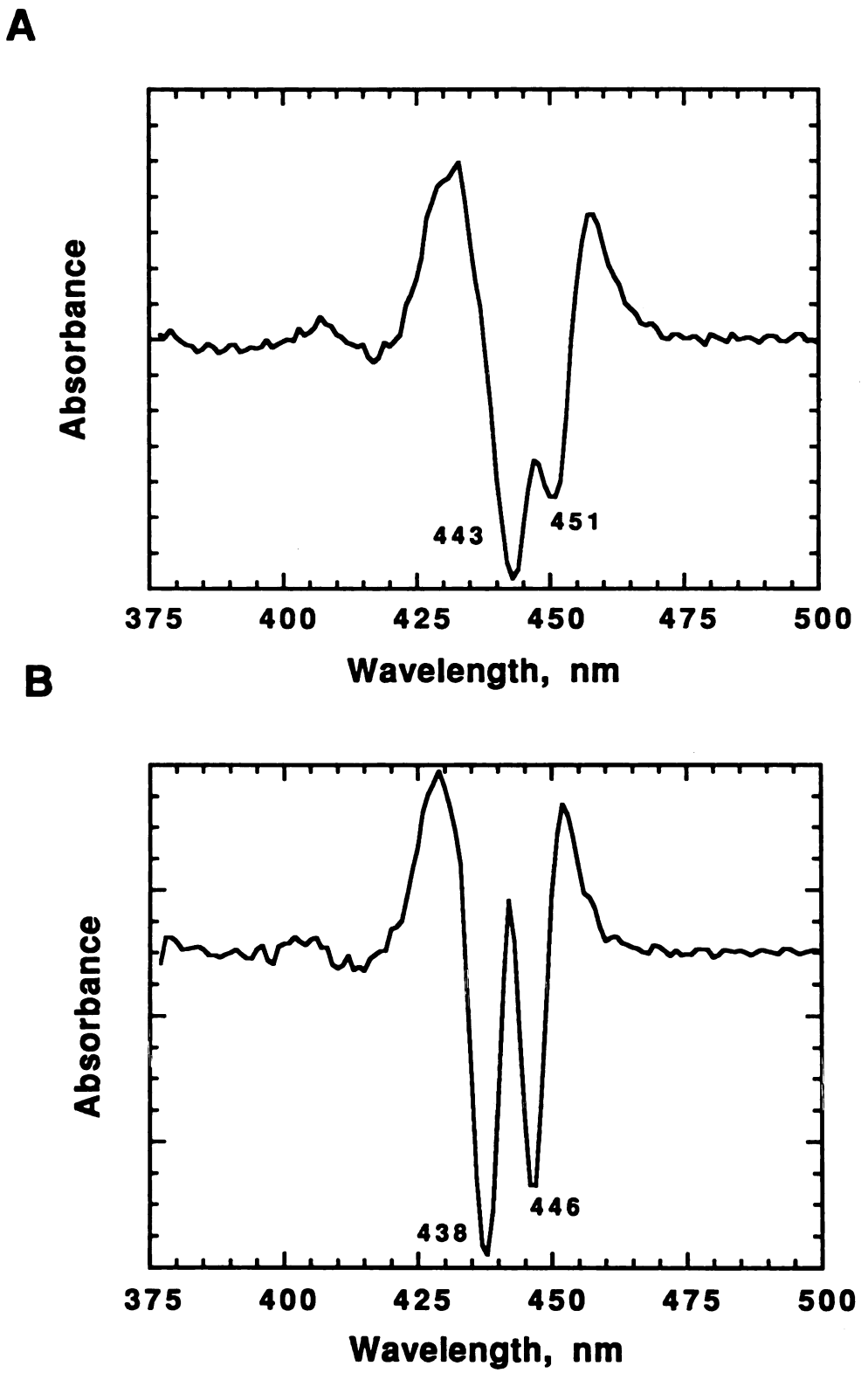

A
Absorbance
443
451
375
400
425
450
475
500
Wavelength, nm
B
Absorbance
438
446
375
400
425
450
475
500
Wavelength, nm

Figure 8.6

Figure 8.7: Effect of CO binding on the absorption spectra of dithionite-reduced wheat germ cytochrome oxidase. CO binding was performed as described in section 8.2.2. The Soret peak shifts from 442 nm in the reduced to 434 nm in the CO-bound form. Oxidase was 8.0 μM in FPLC buffer (described in the legend of Figure 6.3).

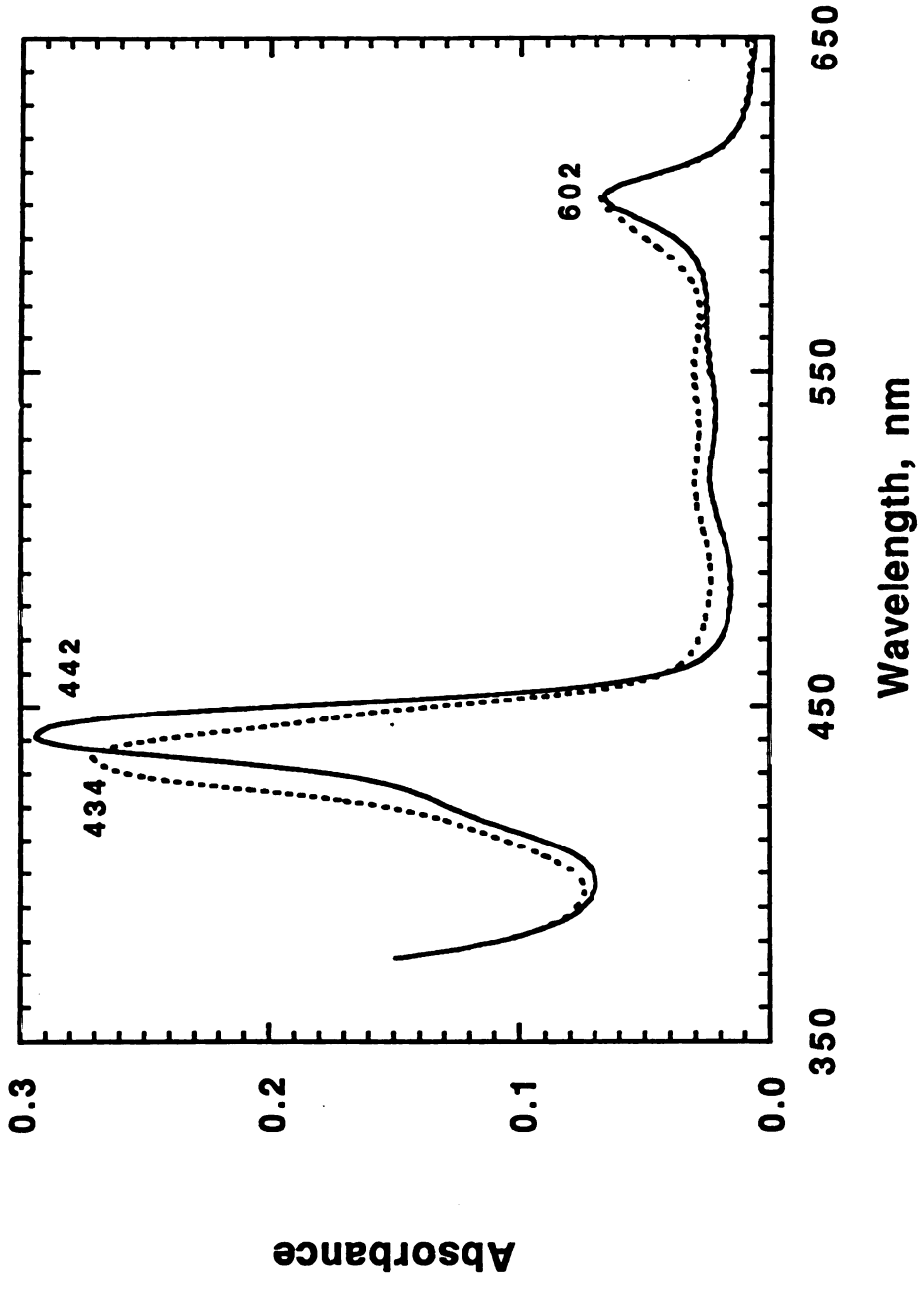

Absorbance
0.3
0.2
0.1
0.0
350
450
550
650
Wavelength, nm
442
434
602

Figure 8.7

Figure 8.8: Second derivative analysis of absorption spectra of CO-bound cytochrome oxidase from wheat germ (**A**) and beef heart (**B**). The maxima near 429 nm in both cases is due to the CO-bound heme a_3, while the other two maxima (438 and 448 nm in wheat germ, 443 and 451 in beef heart oxidase) are due to reduced heme a. Wheat oxidase was as described in Figure 8.7; beef heart oxidase was 5.4 μM in FPLC buffer (described in the legend of Figure 6.3).

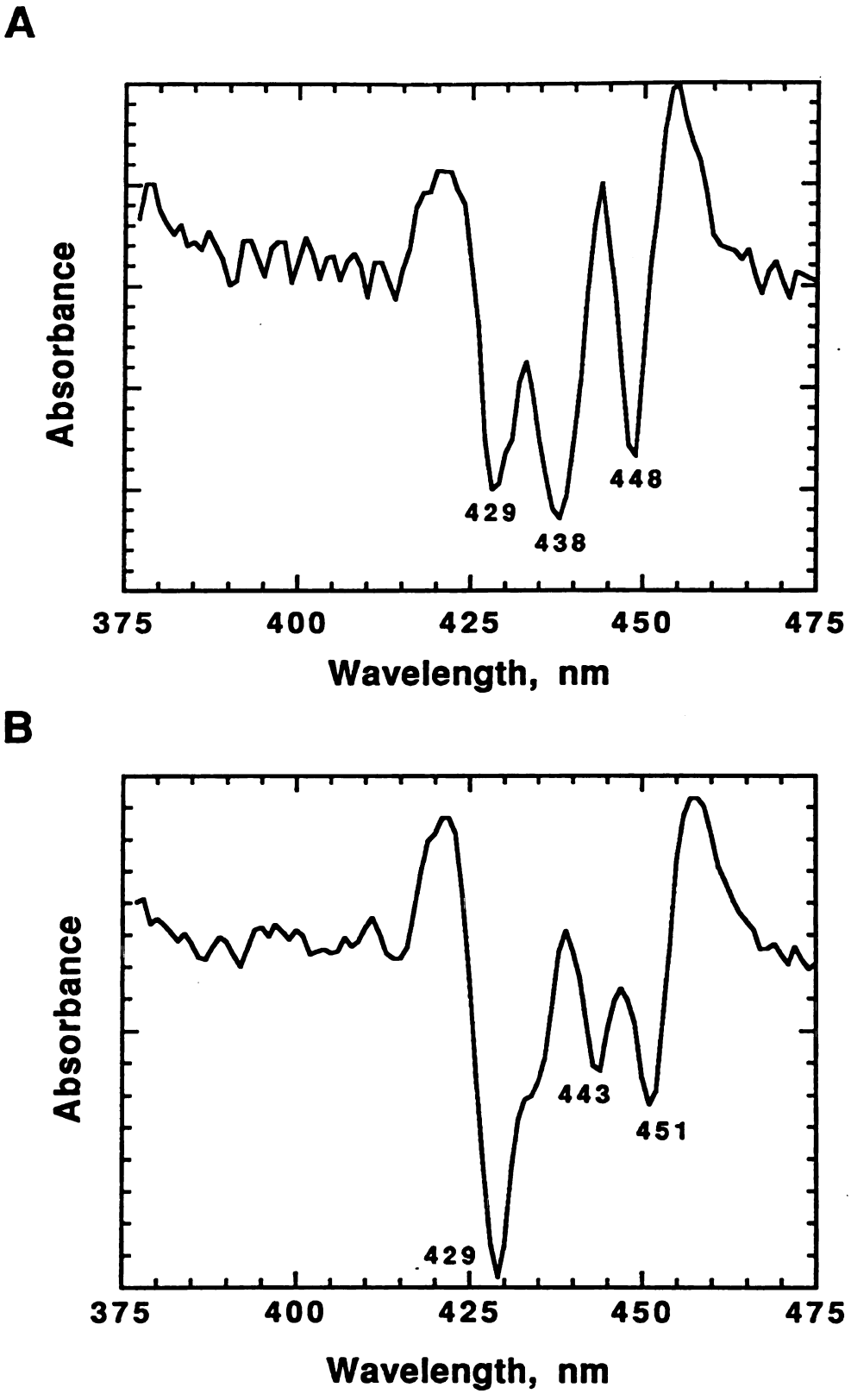

A
Absorbance
429
438
448
375
400
425
450
475
Wavelength, nm
B
Absorbance
429
443
451
375
400
425
450
475
Wavelength, nm

Figure 8.8

the environment of heme *a* relative to beef heart oxidase. Interestingly, CO appears to bind stably in wheat and is not removed by bubbling with air, in contrast to the report of Naik *et al.* (1991) using whole plant tissue.

8.3.3 Resonance Raman spectroscopy

Hemes *a* and a_3 of wheat germ cytochrome *c* oxidase were investigated by resonance Raman spectroscopy. By comparing our results to those from the well-characterized beef heart enzyme (see Babcock, 1988, for a comprehensive review of resonance Raman studies of cytochrome oxidase), we were able to infer structural features of the chromophores in the plant enzymes.

It has already been shown that almost all vibrational bands of the beef heart cytochrome oxidase have direct analogs in the maize system (Dutch *et al.*, 1987). The wheat (Figure 8.9, 8.10) and maize spectra are very similar, suggesting that the hemes in the maize and wheat germ enzymes have similar environments. Therefore, we decided to focus on the plant enzyme from wheat germ for comparison with the mammalian enzyme, since we could produce greater quantities of enzyme in pure form and therefore obtain spectra with less noise and greater resolution.

The strategy for the assignment of the resonance Raman spectra of wheat germ cytochrome oxidase takes advantage of the selective enhancement of heme *a* and heme a_3 modes as a function of redox state and excitation wavelengths (reviewed by Babcock, 1988). The intensity of the weak Raman effect is enhanced by absorption of light; therefore, modes associated with either the reduced or oxidized state will be enhanced when the excitation wavelength matches the absorption wavelength maxima for that state. Thus, fully oxidized samples excited at 406.7 nm show vibrations

Figure 8.9: Resonance Raman spectra of wheat germ cytochrome oxidase: (a) oxidized enzyme, excited at 406.7 nm (10 mW); (b) dithionite-reduced enzyme, excited at 406.7 nm (5 mW); (c) dithionite-reduced enzyme, excited at 441.6 nm (10 mW). Further experimental details are described in section 4.2.2. Of particular interest are the 1657 cm^{-1} mode in (a) due to the oxidized formyl (absent in the reduced) and the appearance of 1610 cm^{-1} in (c) due to the reduced formyl. The 1627 cm^{-1} mode in (c) corresponds to the vinyl group in the reduced heme *a*.

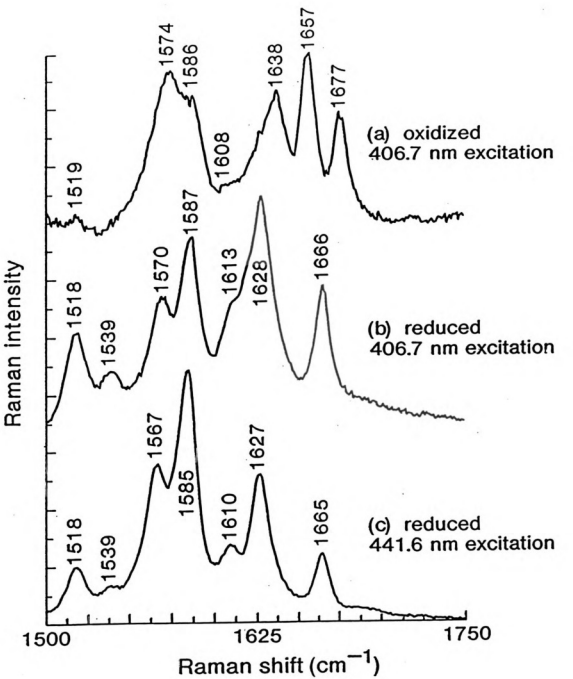

1574
1586
1638
1657
1677
1608
1519
(a) oxidized
406.7 nm excitation
1587
1570
1613
1628
1666
1518
1539
(b) reduced
406.7 nm excitation
1567
1585
1627
1610
1665
1518
1539
(c) reduced
441.6 nm excitation
Raman intensity
1500
1625
1750
Raman shift (cm^{-1})

Figure 8.9

Figure 8.10: Resonance Raman spectra of mixed-valence, CN^--treated wheat germ cytochrome oxidase excited at: (a) 441.6 nm (10 mW); and (b) 602 nm (100 mW). Further experimental details are described in section 8.2.2. The presence of both the 1610 and 1628 cm^{-1} modes confirms that they are due to heme a, not a_3 formyl and vinyl groups respectively. The enhancement of the 1618 cm^{-1} band with visible (602 nm) excitation only indicates that it is composed of a C–C stretching vibration of the porphyrin core (υ_{10} mode, see Babcock, 1988).

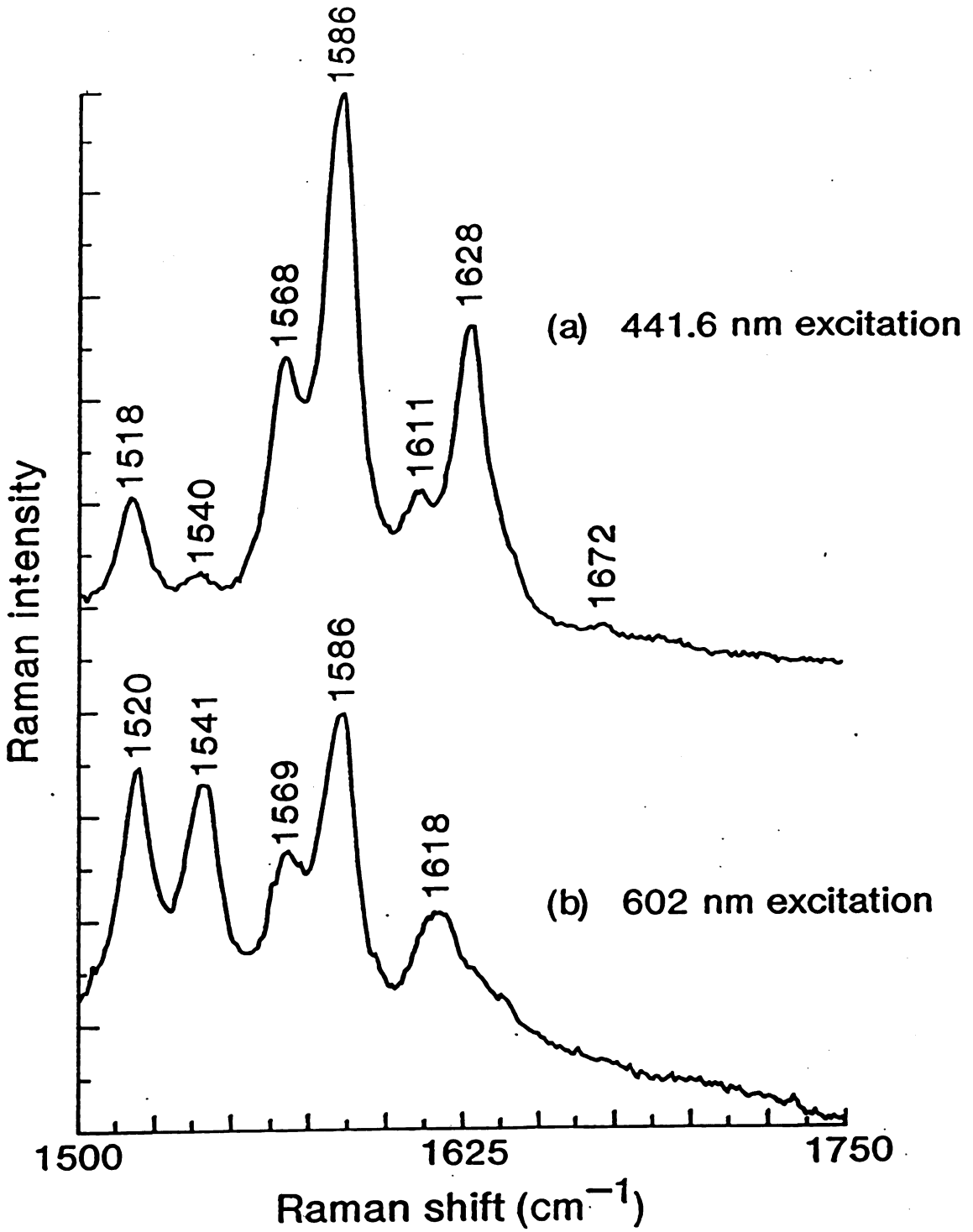

1586
1568
1628
1518
1611
1540
1672
(a) 441.6 nm excitation
Raman intensity
1520
1541
1586
1569
1618
(b) 602 nm excitation
1500
1625
1750
Raman shift (cm^{-1})

Figure 8.10

due to oxidized hemes *a* and a_3. Likewise, fully reduced samples probed at 406.7 and in particular 441.6 nm bring out vibrations from reduced hemes *a* and a_3. The CN^--treated, mixed-valence samples exist as $a^{2+}(a_3{}^{3+}CN^-)$ and, consequently, when probed at 441.6 and 602 nm, allow for the observation of reduced heme *a* modes only, since reduced heme *a* but not oxidized a_3 absorbs light significantly at those wavelengths.

We can assign the 1665 cm^{-1} mode to a heme a_3 vibration because it is enhanced by Soret excitation of the fully reduced enzyme (Figure 8.9b,c) but not by Soret or visible excitation of the mixed-valence enzyme (Figure 8.10a,b). A comparison with the resonance Raman spectra of mammalian cytochrome oxidase further indicates that the 1665 cm^{-1} mode is comprised mostly of a C=O stretching vibration of the formyl group of heme $a_3{}^{2+}$.

Since the 1610 and 1628 cm^{-1} modes appear in both the reduced and mixed-valence (Figures 8.9c, 8.10a), they must be due to a^{2+} and not $a_3{}^{2+}$. These vibrations can be assigned by analogy to the RR spectra of mammalian cytochrome oxidase as arising from a vinyl C=C stretch (1628 cm^{-1}) and a formyl C=O stretch (1610 cm^{-1}). The latter vibrates at the same frequency as in beef heart, but shows weaker enhancement.

The remaining assignments of our RR spectra are summarized in Table 8.1, where data from wheat germ and beef heart cytochrome oxidases are compared. In the table, υ denotes a stretching vibration; C=O and C=C refer to the formyl and vinyl groups, respectively. The numbered vibrations are composed mostly of C–C stretches of the porphyrin ring (see Kitagawa and Osaki (1987) for a review). Modes υ_2, υ_3, υ_{10}, and υ_{19} vary with the size of the porphyrin central core. Mode υ_4 reflects the extent of oxidation of the Fe ion. The numbering system adopted here is that of Abe (1986). Of particular note, as discussed below, are the differences between the plant

Table 8.1: Assignment of high-frequency vibrations (cm^{-1}) in plant cytochrome oxidase and comparisons with the mammalian enzyme.

	Reduced				Oxidized			
	Wheat Germ		Beef Heart		Wheat Germ		Beef Heart	
Mode	a^{2+}	a_3^{2+}	a^{2+}	a_3^{2+}	a^{3+}	a_3^{3+}	a^{3+}	a_3^{3+}
$\upsilon_{C=O}$	1610	1665	1610	1665	1657	1677	1650	1676
$\upsilon_{C=C}$	1627	1627	1623	1623				
υ_{10}	1618	n.o.[a]	1614	1607	1638	n.o.	1641	1615
υ_2	1587	n.o.	1585	1579	1586	1576	1590	1572
υ_{19}	1586		1586					
$\upsilon_{38}(1)$	1567		1567					
$\upsilon_{38}(2)$	1541		1546					
υ_{11}	1518		1519					
υ_3	1492	n.o.	1491	1464	1503	1477	1506	1478
υ_4	1356	1361	1354	1356	1371	1371	1373	1373

[a]n.o. = not observed due to overlapping bands.

and mammalian enzymes with respect to the frequency of the oxidized heme *a* formyl vibration (1657 cm^{-1} versus 1650 cm^{-1} respectively) and the reduced heme *a* vinyl vibration (1627 cm^{-1} versus 1623 cm^{-1} respectively).

8.4 Discussion

8.4.1 Cu_A environment

The similarity of the *g*=2 EPR signal and the 830 nm absorption band of wheat and maize to beef heart cytochrome oxidases suggests that Cu_A has the same ligands in both enzymes. Thus it is unlikely that an amino acid substitution has taken place at the Cu_A site. This result supports the evidence that mRNA editing has occurred at codon 228 of subunit II to restore the conserved cysteine at this site (Covello and Gray, 1989). Amino acid sequence information from wheat oxidase subunit II is necessary to confirm this observation; however, as discussed in chapter 7, the confirmation of editing at other sites makes it highly likely that this site is edited. Since the EPR and visible spectra indicate a normal Cu_A, wheat oxidase does not challenge the theory of Gelles *et al.* (1986) regarding a central role of this metal in proton pumping. We have been unable to demonstrate the enzyme's proton pumping capabilities, but the normal ADP/O ratios measured for intact wheat seedling mitochondria suggest that it does pump protons. Furthermore, purified enzyme reconstituted into asolectin vesicles has good respiratory control ratios (5–6, data not shown).

8.4.2 Visible absorption spectra

The blue-shift observed in the α and Soret peaks of plant oxidases has previously been attributed to heme *a*, since low temperature difference spectra of heme *a* were different from beef heart, while heme a_3 was the same (Lance and Bonner, 1968). We also observe differences in absorption due to heme *a* in spectra with CN or CO bound to heme a_3. Although we were unable to perform our spectra at low temperature, we observe a small splitting of the Soret heme *a* difference peak, with contributions of the two components at similar wavelengths to those observed in whole mitochondria (Lance and Bonner, 1968). The reliability of our result would be greater if the experiment were in fact done at low temperature, and if the problem of the reduction of heme *a* upon CN binding were solved. This problem is a particularly difficult one because after ferricyanide reduction, the original source of electrons in the protein should have been oxidized as well. Since beef heart oxidase did not show this CN-induced reduction of heme *a* under the same conditions, it seems unlikely that the KCN itself is the electron source; however, it could be the source of electrons for reduction of a contaminant of the plant enzyme that is not present in the beef heart enzyme. Alternatively, if the redox potential of the wheat heme *a* were high enough, electrons might be extracted from the buffer, but previous reports have shown the redox potential of heme *a* to be slightly lower in plant mitochondria (Dutton and Storey, 1971). Possibly the binding of CN to a_3 causes some change in heme *a* that alters its redox potential. Further studies will be required to evaluate these possibilities; nevertheless, this unusual behavior of heme *a* in plant oxidase undoubtedly reflects some unique aspects of the heme *a* environment.

The environment of heme a_3 may also be altered in the plant oxidase, as suggested by CO-spectra, but we do not yet have direct evidence for this.

8.4.3 Resonance Raman spectra

Two main differences are observed between the RR spectra of beef heart and wheat oxidase. Some vibrational modes may be more strongly enhanced in one enzyme, for instance the 1610 cm^{-1} formyl mode in beef heart oxidase. Others modes shift frequency, such as the vinyl mode of the wheat enzyme. However, interpretation of frequency and intensity changes in RR spectra remain somewhat speculative.

8.4.3.1 Vinyl modes

The vibrational bands arising from the C=C stretch of the vinyl groups of both heme *a* and a_3 occur at higher frequency in the plant enzymes (1627 cm-1) than in the mammalian enzyme (1623 cm^{-1}). This may be due to the vinyl group and the porphyrin not being coplanar, reducing conjugation between the two π systems (Gersonde *et al.*, 1989). The vinyl group may be rotated more out of the porphyrin plane in the plant heme *a* than in mammalian heme *a*.

8.4.3.1 Formyl modes

The vibrational mode of the formyl group of heme *a* is altered in both frequency and intensity in the plant enzyme. The frequency of the C=O vibration reflects interactions between the heme *a* formyl group and its environment. The interaction may be a hydrogen bond to the protein (Babcock and Callahan, 1983) or resonance energy transfer with a nearby water molecule (Sassaroli *et al.*, 1989). The heme *a*–protein interactions are of similar strength in the reduced plant and the mammalian oxidases, since the formyl C=O vibrations both occur at 1610 cm^{-1}. In the fully

oxidized form, however, the interaction is weaker in plant heme *a* ($\upsilon_{C=O}$ = 1657 cm^{-1}) than in mammalian heme *a* ($\upsilon_{C=O}$ = 1650 cm^{-1}).

The RR spectrum of plant reduced heme *a* reveals another unique feature: the 1610 cm^{-1} formyl C=O mode is strongly enhanced in the beef heart enzyme but only weakly enhanced in the reduced forms of the maize and wheat germ enzymes. Enhancement of the C=O vibration may be determined by the degree of conjugation of the formyl group with the porphyrin π system (dePaula *et al.*, 1990). Therefore the formyl of heme *a* in the plant oxidase may be in a different conformation than in beef heart, supporting our earlier spectral data that heme *a* is in a different protein environment. Alternative explanations are considered below.

8.4.4 Reconciling the absorption and RR data

We have shown that the heme *a* absorption bands of reduced plant oxidases are blue-shifted with respect to reduced mammalian cytochrome oxidases. According to the model put forth by Babcock and Callahan (1983), this means that the hydrogen bond between the protein and the formyl group of heme *a* is weaker in the plant system. However, according to the frequency of the formyl C=O stretching vibration (1610 cm^{-1}), the strength of this putative hydrogen bond is similar in plant and mammalian oxidases. Because the 1610 cm^{-1} intensity is significantly lower in plants, dePaula *et al.* (1990) has suggested that the formyl group conjugates only weakly with the porphyrin group in plant oxidases because it is not coplanar with the porphyrin ring. However, there may be other explanations.

In oxidase from wild-type *Rhodobacter sphaeroides*, the α peak occurs at 607 mn, while the 1610 cm^{-1} is more strongly enhanced than in beef heart (Hosler *et al.*, 1992). Therefore, perhaps the intensity of the

1610 cm^{-1}, not its frequency, relates to the absorption maxima of the α peak. However, the α peak of a mutant substituting phenylalanine for tyrosine at residue 414 of subunit I is shifted to 611 nm while the intensity of the 1610 cm^{-1} mode is unchanged from wild-type (Fetter *et al.*, 1992). Thus, it is likely that the position of the heme *a* absorption bands depend on more factors than just the environment of the formyl group.

Another possible factor in the differences observed in the intensity of the 1610 cm^{-1} mode might be the excitation wavelength. Only one component of the heme *a* absorbance might be involved in this vibration, perhaps the lower wavelength, 438 nm in wheat and 443 nm in beef heart. Thus the excitation wavelength (441.7 nm) is closer to "resonance" with the beef heart at 443 nm than the wheat at 438 nm, enhancing its formyl vibration more than the wheat. If this is so, then the wheat heme *a* formyl may not be very different from beef heart in bond strength and degree of conjugation to the porphyrin ring. At any rate, the available data suggests that other factors besides the heme *a* formyl hydrogen bond strength must be considered in interpreting the position of the maximum of the α band of cytochrome oxidase.

8.4.5 Implications for proton pumping activity

Our results indicate that the nature of the interaction between the formyl C=O group of heme *a* and its environment may be different in the plant and mammalian cytochrome oxidases. The formyl–protein interactions are of similar strength in the reduced forms of the plant and mammalian enzymes, but in the oxidized form the C=O group vibrates at a higher frequency in wheat germ compared to the beef heart enzyme. Thus the change in bond energy is greater for the plant enzyme between the

oxidized and reduced states. According to Babcock and Callahan (1983), this suggests that the wheat enzyme would be an effective proton pump (Babcock and Callahan, 1983). Whether of not this energy change is involved in proton pumping, it appears to be a conserved feature of the oxidase which may have functional significance.

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