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CONVERSION OF THE MITOCHONDRIAL GENE FOR MAMMALIAN CYTOCHROME OXIDASE SUBUNIT II TO A UNIVERSAL EQUIVALENT AND EXPRESSION IN <u>E. COLI, IN VITRO, AND IN XENOPUS</u> OOCYTES

Ву

Jianli Cao

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Biochemistry

ABSTRACT

CONVERSION OF THE MITOCHONDRIAL GENE FOR MAMMALIAN CYTOCHROME OXIDASE SUBUNIT II TO A UNIVERSAL EQUIVALENT AND EXPRESSION IN <u>E. COLI, IN VITRO, AND IN XENOPUS</u> OOCYTES

By

Jianli Cao

Cytochrome oxidase is a multisubunit enzyme that is intrinsic to the inner mitochondrial membrane (Tzagoloff, 1982). It is capable of transporting electrons from cytochrome <u>c</u> to molecular oxygen and translocating protons across the mitochondrial membrane to establish a transmembrane electrochemical gradient, which in turn is used for the synthesis of ATP (Wikstrom, 1977, 1984). Although each of the 13 peptides in the mammalian cytochrome oxidase complex is likely to have an important role, there is evidence that the mitochondrially encoded subunits comprise the essential catalytic unit (Kagawa, 1967; Thompson et al., 1982; Suarez et al., 1984). To begin to assess the independent structural and functional characteristics of these subunits, the cloned gene for rat liver cytochrome \underline{c} oxidase subunit II (coxII) has been converted into its "nuclear" equivalent (ncoxII) by sitedirected mutagenesis. This involved synthesizing 12 oligonucleotides to achieve the 18 changes needed (13 ATA codons to ATG codons and 5 TGA codons to TGG codons).

To overexpress the converted subunit II gene in \underline{E} .

<u>coli</u>, several different expression vectors were used with different promoters and ribosome-binding sequences. A striking increase in the level of expression resulted when subunit II was fused to other <u>E</u>. <u>coli</u> genes. A cro- β -gal-COXII fusion protein was characterized and used for production of antibodies to subunit II, but due to its denatured state it is not suitable for the study of structure-function relationships.

Messenger RNA transcribed from the $\underline{ncox}II$ gene with $\underline{SP6}$ RNA polymerase was expressed in an $\underline{in \ vitro}$ translation system using a rabbit reticulocyte lysate, and in <u>Xenopus</u> <u>laevis</u> oocytes. In both systems, the newly made subunit II is inserted into membranes and can be extracted with a mild detergent, laurylmaltoside. In the <u>in vitro</u> translation system, the production of subunit II peptide is stimulated by the addition of artificial membranes providing a system from which COXII may be purified for further studies. These results indicate that the subunit II peptide can be synthesized in a membrane-bound, nondenatured form in the absence of other subunits of cytochrome <u>c</u> oxidase, leading to the possibility of structure-function analysis of the independent peptide. Dedicated to my mother and father

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LIST OF ABBREVIATIONS

bp	Base pair(s)
BSA	Bovine serum albumin
<u>cox</u> II	Gene for cytochrome oxidase subunit II
COXII	Cytochrome oxidase subunit II peptide
DCCD	N,N'-dicyclohexylcarbodiimide
EDTA	Ethylene-diaminetetraacetic acid
FPLC	Fast protein liquid chromatography
GalK	Galactokinase
HEPES	4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid
HPLC	High performance liquid chromatography
IPTG	Isopropyl-ß-D-thiolgalactopyranoside
<u>Lac</u> Z	Gene for ß-galactosidase
LB medium	Luria-Bertani medium
<u>ncox</u> II	Nuclear form of gene for cytochrome oxidase subunit II
PMSF	Phenylmethylsulfonyl fluoride
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TMPD	N,N,N'N'-tetramethyl-p-phenylenediamine
Tris	Tris (hydroxymethyl) aminomethane
TTBS	Tris buffered saline plus 0.05% Tween-20
X-gal	5-Bromo-4-chloro-3-indolyl-ß-D- galactopyranoside
ß-gal	ß-galactosidase

CHAPTER 1

LITERATURE REVIEW

All living organisms require a continual input of free energy for their growth and the performance of various cellular activities. The major source of energy in aerobic cells is derived from oxidative phosphorylation. In this process, reducing equivalents (in the form of NADH and FADH₂) are generated from oxidation of carbohydrates, fatty acids and proteins, and then fed into the respiratory chain, through a series of electron transfer complexes that span the mitochondrial membrane. Electron transfer through the complex is coupled to the translocation of protons across the membrane. The electrochemical gradient generated is then used to drive the synthesis of ATP from ADP and Pi, according to the chemiosmotic mechanism of Mitchell (1961). Electron transfer reactions play an important role in bacterial energy conservation as well as in eukaryotic cells.

The mitochondrial electron transport chain consists of four major complexes, in addition to cytochrome \underline{c} and coenzyme Q, both of which function in electron transfer by acting as shuttles between the complexes (Keilin and

Hartree, 1945; Barlow and Margoliash, 1966; Hauska, 1977; Futomi et al., 1979). The first enzyme, NADH-coenzyme Q reductase or Complex I, catalyzes the oxidation of NADH and the reduction of coenzyme Q to coenzyme QH₂; the second enzyme, succinate-coenzyme Q reductase or Complex II, oxidizes succinate which is produced in the TCA cycle, resulting in the formation of fumarate and coenzyme QH₂; electrons from coenzyme QH_2 are transferred to cytochrome <u>c</u> in a reaction catalyzed by Complex III, the coenzyme QH₂cytochrome c reductase; the last member in the sequence is cytochrome oxidase or Complex IV, which transfers reducing equivalents from cytochrome c to molecular oxygen, and leads to the formation of water (Tzagoloff, 1982). All these complexes are multisubunit, intrinsic membrane proteins that have been difficult to study by classical biochemical approaches.

The use of molecular biology and genetic engineering tools to study the proteins involved in mitochondrial energy transduction has also been severely limited because of the unusual nature of the mitochondrial genome and its unique genetic code, as well as the apparent lability of the enzymes involved in its transcription and translation. Some of these special characteristics are described below.

Organization and Expression of the Mitochondrial Genome

Mitochondria contain circular duplex DNA which is located in the matrix compartment and is attached to the inner mitochondrial membrane (Darnell et al., 1986). It has a molecular weight ranging from 1×10^7 to 5×10^7 daltons depending on the organism. Mammalian mtDNA has a small genome size with molecular weight of about 1×10^7 daltons. The amount of mitochondrial DNA is less than 1% of the total nuclear DNA (Clayton, 1982). Mitochondrial DNAs from human, bovine, and murine cells have been sequenced (Anderson et al., 1981; Bibb et al., 1981); they exhibit some similarities of gene organization among themselves, as well as a number of unique features compared to the nuclear genome. First, the mammalian mtDNA sequence is very compact (Van Tuyle and McPherson, 1979). No evidence for intervening sequences has been found in genes coding for various poly(A)-containing mRNAs. Many of the genes that code for proteins are separated by tRNA genes. For some of the mRNAs, stop codons are formed through a posttranscriptional processing event involving cleavage at the 5' end of the tRNA and simultaneous polyadenylation of the 3' end of the mRNA; formation of the TAA stop codon occurs by addition of the adenine residues (Attardi et al., 1980). Second, mitochondria use a genetic code that differs somewhat from the universal nuclear genetic code. In particular, in mitochondria UGA codes for tryptophan and not for termination, AUA codes for methionine and not for

isoleucine, and the pair AGA/AGG that normally code for arginine may be used for termination. Third, the pattern of codon usage in mitochondrial DNA is biased in favor of A+T rich codons, which is reflected in the lower G+C content of mitochondrial DNA. The two strands of mammalian mtDNA can be separated into heavy (H) and light (L) strands by buoyant density gradient centrifugation because of the uneven base The genes that are composition (Kasamatsu et al., 1971). encoded by the mtDNA have been identified, including 22 mitochondrial tRNAs (Lynch and Attardi, 1976; Angerer et al., 1976; Barrell et al., 1980), 2 mitochondrial rRNAs (Crews and Attardi, 1980), the three largest subunits of cytochrome oxidase (Hare et al., 1980), subunits 6 and 8 of ATPase (Macreadie et al., 1983), cytochrome b apoprotein (C.-J. Doersen, in preparation), and seven components of the respiratory chain NADH dehydrogenase (Chomyn et al., 1985; Ise et al., 1985). The organization of rat liver mitochondrial DNA is summarized in Figure 1. However, note that the majority of mitochondrial proteins are encoded in the nucleus, synthesized on ribosomes in the cytoplasm, and imported into the organelle for assembly with the mitochondrial counterparts, e.g. F₁F₀-ATPase, NADH dehydrogenase, and cytochrome oxidase (Schatz and Mason, 1974; Tzagoloff et al., 1979; Hay et al., 1984; Hurt and Van Loon, 1986).

Mitochondria have a complete and independent protein

Figure 1. Structure of the genetic map of rat liver mitochondrial DNA (from Grosskopf and Feldmann, 1981). O, the origin of mitochondrial DNA replication; URF1, URF2, URF3, URF4, URF4L, URF5, and URF6, coding regions for seven components of NADH dehydrogenase; URFA6L, coding region for ATPase subunit 8; ATPase6, subunit 6 of ATPase; COXI, COXII, and COXIII, coding regions for the three largest subunits of cytochrome oxidase; cytb, coding region for cytochrome <u>b</u> apoprotein; black circles represent the coding regions for 22 mitochondrial tRNAs; coding regions for the two mitochondrial rRNAs are also indicated.



synthesis system that is responsible for the synthesis of proteins encoded by the mitochondrial genome. This differs from the cytoplasmic system not only in details of the translation apparatus (Ashwell and Work, 1970) but also in susceptibility to antibiotics. Translation in mitochondria is sensitive to antibiotics such as erythromycin and chloramphenicol but is resistant to cycloheximide (Beattie et al., 1967; Lamb et al., 1968; Mahler et al., 1968). Mitochondrial protein synthesis has been demonstrated in both isolated mitochondria (Bhat et al., 1981; Amuro et al., 1982; McKee and Poyton, 1984) and in cultured mammalian cells in the presence of cycloheximide (Chomyn et al., 1985), but a mitochondria-free translation system has not been obtained. Due to the unique fact that mammalian mitochondrial mRNAs lack 5' untranslated regions upstream of the initiation codon as compared with eukaryotic and prokaryotic mRNAs (Shine and Dalgarno, 1974; Steitz and Steege, 1977; Kozak, 1983), questions remain as to the molecular mechanisms whereby these mitochondrial mRNAs bind to their ribosomes. Recent studies show that in the absence of auxiliary initiation factors or initiator tRNA, cytochrome oxidase subunit II mRNA (having a single extra nucleotide 5' to the initiation AUG codon because of its method of preparation) binds to the small 28S ribosomal subunit only, but it does not bind to the large 39S subunit nor to the complete 55S mitochondrial ribosomes (Liao and

Spremulli, 1989). The results of Denslow <u>et al</u>. (1989) show that the binding of mitochondrial mRNAs to ribosomes requires initiation factors for the proper recognition and melting of the secondary structure in the 5'-terminal region of the mRNAs. The AUG at the 5'-terminal of mitochondrial mRNA is not necessary for binding.

The assembly of mitochondrially coded proteins into their native complexes requires the presence of the cytoplasmically-synthesized subunits which are imported into mitochondria in an electrochemical potential-dependent manner (Eilers and Schatz, 1988; Roise and Schatz, 1988). Other important components of the synthetic machinery including the translational interaction factors must also be imported. This may explain why the synthesis of cytochrome oxidase subunit II requires an electrochemical potential across the inner mitochondrial membrane (Clarkson and Poyton, 1989) even though it does not have to be imported This requirement for a membrane potential, along itself. with lability of the enzymes involved (Bolden et al., 1977), may also account for why a soluble translation system without intact mitochondria is not yet available.

Structure of Cytochrome Oxidase

<u>Subunit Composition</u>. Cytochrome oxidase in eukaryotes consists of 7 to 13 different polypeptides, depending on the

organism. Cytochrome oxidase from <u>Dictyostelium discoides</u> has seven subunits (Bisson and Schiavo, 1986), the yeast enzyme has nine subunits (Power <u>et al</u>., 1984), and mammalian cytochrome oxidase has as many as thirteen different subunits (Kadenbach and Merle, 1980; Capaldi <u>et al</u>., 1988). All thirteen subunits of beef heart cytochrome oxidase have been sequenced, either by direct peptide sequencing (Tanaka <u>et al</u>., 1979; Meinecke <u>et al</u>., 1984), or indirectly by sequencing of the DNA (Anderson <u>et al</u>., 1982; Lightowlers <u>et</u> <u>al</u>., 1989). On the other hand, cytochrome oxidase from prokaryotes has a much simpler peptide composition, consisting of only two or three subunits. Oxidase from <u>Paracoccus dentrificans</u> has three subunits (Haltia <u>et al</u>., 1988); that from <u>Thermus thermophilus</u> has two (Yoshida and Fee, 1984).

The three largest subunits of eukaryotic cytochrome oxidases are coded by the mitochondrial genome and are synthesized in mitochondria. The rest of the subunits are coded by the nuclear genome, synthesized on cytoplasmic ribosomes, and imported into mitochondria where they are assembled into the final oxidase complex (Schatz and Mason, 1974; Tzagoloff <u>et al</u>., 1979; Hay <u>et al</u>., 1984; Hurt and Van Loon, 1986). The actual subunit composition of cytochrome oxidase has been the subject of long controversy because of the possibilities of contaminating polypeptides (Briggs and Capaldi, 1977; Downer <u>et al</u>., 1976) or loss of subunits or

of prosthetic groups during purification (Saraste <u>et al</u>., 1981). This is true even for the enzyme from <u>Paracoccus</u> <u>denitrificans</u>, which was considered to be a two subunit enzyme until a gene coding for a third subunit was isolated and sequenced (Saraste <u>et al</u>., 1986). Using a modified preparation method, a three subunit cytochrome oxidase was isolated from <u>P</u>. <u>denitrificans</u> (Haltia <u>et al</u>., 1988). Thus the composition of cytochrome oxidase can "vary" depending on the methods used for purification.

Tissue-specific and development-specific isozymes of mammalian cytochrome oxidase have been reported (Bisson and Schiavo, 1986; Kadenbach <u>et al</u>., 1982, 1983). These isozymes are characterized by differences in subunit size (Kadenbach <u>et al</u>., 1982), cysteine content (Stroh and Kadenbach, 1986), N-terminal amino acid sequence (Kadenbach <u>et al</u>., 1982, 1983), and immunological reactivities (Kuhn-Nentwig and Kadenbach, 1985) in the nuclear coded subunits, whereas no differences were found in the mitochondrially coded subunits. Therefore, these findings support the idea that mitochondrial encoded subunit are the catalytic unit of the enzyme.

<u>Prosthetic Groups</u>. Cytochrome oxidase contains four redox-active metal centers per monomer, two heme groups and two copper atoms (Caughey <u>et al.</u>, 1975). The heme groups are designated cytochrome <u>a</u> and <u>a</u>₃, so cytochrome oxidase is

also called cytochrome <u>aa</u>₃. The two hemes have different locations and thus function differently. Cytochrome <u>a</u> is usually of low spin, while cytochrome <u>a</u>₃ is of high spin and is directly involved in binding of molecular oxygen. Cytochromes <u>a</u> and <u>a</u>₃ have unique optical spectra in the reduced and oxidized forms (Vannest, 1966; Halaka <u>et al</u>., 1981). Fully oxidized cytochrome oxidase has maximal absorbances at 420 nm (Soret band) and 598 nm (α -band), while the fully reduced form has maximal absorbances at 445 nm (Soret band) and 605 nm (α -band). The 605 nm band of the reduced enzyme is mainly due to ferrous heme <u>a</u>; the band at 445 nm contains about equal contributions from both hemes <u>a</u> and <u>a</u>₃.

The two copper atoms are designated as Cu_A and Cu_B (Blair <u>et al</u>., 1983). Cu_A exhibits an absorbance maximum at 820-840 nm in the oxidized enzyme and is EPR detectable. It is believed that Cu_A and heme <u>a</u> together are involved in accepting the electrons transferred from cytochrome <u>c</u>. Cu_B is located very close to heme <u>a</u>₃ (about 4 Å separation, Powers <u>et al</u>., 1981), so that the prosthetic groups are strongly coupled antiferromagnetically (Tweedle <u>et al</u>., 1978) with sulfur acting as a bridging ligand (Powers <u>et</u> <u>al</u>., 1981). Thus Cu_B is usually not detectable by EPR. Cu_B -heme <u>a</u>₃ forms a binuclear center which is the binding site for O₂, CO, NO, and HCN (Malmstrom 1982; Wikstrom <u>et</u> <u>al</u>., 1981a)

Some recent studies suggest that there may be a third copper shared between two monomers in a cytochrome oxidase dimer. This has been designated as Cu. (Einarsdottir and Caughey, 1985; Yewey and Caughey, 1987; Steffens et al., 1987). The existence and function of the third copper atom is still equivocal. There is also evidence that many preparations of cytochrome oxidase contain one Zn and one Mg per <u>aa</u>, monomer (Einarsdottir and Caughey, 1985). It is postulated that Zn may be associated with proton translocation, based on the finding that when Zn is displaced the enzyme loses its proton pumping activity (Nilsson et al., 1988). It has been proposed that Zn may have an important functional role in facilitating proton donation at or near the dioxygen binding pocket, thereby promoting 0-0 bond cleavage and preventing partially reduced O₂ products such as superoxide, hydrogen peroxide, and hydroxyl radical from leaving the reaction center (Einarsdottir et al., 1988). Huther and Kadenbach (1986) proposed that Mg may be associated with ATP binding, based on evidence suggesting a specific ATP binding site on the enzyme that alters the kinetics of the cytochrome c reaction (Montecucco et al., 1986). In general, the roles for Cu,, Zn and Mg remain hypothetical.

Location of Prosthetic Groups. Freedman et al. (1979) reported that more than 90% of the heme groups were associated with subunit I, II, and IV in an electrophoretic analysis, while Yu and Yu (1977) have isolated a hemecontaining subunit equal in size to subunit V. However, these results are difficult to interpret due to the denaturing conditions used in the course of preparation analysis, since heme <u>a</u> is very easily dissociated from its native environment and may interact with any other suitable binding site. Using polyacrylamide gel electrophoresis under less denaturing conditions (lithium dodecyl sulfate at 0° C), Cu was detected on subunit II, and hemes were found associated with subunit I and II in equal amounts (Winter <u>et</u> al., 1980).

On the basis of a number of lines of evidence indicating EPR and ENDOR data (Blumberg and Peisach, 1979; Hoffman <u>et al</u>., 1980; Scott <u>et al</u>., 1981; Stevens <u>et al</u>., 1982), similarities to other copper binding proteins, conservation of amino acid residues available for reaction with ligands (Holm <u>et al</u>., 1987), and chemical modification studies (Hall <u>et al</u>., 1988), the Cu_A binding site has been assigned to subunit II (Martin <u>et al</u>., 1988), while Cu_B, heme <u>a</u>, and <u>a</u>₃ appear to be associated with subunit I (Holm <u>et al</u>., 1987). In <u>Paracoccus denitrificans</u>, it was reported that both hemes and coppers are located in subunit I (Müller <u>et al</u>., 1988). Müller <u>et al</u>. (1988) obtained a one-subunit enzyme of <u>P</u>. <u>dentrificans</u> by a sequential two-step proteolytic digestion of the normal two-subunit enzyme. The

single subunit enzyme exhibits spectral properties indistinguishable from the two-subunit enzyme and retains significant enzymatic activity. However, this approach has not been attempted with oxidases from other systems, and it is too early to draw any conclusion based on one experiment.

Three-Dimensional Structure. While only limited progress has been made in obtaining crystals suitable for Xray crystallography of a large, membrane-bound protein such as cytochrome oxidase (Yoshikawa et al., 1988), electron diffraction studies on two dimensional crystalline sheets have provided useful information about the spatial structure of the enzyme. By using image reconstruction, it has been deduced that the \underline{aa}_3 monomer is about 110 Å long and resembles a capital letter "Y" (Figure 2). The two arms of the Y are 55 Å in length, have a center-to-center separation of 40 Å, and are on the matrix side of the inner mitochondrial membrane (M-domains). The stem of the Y is the large domain on the cytoplasmic side of the membrane (Cdomain) which contains the cytochrome <u>c</u> binding site. Twodimensional crystals that have been prepared from cytochrome oxidase-lipid vesicles indicate a dimer form with contacts through the hydrophilic domains of the monomers (Henderson <u>et al.</u>, 1977; Deatherage <u>et al</u>., 1982).

Topological Orientation of Subunits. The orientation

Figure 2. Three dimensional structure of cytochrome oxidase. See text for details (from Thompson, 1984).

•

of the polypeptides of cytochrome oxidase in the inner mitochondrial membrane has been studied by a number of researchers using a variety of techniques. These include: chemical labeling with hydrophilic modifying reagents (Schneider et al., 1972; Ludwig et al., 1979); proteinase digestion (Jarausch and Kadenbach, 1985b; Zhang et al., 1988); nearest-neighbor cross-linking (Jarausch and Kadenbach, 1985a; Briggs and Capaldi, 1977); subunitspecific antibody probing (Kuhn-Nentwig et al., 1985); radioactive labeling (Zhang et al., 1984); and cross-linking of cytochrome c to cytochrome oxidase by carbodiimide (Millett et al., 1982, 1983). Some common features deduced from these experiments are as follows. Subunits I-III along with the C-terminal portions of the nuclear encoded subunits IV, Vb, VIb, VIc, and VIII are exposed on the cytoplasmic side of the membrane, and form the C domain. Part of subunit II, and the N-terminal regions of IV, Vb, VIb, and VIc are exposed on the matrix side and form the two M Therefore, subunits II, IV, Vb, VIa have a domains. transmembrane orientation (Jaraush and Kadenbach, 1985a,b). Subunit II is directly involved in cytochrome c binding (Millett et al., 1982, 1983). The function of some of the nuclear-coded subunits may be to regulate the binding of cytochrome oxidase to its substrate cytochrome c (Kadenbach, 1986).

Function Of Cytochrome Oxidase

Electron Transfer and Proton Translocation. Cytochrome oxidase catalyses the reduction of molecular oxygen to water. The reaction can be expressed as follows:

(1)

 $4Cytc^{2+} + 4H^{+} + 0_{2} ----> 4Cytc^{3+} + 2H_{2}0$ The four electrons are transferred from cytochrome <u>c</u> to dioxygen and two protons are translocated from the matrix side to the cytoplasmic side of the inner mitochondrial membrane (Wikstrom et al., 1981a). This electron transfer process is quite complicated: the reaction is very fast and generally takes place over a large molecular distance, the structural changes in the redox sites that accompany the electron transfer are very subtle, and electron transfer is coupled to proton pumping. The process appears to involve conformational control of electron flow (Gray and Malmstrom, 1989). Due to these complexities, the exact mechanisms whereby electrons are transferred from cytochrome \underline{c} via cytochrome oxidase to molecular oxygen are not clear. However, it is known that electrons from cytochrome c first reduce Cu_A or cytochrome <u>a</u>, that Cu_A and cytochrome <u>a</u> are in rapid equilibrium (Antalis and Palmer, 1982), and that electrons from these reduced centers are transferred in turn to the \underline{a}_3 -Cu_R binuclear center, which is the dioxygen binding and reducing site (Malmstrom, 1982; Hill et al., 1986; Naqui <u>et al.</u>, 1986).

Lehninger (1954), and Maley and Lardy (1954) demonstrated that oxidation of cytochrome \underline{c} by O_2 is coupled to oxidative phosphorylation. Mitchell (1966) proposed that the "redox loop" serves as a device for achieving proton translocation. The redox loop is defined as a system that uses the electron carrying centers to transport hydrogen atoms vectorially across the membrane, leaving protons on one side of the membrane. By such a mechanism, a net translocation of protons should result in a $H^+/e^$ stoichiometry of unity. However, using intact mitochondrial membranes, a stoichiometry of $2H^+/e^-$ has been observed by many researchers in this field (Azzone et al., 1979; Reynafarje et al., 1982; Lehninger et al., 1985). Casting doubt on the redox loop mechanism as originally formulated. The role of cytochrome oxidase in energy conservation was debated for many years because it appeared not to have any redox centers capable of translocating protons by a redox loop process. Yet it is now generally accepted that cytochrome oxidase not only consumes one proton per electron in the production of water, but also translocates one proton per electron from the matrix to the cytosolic side of the inner membrane, overall an reaction that can be written as:

 $4Cytc^{2+} + 8H_{M}^{+} + O_{2} ----> 4Cytc^{3+} + 2H_{2}O + 4H_{C}^{+}$ (2)

This conclusion is based on studies with purified cytochrome oxidase incorporated into lipid vesicles. With such a preparation, a H^+/e^- ratio close to one is observed

(Krab and Wikstrom, 1978; Casey <u>et al</u>., 1979; Sigel and Carafoli, 1980; Prochaska <u>et al</u>., 1981; Thompson <u>et al</u>., 1985). The true stoichiometry of the process may in fact be greater, since evidence from some studies with reconstituted lipid vesicles shows that the stoichiometry varies depending on the turnover rate of the enzyme (Proteau <u>et al</u>., 1983). These results again suggest that a mechanism other than the "redox loop" must be involved in proton translocation.

The mechanism whereby proton translocation is coupled to electron transfer in cytochrome oxidase is not fully understood. It was suggested that subunit III might play an important role in this process based on the observations that removal of subunit III resulted in the loss of proton translocation activity (Penttila and Wikstrom, 1981; Penttila, 1983; Thelen <u>et al</u>. 1985); similar loss of function is seen if DCCD is bound to subunit III (Prochaska <u>et al</u>., 1981). However, more recent studies show that significant levels of proton translocation remain after removal of subunit III (Puettner <u>et al</u>., 1985; Thompson <u>et</u> <u>al</u>., 1985; Sarti <u>et al</u>., 1985; Finel and Wikstrom, 1986; Gregory and Ferguson-Miller, 1988, 1989). The latter results indicate that subunit III is not essential to the proton translocation process.

Wikstrom (1988) proposed that the redox centers generate a charge separation that drives the vectorial movement of protons. This idea is supported by the

observation of an energy-dependent spectral shift in cytochrome \underline{a}_3 which may correspond to a transition of the cytochrome into a "high energy" state directly involved in proton translocation (Wikstrom, 1977; Wikstrom and Saari, 1977; Wikstrom and Krab, 1978, 1979). In addition, conformational changes in cytochrome oxidase during redox activity are evidenced by large changes in the kinetics of cyanide binding to cytochrome \underline{a}_3 (Jones <u>et al</u>., 1984). As yet, however, there is no model that will satisfactorily explain the molecular mechanism of coupling of electron transfer and proton translocation in this complicated system.

Binding of Cytochrome c. Cytochrome c is a small water-soluble molecule that acts as an electron transfer shuttle between complexes III and IV in the mitochondrial respiration chain (Barlow and Margoliash, 1966). The kinetics of the cytochrome <u>c</u>-cytochrome oxidase reaction have been intensively studied. It is generally accepted that there are two binding sites on the enzyme for cytochrome <u>c</u>. Under steady-state condition, a biphasic kinetic plot is obtained, which can be rationalized in terms of two processes involving Michaelis-Menten kinetics corresponding to high and low affinity sites on the enzyme (Ferguson-Miller <u>et al</u>., 1976, 1978). Several hypotheses have been put forth to explain the two cytochrome <u>c</u> binding

In one model each site is catalytically active and sites. serves as a site for transfer of electrons to oxidase (Ferguson-Miller et al., 1976, 1978; Errede and Kamen, 1978). Alternatively, the low affinity site may transfer electrons to cytochrome <u>c</u> bound to the high affinity site, which in turn transfers electrons to heme a/Cu_{a} (Nicholls, 1965; Brooks and Nicholls, 1982). Another model involves only one catalytic site per cytochrome oxidase monomer with a second cytochrome <u>c</u> acting in a regulatory capacity (Speck et al., 1984; Sinjorogo et al., 1986). Results from crosslinking experiments (Bisson et al., 1980, 1982) and spectral perturbation measurements (Michel and Bosshard, 1984) favor only one catalytic site for cytochrome <u>c</u> per <u>aa</u>, unit (Margoliash and Bosshard, 1983). The high affinity binding site may be located on the cytoplasmic side of subunit II, as demonstrated by cross-linking (Briggs and Capaldi, 1978) and chemical modification (Millett et al., 1983) studies. The low affinity binding site has been postulated to involve negatively charged cardiolipin that is tightly bound to the enzyme (Bisson et al., 1980; Vik et al., 1981; Fuller et <u>al.</u>, 1981). Fuller <u>et al</u>. found that at least 2 molecules of tightly associated cardiolipin were essential for the low affinity binding of cytochrome \underline{c} on the enzyme. However, this is not the case for rat liver cytochrome oxidase that is purified in the presence of laurylmaltoside (Thompson and Ferguson-Miller, 1983; Gregory, 1989). This enzyme can be
prepared with less than 1 mole of cardiolipin per mole of aa_3 , and still retains the ability to interact with cytochrome <u>c</u> with two kinetic phases similar to those observed with the membrane-bound enzyme under the same conditions. Obviously, the nature of the low affinity binding site for cytochrome <u>c</u> on cytochrome oxidase remains to be defined both structurally and functionally.

Molecular Weight and Function. The issue of whether cytochrome oxidase exists as a dimer in the mitochondrial membrane and whether the dimer has any functional significance has been hard to resolve because of the tendency of the enzyme to exist in a variety of aggregation states that are difficult to separate and obtain in pure form. The mammalian aa₃ monomer contains two heme groups, two copper atoms, and one copy of each of the 13 polypeptides, with a molecular weight of approximately 203,000 daltons (Buse et al., 1982). Cytochrome oxidase from bovine heart was reported to be mainly dimeric in Triton X-100 (Robinson and Capaldi, 1977) but monomeric at a high detergent to protein ratio (Robinson and Talbert, 1986; Georgevich et al., 1983), or in the presence of laurylmaltoside (Rosevear et al., 1980; Ferguson-Miller, 1982; Suarez et al., 1984). The monomeric form is active in electron transfer (Suarez et al., 1984) and shows normal biphasic kinetics. Cytochrome oxidase from shark heart

exists as a monomer (129-160 Kd) and also shows electron transfer activity comparable to that of the dimer form (Georgevich <u>et al.</u>, 1983). It is still not clear whether a dimer of cytochrome oxidase is an artifact of purification, or if it is the active form <u>in vivo</u>.

Radiation inactivation analysis of mammalian cytochrome oxidase reveals a functional unit of 70 to 110 kd for the beef heart and rat liver enzymes (Kagawa, 1967; Thompson <u>et</u> <u>al</u>., 1982; Suarez <u>et al</u>., 1984), which could correspond to subunits I+II (83 kd). Mammalian cytochrome oxidase lacking subunit III (and several of the small nuclear subunits, Vb, VIa, and VIb) still has high electron transfer and proton pumping activities (Thompson and Ferguson-Miller, 1983). Bacterial cytochrome oxidases, which contain only two or three subunits having a total molecular weight of 80-100 kd, perform functions similar to those of the mammalian enzymes. All these results suggest that the basic electron transfer and proton pumping activity of cytochrome oxidase may reside in the large, mitochondrially encoded subunits, I and II.

<u>Mitochondrially Coded Subunits and</u> <u>Bacterial aa₃-Type Oxidases</u>

Cytochrome <u>c</u> oxidases of aerobic bacteria are located in the cytoplasmic membranes (Poole, 1983; Ludwig, 1980). Bacterial cytochrome oxidases of the <u>aa</u> type have been

isolated from different sources including: <u>Paracoccus</u> <u>denitrificans</u> (Ludwig and Schatz, 1980; Raitio <u>et al.</u>, 1987); <u>Rhodopseudomonas sphaeroides</u> (Gennis <u>et al.</u>, 1982); and <u>Thermus thermophilus</u> (Fee <u>et al.</u>, 1980; Sone and Yanigita, 1982). These enzymes have similar metal centers to the mammalian enzymes, but contain only two or three different subunits.

Most of the information about bacterial cytochrome oxidase is from the study of P. denitrificans. The genes that encode the three subunits have been sequenced (Raitio et al., 1987) revealing significant homology to the three largest subunits of the mammalian enzyme. The bacterial enzyme contains two heme groups and two copper atoms (Ludwig and Schatz, 1980) with properties similar to those of eukaryotic oxidases (maximal absorbancies in the visible region for the oxidized form, 424 nm and 602 nm; for the reduced form, 445 nm and 605 nm). The molecular weight of the <u>P</u>. <u>denitrificans</u> enzyme is close to the sum of the three mitochondrially encoded eukaryotic subunits, and the subunits from P. denitrificans have polarity indexes that approximate those of the largest subunits from the bovine heart enzyme (Ludwig and Schatz, 1980; Raitio et al., 1987). Both subunit I of P. denitrificans and cytochrome oxidase subunit I of beef heart and yeast are very hydrophobic, tend to aggregate, and run as diffuse bands on SDS-gels without Subunit III of both <u>P</u>. <u>denitrificans</u> and mammalian urea.

cytochrome oxidase can be removed without destroying electron transfer activity (Penttila, 1983; Bill and Azzi, 1982) though the rates are lower (Finel and Wikstrom, 1986; Puettner <u>et al.</u>, 1985; Sarti <u>et al.</u>, 1985). Subunit III in both prokaryotes and eukaryotes reacts with DCCD to produce a covalent adduct.

Thus these results with bacterial cytochrome oxidases further substantiate the notion that studies of the mitochondrially encoded subunits of the mammalian enzyme may offer possibilities for exploring the mechanism of cytochrome oxidase function in eukaryotic cells.

<u>Subunit II</u>

The subunit II gene of rat liver is a segment of mitochondrial DNA (683 bp) (Grosskopf and Feldmann, 1981) that codes for a peptide containing 227 amino acids and has a molecular weight of about 26 kd. A schematic model of subunit II has been presented by Millett <u>et al</u>. (1983) (Figure 3). Two hydrophobic segments are proposed to traverse the lipid bilayer (Jarausch and Kadenbach, 1985b; Steffens and Buse, 1979) and form a "hairpin" structure that anchors the subunit to the membrane, with most of the Cterminal region exposed on the cytoplasmic side of the membrane (Ludwig <u>et al</u>., 1979); this region is thought to be involved in the binding of cytochrome <u>c</u> (Bisson <u>et al</u>., Figure 3. Proposed model of COXII. The capital letters represent the strictly invariant amino acid residues. The amino acid residues that are proposed to be involved in Cu_A binding and cytochrome <u>c</u> binding (labeled with arrows) are also indicated (from Millett <u>et al.</u>, 1983).



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1980; Fuller <u>et al</u>., 1981; Millett <u>et al</u>., 1982, 1983). Based on sequence homologies between the C-terminal domain of subunit II and the Cu-binding site of blue copper proteins, Steffens and Buse (1979) proposed that subunit II contained one of the copper atoms, specifically Cu_A (Wikstrom <u>et al</u>., 1981a). The binding site for Cu_A may involve two histidine and two cysteine groups; these residues are identified mainly on the basis of their conservation in known subunit II sequences and from interpretation of various physicochemical analyses (e.g. chemical modification and EPR studies) (Nilsson <u>et al</u>., 1988; Larsen <u>et al</u>., 1989; Gelles <u>et al</u>., 1986). Thus their participation in Cu_A binding remains to be firmly established.

Subunit II has also been proposed to contain heme <u>a</u> (Winter <u>et al</u>., 1980). This idea is supported by the fact that heme <u>a</u> is one of the first acceptors of electrons from cytochrome <u>c</u> and has fast electron equilibration with Cu_A (Wilson <u>et al</u>., 1980; Antalis and Palmer, 1982, Gelles <u>et</u> <u>al</u>., 1986). However, due to the lack of a sufficient number of conserved histidines, it is unlikely that COXII contains both heme <u>a</u> and Cu_A (Blumberg and Peisach 1979, Babcock <u>et</u> <u>al</u>., 1979). On the other hand, it was suggested that a conserved cluster of aromatic amino acids (between residues 128 and 137) may facilitate electron transfer between heme <u>a</u> and Cu_A even if they are not in the same subunit (Steffens

and Buse, 1979; Millett et al. 1984).

Subunit II also contains the high affinity binding site of cytochrome <u>c</u> (Capaldi <u>et al</u>., 1982). Cross-linking (Bisson and Montecucco, 1982) and chemical-labelling (Millett <u>et al</u>., 1983) studies implicate invariant, negatively-charged residues, Asp^{112} , Glu^{114} , and Glu^{198} in the electrostatic association with cytochrome <u>c</u>. These results do not rule out the possibility that other subunits, including some of the smaller peptides (Jarausch and Kadenbach, 1985b), may also be involved in cytochrome <u>c</u> binding.

To elucidate the mechanism of electron transfer and proton translocation by cytochrome oxidase, an essential first step is the location of the redox centers and definition of the residues that are directly involved in interaction with these prosthetic groups and with the substrate, cytochrome <u>c</u>. Although a large amount of information has been accumulated on this subject through the use of chemical and physical methods, the results are still ambiguous. The use of oligonucleotide-directed, sitespecific mutagenesis of the putative amino acid residues involved should shed light on these matters in a less equivocal way. To begin such studies, we chose to convert the mitochondrially coded subunit II gene (<u>cox</u>II) into its "nuclear" equivalent (<u>ncox</u>II), and to find a system in which it could be expressed in significant amounts, in a

nondenatured, membrane associated form that would permit purification for functional and structural studies.

CHAPTER 2

CONVERSION OF THE RAT LIVER CYTOCHROME OXIDASE SUBUNIT II GENE INTO A NUCLEAR EQUIVALENT BY SITE-DIRECTED MUTAGENESIS

INTRODUCTION

Mammalian cytochrome \underline{c} oxidase is a complex, multisubunit enzyme whose functional properties have yet to be defined in terms of the roles of individual peptides. As previously discussed (Literature Review) at least 2 copper atoms and 2 heme groups (Wikstrom et al., 1981b) are required for activity. Results from a variety of studies (Winter et al., 1980; Blumberg and Peisach, 1979; Stevens et al., 1982) indicate that subunit I probably contains one of the copper atoms and two heme groups, while subunit II may contain the other copper, Cu_A . However, there is still considerable uncertainty concerning the number and location of the metal centers (Müller et al., 1988; Einarsdottir and Caughey, 1985). This and other issues regarding the properties of each subunit might be resolved if the individual subunits could be isolated and characterized, but so far the subunits have been separated only under conditions that cause irreversible denaturation of the

enzyme and loss of the prosthetic groups. Thus analysis of the individual subunits and reconstitution of an active form from component parts has not been achieved.

A different approach to investigate structure-function relationships in the enzyme involves use of the techniques of molecular biology. Cloning and expression of individual subunits of multisubunit complexes has been achieved in several cases (Fujita et al., 1986; Garboczi et al., 1988). For mitochondrially encoded proteins, progress in this area has been hampered by the fact that mitochondria use a somewhat different genetic code than the universal genetic one. In mitochondria, UGA codes for tryptophan, not for termination, while AUA codes for methionine, not for isoleucine; the pair of codons AGA/AGG normally used for arginine may be used for termination instead (Anderson et al., 1981). Translation of mitochondrial polyadenylated mRNA in an E. coli cell-free translation system leads to production of only aberrant proteins (Pakmanaban et al., 1975; Scragg and Thamos, 1975). In vitro suppression of the UGA stop codons in a mitochondrial mRNA results in the synthesis of a full length subunit II but with incorrect amino acid substitutions at the UGA sites (De Ronde et al., So far, a mitochondrially-derived translation system 1980). has not been developed, except using intact coupled organelles (Clarkson and Poyton, 1989).

A yeast mitochondrial DNA intron containing an open

reading frame was cloned in <u>E</u>. <u>coli</u>, after it had been converted to a "nuclear" gene by oligonucleotide directed site-specific mutagenesis; it was found to be expressed as a double-strand specific endonuclease (Colleoux <u>et al</u>., 1986). Since cloned and sequenced rat liver mitochondrial genomic DNA was available (Grosskopf and Feldmann, 1981), I used a similar approach with the mitochondrial cytochrome oxidase subunit II. This chapter reports on the strategy that was used for subcloning and site-directed mutagenesis of the subunit II gene of rat liver cytochrome oxidase.

EXPERIMENTAL PROCEDURES

<u>Materials</u>. Rat liver mitochondrial DNA clones were generously provided by Drs. R. Grosskopf and H. Feldmann of the Federal Republic of Germany. <u>E</u>. <u>coli</u> strain JM101 and the replicative form of M13mp18 vector were from Bethesda Research Laboratories. Restriction enzymes and synthetic linkers were purchased from Bethesda Research Laboratories or from Boehringer Mannheim. Oligonucleotides were synthesized by the Macromolecular Structure Facility of Michigan State University. Reagents for bacterial growth media were from Difco, and other chemicals were from Sigma and were the highest grade available.

General Recombinant DNA Techniques. All restriction enzyme digestion reactions were performed according to the supplier's specifications. For DNA fragment isolation, the restriction digest was electrophoresed through a 5% polyacrylamide gel in TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA, pH 8.3) or a 0.8-1% agarose gel in TEAC buffer (40 mM Tris, 20 mM sodium acetate, 18 mM NaCl, and 2 mM EDTA). Gels were stained with ethidium bromide (0.5 ug/ml) in TBE or TEAC and visualized by UV-illumination. The bands containing the desired fragments were cut out, electro-eluted, and concentrated by ethanol precipitation.

Subcloning the coxII Gene. The plasmid pMD (Grosskopf and Feldmann, 1982) containing the rat liver coxII gene (see Figure 4 below) was linearized by partial digestion with HindIII. The linearized plasmid was treated with 1 unit Bal31 nuclease per ug of DNA at 37°C, and aliquots were taken at different times to produce fragments of various lengths. The ends of shortened fragments were filled in by Klenow fragment of E. coli DNA polymerase I (Maniatis et al., 1982) and synthetic <u>Hind</u>III linkers were ligated to the blunt-ended DNA fragments. Following <u>Hind</u>III digestion, fragments of about 700 bp were isolated and ligated to the plasmid pBR322, which had been linearized with <u>Hind</u>III and dephosphorylated with alkaline phosphatase by using 2 units per ug of DNA for 1 h at 37° C. The ligation reaction was performed by using T_4 DNA ligase at 16°C overnight. The ligation mixture was used to transform competent E. coli strain HB101 cells according to the procedure of Hanahan (1980). Aliquots of cells were spread on LB plates containing 50 ug/ml ampicillin and plates were incubated at 37°C overnight. Recombinant plasmids (pBROXII) from ampicillin resistant colonies were prepared by the alkaline-SDS method (Maniatis et al., 1982) and were screened for the coxII gene by restricting the DNA with EcoRI, HapII, and HinfI.

Synthesis of Oligonucleotides. To convert the coxII

Figure 4. Subcloning the <u>cox</u>II gene. Plasmid pMD was partially digested using <u>Hind</u>III, and the linearized DNA was purified and digested with <u>Bal</u>31. <u>Hind</u>III linkers were ligated to the shortened fragments after the ends were filled-in by Klenow fragment. After <u>Hind</u>III digestion, fragments about 700 bp in length were isolated and ligated to <u>Hind</u>III digested pBR322. The black bars represent the <u>cox</u>II gene, and the relevant restriction sites are indicated.



gene into its corresponding "nuclear" form, twelve different oligonucleotides were synthesized (Table 1) to change the thirteen TGA codons into TGG codons and five ATA codons into ATG codons. To facilitate the cloning of the <u>ncox</u>II gene into different expression vectors, a <u>Nco</u>I site was introduced at the initiation codon region by site-directed mutagenesis in which the A of the original sequence, ACATGG, was replaced by a C (Table 1).

Results from the expression of the yeast mitochondrial intron reading frame in <u>E</u>. <u>coli</u> show that the codon usage pattern affects the level of expression (Colleaux <u>et al</u>., 1986). Thus three additional oligonucleotides were synthesized to change three arginine codons in <u>cox</u>II (one CGG and two CGA that are rarely used in <u>E</u>. <u>coli</u> to the more commonly used codons CGC). This was done to permit a test of whether the presence of rare codons might hinder synthesis of the COXII peptide in <u>E</u>. <u>coli</u>.

<u>M13 Site-Directed Mutagenesis</u>. A 700 bp fragment containing the complete <u>cox</u>II coding sequence was obtained by <u>Hind</u>III digestion of the plasmid pBROXII. The <u>cox</u>II gene was cloned into the M13mp18 replicative form which had been digested with <u>Hind</u>III (Figure 5). Single-strand recombinant M13 DNA was isolated from recombinant colorless plaques according to Messing (1983) and used as template for sitedirected mutagenesis (Zoller and Smith, 1983) (Figure 6).

Figure 5. Cloning the <u>cox</u>II gene into M13mp18. Plasmid pBROXII was digested with <u>Hind</u>III and the DNA fragment containing the <u>cox</u>II gene was isolated and cloned into M13mp18 at the <u>Hind</u>III site. Black bars represent the <u>cox</u>II gene, MCS denotes the multiple cloning site.



There are three unique restriction sites in the <u>cox</u>II gene (Figure 7): from 5' to 3', these are <u>Eco</u>RI, <u>Hinf</u>I, and <u>Hap</u>II. The 18 nucleotides to be changed in the <u>cox</u>II gene actually were altered in parallel using four M13 clones. Clones I, II, III, and IV contained the sequences between the 5' <u>Hind</u>III and <u>Eco</u>RI sites, <u>Eco</u>RI and <u>Hinf</u>I sites, <u>Hinf</u>I and <u>Hap</u>II sites, and <u>Hap</u>II and <u>Hind</u>III sites, respectively (as indicated in Figure 7).

Approximately 1 ug (0.5 pmol) of the purified singlestrand DNA was annealed with a 50-fold excess (molar ratio) of synthetic oligonucleotide which may contain one, two, or even three mismatched bases (Table 1). Annealing reactions were performed in 10 ul of 20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 50 mM NaCl, 1 mM dithiothreitol by boiling the mixture for 5 min and allowing it to cool to room temperature. Ten ul of buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10 mM dithiothreitol) containing 1 mM each of the four dideoxynucleoside triphosphates, 5 units of Klenow fragment of E. coli DNA polymerase I and 10 units T4 DNA ligase were added to the annealing mixture and the reaction was incubated at 16°C for 6 to 12 h. About 10 ul of the elongation-ligation reaction was used to transform competent JM101 cells, and serial dilutions were plated onto YT (8 g bacto tryptone, 5 g bacto yeast extract, and 5 g sodium chloride per liter) plates. For each 100x15 mm plate, 3 ml of 0.8% melted bacto-agar, 0.2 ml of fresh stationary phase

Figure 6. Simplified method for site-directed mutagenesis based on the approach of Zoller and Smith (1983). The oligonucleotide primer was annealed to single strand recombinant M13, elongated by Klenow fragment, and ligated with T4 DNA ligase. The mixture was used to transform competent JM101 cells and plated on YT plates which were incubated at 37°C overnight. Plates having about 500 isolated colonies were used to screen for mutants by differential hybridization.



JM101, 50 ul of X-gal (10 mg/ml), and 10 ul of IPTG (24 mg/ml) were mixed and plated onto the YT plate. Plates were incubated at 37°C overnight and were replicated onto nitrocellulose papers BA85 (Schleicher & Schuell), which were denatured in 0.1 N NaOH, 1.5 M NaCl for 30 second, and neutralized in 0.2 M Tris-HCl, 2X SSC for 1 min.

Desired clones were identified by differential hybridization. After being air dried, the filters were baked in a vacuum oven at 80°C for 2 h and prehybridized in 6X SSC, 10X Denhardt's (1% Ficoll, 1% polyvinylpyrrolidone, and 1% BSA), 0.2% SDS and 100 ug/ml denatured calf thymus DNA at 67°C for 3 h. Hybridization was performed in 6X SSC, 10X Denhardt's, plus ³²P-labeled oligonucleotide (specific activity >10⁶ cpm/ug) at room temperature overnight. The oligonucleotides had been radioactively labeled using polynucleotide kinase and $[\gamma^{-32}P]ATP$ according to the protocols of Maniatis et al. (1982). Following hybridization, the filters were washed with excess 6X SSC with four changes at room temperature. The filters were air dried and autoradiographied at -70° C for four hours with an intensifying screen. To distinguish mutants from the wild type DNA, the filters were washed at a higher temperature that was calculated from an empirical formula (Meinkoth and Wahl, 1984) and autoradiographed (Figure 8). Positive phage plaques were isolated and sequenced by the M13 dideoxy chain termination method (Sanger et al., 1977).

After all clones were sequenced, the replicative forms of the mutant phages were isolated and DNA regions containing the altered codons were obtained by digesting the phage DNA with appropriate restriction enzymes. The four sections of the altered <u>cox</u>II gene were ligated and cloned into M13mp18 in proper order. The final recombinants were checked by sequencing by both the Maxam-Gilbert (1980) and dideoxy chain termination (Sanger <u>et al.</u>, 1977) methods.

RESULTS AND DISCUSSION

The conversion of the coxII gene to a nuclear form was achieved by site-directed mutagenesis in phage M13 (Zoller and Smith, 1983). The coxII gene was cloned into M13mp18 and 12 oligonucleotides that cover the 18 necessary changes were synthesized. Additional mutations were also made to better adapt to the codon usage pattern of E. coli: oligonucleotides were synthesized to change the three arginine codons in coxII that are rarely found in E. coli to a more commonly used codon (Kiesewetter et al., 1987). In addition, one oligonucleotide was synthesized to introduce a unique restriction site NcoI (CCATGG) at the natural initiation codon of the coxII gene. This permits ligation of ncoxII into E. coli expression vectors, such as pDR540, pKK233-2, and pCQV2, in such a way that translation initiation is forced to occur at the desired ATG.

Both the wild type and mutant rat liver coxII genes were completely sequenced by the Maxam-Gilbert chemical and M13 dideoxy chain termination methods. The results confirm that the <u>ncoxII</u> sequence was identical to that of the wild type <u>coxII</u> except for the A to G changes at the appropriate positions (Figure 8). Comparison of the wild type gene sequence with the published rat liver <u>cox</u>II sequence (Grosskopf and Feldmann, 1981) revealed four disagreements (Figure 7). The previously reported <u>cox</u>II sequence showed

Figure 7. The complete nucleotide and deduced amino acid sequence of rat liver coxII. Bases changed to convert the mitochondrial gene to a nuclear gene are indicated below the mitochondrial codons. All the residues represent changes from ATA and TGA to ATG and TGG respectively; three underlined bases represent the changes of arginine codons that are rare in <u>E</u>. <u>coli</u>; four boldface bases are different from the published sequence and the corrected amino acid is underlined; the restriction sites used in experimental constructions are also underlined. The numbering system is that corresponding to the bovine subunit II gene (Steffens and Buse, 1979).

10 20 MAYPFQLGLQDATSPIMEEL A<u>CATGG</u>CTTACCCATTTCAACTTGGCTTACAAGACGCCACATCACCAATC<u>ATA</u>GAAGAACTT 60 C NCOI G T N F H D H T L M I V F L I S S L V L Y **ACAAACTTTCATGACCACACCCTAATAATTGTATTCCTCATCAGCTCCCTAGTACTTTAT** 120 G I I S L M L T T K L T H T S T M D A Q E ATTATTTCACTA<u>ATA</u>CTAACAACAAAACTAACACACACAAGCACA<u>ATA</u>GACGCCCATGAA 180 G G V E T I W T I L P A V I L I L I A L P S GTAGAAACAATT<u>TGA</u>ACAATTCTCCCAGCTGTCATTCTTATTCTAATCGCCCTTCCCTCC 240 L R I L Y M M D E I N N P V L T V K T M CTACGAATTCTATACATAATAGACGAGATTAATAACCCAGTTCTAACAGTAAAAACTATA 300 <u>C</u> <u>Eco</u>RI GG G H Q W Y W S Y E Y T D Y E D L C F D S $\mathbf{GGACACCAA} \underline{\mathbf{TGA}} \mathtt{TAC} \underline{\mathbf{TGA}} \mathtt{AGCTATG} \underline{\mathbf{AGTATG}} \mathtt{AGTATG} \mathtt{AGACCTATG} \mathtt{CTTT} \underline{\mathbf{GACTC}} \mathtt{C}$ 360 G G HinfI Y M I P T N D L K P G E L R L L E V D N $\texttt{TAC}\underline{\texttt{ATA}}\texttt{ATCCCAACCAATGACCTAAAAC}\underline{\overline{\texttt{T}}}\texttt{AGGTGAACTTCGCTTATTAGAAGTTGATAAT}$ 420 R V V L P M E L P I R M L I S S E D V CGGGTAGTCTTACCAATAGAACTTCCAATTCGTATACTAATCTCATCCGAAGACGTCCTG 480 G Ç H S W <u>A</u> I P S L G L K T D A I P G R P N CACTCATGACCCATCCCTTCACTAGGGTTAAAAAACCGACGCAATCC<u>CCGG</u>CCGCCCGAAC 540 GG HapII Q A T V T S N R <u>P</u> G L F Y G Q C S E I C 600 <u>c</u> **c** G S N H S F M P I V L E M V P L K Y F E GGCTCAAATCACAGCTTC<u>ATACT</u>CATTGTACTAGAA<u>ATA</u>GTGCCTCTAAAATATTTCGAA 660 GĒ G NWSASMI* AACTGATCAGCTTCTATAATTTAA 684 G Ð

leu at position 130 (codon, C<u>T</u>A), pro at position 164 (codon <u>C</u>CC), leu at position 189 (C<u>T</u>C), and leu at position 208 (codon, C<u>T</u>C). Our sequencing data indicate that the correct codons should be CCA, GCC, CCC, and CCC, which would place pro, ala, pro, and pro at positions 130, 164, 189, and 208. These results are consistent with a later published rat liver <u>cox</u>II sequence (Brown and Simpson, 1982). Comparison of the rat liver COXII peptide sequence with those of other known mammalian sequences (human, bovine, and mouse), strongly supports these results, since the residues in question are conserved in all the mammalian proteins.

Our results show that an oligonucleotide of length 17 residues or even of length 15, complementary except for one mismatch to the <u>cox</u>II DNA sequence, can successfully induce a mutation at the specific site; up to three base changes have been achieved simultaneously by using a longer oligonucleotide (35 bases) as primer. The calculated dissociation temperature for the template-oligonucleotide hybrids generally agreed well with the actual dissociation temperature (see Table 1), which facilitated the mutagenesis procedure. These results also show that 5-10% efficiency of mutagenesis could be achieved, even without using a universal primer in the recombinant M13-primer elongation and ligation reaction (Norris <u>et al</u>., 1983).

In conclusion, a mitochondrially encoded subunit II of rat liver cytochrome oxidase has been converted into the

Figure 8. Portion of a sequencing gel for <u>ncox</u>II DNA, showing two mutations that were introduced by site-directed mutagenesis. These data are for the lower strand of the gene; the changes are at positions 258 and 261. Using a single oligonucleotide, the underlined T residues in panel A were changed into C residues as shown in panel B. These alterations represent changes from A to G in the upper strand, hence in each case conversion of ATA to ATG as desired.



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"nuclear" version by site-directed mutagenesis. The successful conversion of <u>cox</u>II makes it feasible to express this gene as a correct peptide using conventional expression systems.

Table 1 Sequence of synthesized oligonucleotides

	Oligonucleotide	Length	Codons Changed and Position		Dissociation Temperature	
Ро	3' atatagaa <u>g</u> gtaccgaa5'	17b	TTC-TCC	0	(a) 45°C	(b) 48°C
P1	3' ggttagta <u>c</u> cttcttga5'	17b	ATA-ATG	17	50°C	50°C
P2	3' tgggatta <u>c</u> taacataa5'	17b	ATA-ATG	29	45°C	45°C
Р3	3' AAGTGATTA <u>C</u> GATTGTTG5'	18b	ATA-ATG	45	50°C	4 7°C
P4	3'CGTGTTA <u>C</u> CTGCGGG5'	15b	ATA-ATG	56	38°C	54°C
P5	3' tgttaac <u>c</u> tgtttaag5'	15b	TGA-TGG	65	40°C	40°C
P6	3'GATATGTACTACCTGCTCTA5	20b	2ATA-ATG	86, 87	50°C	57°C
P7	3' TTGATACCCTGTGGTTACCAT GACCTCGATAC5'	35b	ATA-ATG 2TGA-TGG	100 104, 10	65°C	75°C
P8	3' aggatgta <u>c</u> tagggttg5'	17b	ATA-ATG	122	52°c	52°C
P9	3' AATGGTTACCTTGAAGGTTA AGCATACGATTAGAG5'	35b	2ATA-ATG	146 152	68°C	73°C
P10	3' GTGAGTAC <u>C</u> GGGTAGGG5'	17b	TGA-TGG	163	45°C	60℃C
P11	3' TCGAAGTA <u>C</u> GAGTAACATGA TCTTTA <u>C</u> CACGGAGA5'	35b	2 ATA-A TG	207 213	70°C	76°C
P12	3' CTTTTGACCAGTCGAAGATA CTAAATTTG5'	29b	TGA-TGG	222	60°C	67°C
PR1	3' AGGGATGC <u>G</u> TAAGATAT5'	17b	CGA-CGC	246	45°C	50°C
PR2	3' CTATTAGC <u>G</u> CATCAGAA5'	17b	CGG-CGC	423	45°C	50°C
PR3	3'AGTTTGGC <u>G</u> GGTCCAGA5'	17b	CGA-CGC	564	50°C	57°C

Po represents the oligonucleotide used to create the <u>NcoI</u> site at the 5' end of the <u>ncox</u>II gene; P1-P12 are the oligonucleotides used to change 13 ATA to ATG and 5 TGA to TGG; PR1 to PR3 are the oligonucleotides used to change the three rarely used arginine codons. Data in column (a) are the experimental dissociation temperatures, while column (b) displays the calculated dissociation temperatures according to Meinkoth and Wahl (1984).

CHAPTER 3

EXPRESSION OF RAT LIVER NCOXII IN E. COLI

INTRODUCTION

Having converted the <u>coxII</u> gene of rat liver cytochrome oxidase into its nuclear form by using site-directed mutagenesis, it was necessary to find a system that would synthesize the subunit II independently in a native-like membrane-associated form. If successful, this would permit study of the functional properties of rat liver COXII and the identification of residues involved in the binding of copper and cytochrome \underline{c} .

With the development of recombinant DNA techniques, it has proved possible to produce proteins of limited natural availability, and to engineer novel proteins using <u>in vitro</u> mutagenesis techniques. The expression of cloned genes has been achieved in a variety of prokaryotic and eukaryotic host organisms. With <u>E</u>. <u>coli</u>, in particular, efficient and controlled production of recombinant polypeptides is routine in many laboratories. This chapter summarizes the expression of <u>ncox</u>II in different <u>E</u>. <u>coli</u> strains. We find that <u>ncox</u>II expression is very low when a clone of the ncoxII gene alone is used. A striking increase in the level

of expression resulted when the <u>ncox</u>II gene was fused to a truncated galactokinase gene in the plasmid vector; the fusion protein was found in inclusion bodies in the cell. Fusing <u>ncox</u>II with the ß-galactosidase gene produced even higher yields of the fusion protein. Although these denatured fusion proteins are not the ultimately desired products, they proved useful in making COXII-specific antibodies. A monospecific COXII antibody was isolated that inhibits activity of the native enzyme, reacts very strongly with the denatured COXII on immunoblots, and shows no crossreaction with other subunits of the rat liver cytochrome oxidase.

EXPERIMENTAL PROCEDURES

Materials. Plasmid pDR540 (Russell and Bennett, 1982), and <u>E. coli</u> strains N4830-1 and N99CI⁺ were from Pharmacia P-L Biochemical. <u>E. coli</u> strain MCL22 was obtained from Dr. J. Kaguni of this department. Plasmid pPLEX was obtained from Dr. G. Sczakiel (Sczakiel <u>et al</u>., 1987). Plasmid pEX1 (Stanley and Luzio, 1984) was from P&S Biochemicals, Inc. Goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate, protein molecular weight markers, and nitrocellulose papers were from Bio-Rad. Alkaline phosphatase color development reagents and Tween-20 were from Sigma. Complete and incomplete Freund's adjuvants were from Behring Diagnostic. Other enzymes, chemicals, and recombinant DNA techniques were as described in Chapter 2.

<u>Construction of Various Expression Vectors: pDROXII,</u> <u>pPLEXOXII, and pEXOXII</u>. As shown in Figure 9, the <u>ncox</u>II gene was obtained by digestion of recombinant M13mp18 with <u>NcoI and SmaI</u>, and then was blunt-end ligated (Maniatis <u>et</u> <u>al</u>., 1982) into plasmid pDR540 at the <u>Bam</u>HI site. The constructed expression vector, plasmid pDROXII, contains a <u>tac (trp/lac</u> hybrid) promoter that can be induced by IPTG, followed by the <u>ncox</u>II gene with an <u>Nco</u>I site at its 5'-end and a <u>Bam</u>HI site at its 3'-end. Another expression vector, pPLEXOXII, was made as follows. Plasmid pDROXII was

Figure 9. Construction of expression vectors: pDROXII, pPLEXOXII, pEXOXII. The <u>ncox</u>II gene was obtained by digestion of recombinant M13mp18 DNA (a) with <u>Nco</u>I and <u>Sma</u>I, and was blunt-end ligated into pDR540 (b) at the <u>Bam</u>HI site. The new construct, pDROXII (c), was digested with <u>Bam</u>HI and the <u>ncox</u>II gene blunt-end ligated into plasmid pPLEX (d) at the <u>Mlu</u>I site within the <u>galk</u> gene, resulting in the new expression vector, pPLEXOXII (e). The vector pEXOXII (g) was constructed by digestion of pDROXII (c) with <u>Bam</u>HI, followed by ligation of the <u>ncox</u>II gene into pEX1 (f) at the <u>Bam</u>HI site located at the 3' end of the <u>lacZ</u> gene. The dark bars (a, c, e, g) represent the <u>ncox</u>II gene; promoters, restriction sites, multiple cloning sites (MCS), and other genes are also indicated in this figure.


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digested with <u>Bam</u>HI and the <u>ncox</u>II gene was blunt-end ligated into the <u>gal</u>K gene at the <u>Mul</u>I site (Debouck <u>et al.</u>, 1985) of plasmid pPLEX. The resulting plasmid pPLEXOXII contained the lambda \underline{P}_L promoter and, downstream, the <u>ncox</u>II gene located within the reading frame of the truncated <u>gal</u>K gene. A third expression vector was constructed by digesting pDROXII with <u>Bam</u>HI and ligating the <u>ncox</u>II gene into a plasmid pEX1 at the <u>Bam</u>HI site in the polylinker region; this places <u>ncox</u>II under control of the lambda \underline{P}_R promoter. The sequence of the junction region of vector and <u>ncox</u>II in pDROXII was confirmed by the dideoxy chain termination approach (Sanger <u>et al.</u>, 1977).

<u>Maxicell Detection of Plasmid Encoded Proteins</u>. The expression vector pDROXII was transformed into <u>E</u>. <u>coli</u> strain MCL22 and the expression of COXII was detected by the maxicell technique as described by Sancar <u>et al</u>. (1979). Briefly, plasmids were transformed into the MCL22 cells. An aliquot of a 10 ml culture at OD=0.8 was UV irradiated with a dose of 10-20 J/m^2 . After irradiation, cells were incubated with ³⁵S-methionine for 1 h. About 200 ul of the labeled cells were pelletted and lysates were analyzed by electrophoresis on an 18% urea SDS-polyacrylamide gel.

Expression of the ncoxII Gene as Fusion Proteins. Overnight cultures of cells harboring the recombinant

plasmids pPLEXOXII or pEXOXII were diluted 100-fold with fresh LB medium and grown to OD₆₀₀=0.5 at 30°C, then were shifted to 42°C and grown for 1.5 h at 42°C (Stanley and Luzio, 1984). The phage lambda promoters are induced at elevated temperature, and genes under control of these promoters are expressed. Aliquots of cells were pelletted by centrifugation, lysed with SDS loading buffer (1% SDS, 0.1 M dithiothreitol, 10% glycerol, 0.005% bromphenol blue, 10 mM Tris, pH 6.8) and analyzed by SDS-PAGE.

Purification of the cro-ß-gal-COXII Fusion Protein.

The cro-ß-gal-COXII fusion protein was purified according to Nagai and Thogerson (1987) with modification. Briefly, cells containing pEXOXII (50 g) were thawed and suspended in 50 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 25% sucrose, 1 mM EDTA). Lysozyme (200 mg) dissolved in 20 ml of lysis buffer was added and the solution was incubated on ice for 1 MgCl₂, MnCl₂, and DNase I were added to final h. concentrations of 10 mM, 1 mM, and 10 ug/ml, respectively. After an additional 30 min on ice, 100 ml of detergent buffer (0.2 M NaCl, 1% Nonidet P-40, 20 mM Tris-HCl, pH 7.5, 2 mM EDTA) was added to the lysate, which was then centrifuged at 5,000xg for 10 min. The pellet was completely resuspended in 0.5% Triton X-100, 1 mM EDTA and centrifuged at 5,000xg for 10 min until a tight pellet was obtained. The yield of cro-ß-gal-COXII fusion protein in

the pellet was approximately 500 mg, with an estimated purity of 70%.

This crude preparation was further purified by preparative polyacrylamide gel electrophoresis. About 10 mg of crude fusion protein was loaded onto a 20x15x0.4 cm 7.5% SDS-polyacrylamide gel. After electrophoresis, the gel was briefly washed with distilled water and the protein bands were visualized by immersing the gel in 1 M cold potassium acetate for 15 min. The discrete fusion protein band was excised and washed with water for 30 min to remove the potassium acetate. The gel slice containing the fusion protein was smashed by passage through a 5 ml syringe with an 18 gauge needle, then was soaked in 0.05% ammonium bicarbonate buffer at room temperature for 24 h. Gel pieces were removed by centrifugation. The protein-containing supernatant was lyophilized, and redissolved in water. The $cro-\beta$ -gal protein was purified by the same procedure.

Endolysin-C Digestion. The fusion protein purified as above was dialyzed against 0.05% SDS and 0.05% ammonium bicarbonate buffer in the presence of the anion exchanger DE-52 to remove excess SDS. Following dialysis, the peptide was digested with proteinase endolysin-C (from Boehringer Mannheim) with a protein:enzyme ratio of 1:100 at 37°C for 24 h with gentle shaking. After digestion, the mixture was lyophilized and analyzed by SDS-PAGE and immuno-blotting as

described below.

<u>Amino Acid Sequence Analysis</u>. A 15 kd fragment produced by endolysin-C digestion above was subjected to sequence analysis by automated Edman degradation on an Applied Biosystems 477A Protein Sequencer. PTH derivatives were identified by HPLC.

Preparation of Anti-cro-ß-gal-COXII Serum. Antiserum was raised in New Zealand female rabbits (Carroll and Laughon, 1987). About 0.5 mg of the purified fusion protein (in 0.05% SDS and 0.05% ammonium bicarbonate) was emulsified in Freund's complete adjuvant for initial injection; booster injections were given at days 14 and 21 with 0.1 mg fusion protein in incomplete Freund's adjuvant. The rabbit was given booster injections every eight weeks. On day 28, and at two-week intervals thereafter, blood was collected from the rear ear vein.

Purification of COXII-Specific Antibodies. To purify COXII-specific antibody free of anti- β -gal IgG and any other non-specific IgG, the antiserum was applied to two columns (Burton <u>et al.</u>, 1988). A β -gal affinity column was made by coupling 15-20 mg of β -galactosidase (Sigma Chemical Co.) to 3 ml of Affi-gel 10 (Bio-Rad Laboratories) in 6 ml of 0.1 M HEPES (pH 7.9) and 1% SDS, shaking overnight at room

temperature. Unreacted groups on Affi-gel 10 were blocked with 1.0 M monoethanolamine (pH 7.0) which was added to a final concentration of 150 mM and shaken for 3 h. A cro-ßgal-COXII column was made by the same procedure. Anti-cro-B-gal-COXII serum was first passed five times through the Bgal column and the final flow through then passed five times through the cro-ß-gal-COXII column. The glycine/HCl (pH 2.5) eluate of the ß-gal column was the affinity-selected anti-ß-gal, and the glycine eluate of the ß-gal-COXII column was the affinity-selected anti-COXII. The glycine eluates were neutralized by 1 M HEPES (pH 7.9) and immediately dialyzed against 500 ml PBS at 4° C with three changes. Control immunoglobin IgG was purified by protein-A agarose according to the procedure described by Ey et al. (1978). Antibody concentrations were calculated by assuming that an A₂₈₀ of 1.4 corresponds to 1 mg/ml IgG (Carroll and Laughon, 1987).

SDS-Polyacrylamide Gel Electrophoresis and

Immunoblotting. Routine SDS-PAGE analysis, in 7.5-18.5% gels, was performed according to Laemmli (1970) or Kadenbach <u>et al</u>. (1983). After electrophoresis, the proteins were transferred onto a nitrocellulose filter at 150 mA (4°C) for 1-2 h in transfer buffer (12.5 mM Tris, 96 mM glycine, pH 8.2, 0.02% SDS, 20% methanol). The filter was placed in TBS containing 5% bovine serum albumin for 2 h, and then was

incubated with beef heart cytochrome oxidase antibody or rat liver COXII-specific antibody overnight (1:5000 dilutions from 1 mg/ml antibody stocks). The filter was washed three times with TTBS and incubated with alkaline phosphataseconjugate goat anti-rabbit IgG (1:7000 dilutions of a 1 mg/ml IgG stock from Bio-Rad) for 2 h at room temperature. The filter was washed once with TTBS and twice with TBS. Protein bands reacting with the primary and secondary antibodies were visualized by staining with 100 ml solution of 0.3 mg/ml nitro blue tetrazolium (pre-dissolved in dimethyl formamide) and 0.15 mg/ml 5-bromo-4-chloro-3-indoyl phosphate (pre-dissolved in 70% dimethyl formamide) in carbonate buffer (0.1 M NaHCO₃, 1 mM MgCl₂, pH 9.8).

RESULTS

Maxicell Detection of Plasmid Encoded Protein. The ncoxII gene was cloned into the expression vector pDR540, under the control of the hybrid tac promoter; this permits translation of the <u>ncox</u>II gene starting at its natural AUG initiation codon using a ribosome binding site supplied by the vector. Figure 10 is an autoradiogram of an SDS gel showing the results when this vector is expressed in maxicells. The upper band (42 kd) which is present in all lanes, corresponds to the galk protein. The second band at about 27 kd represents β -lactamase. A third band (26 kd) is equal in size to the COXII peptide, and is not present in the strain containing control plasmid, indicating that the COXII peptide was synthesized by the vector in <u>E</u>. <u>coli</u>. All other bands are present in both the control and expression vector lanes and presumably result from contamination by bacterial protein.

Expression of the ncoxII Gene as a GalK-COXII Fusion Protein. As shown in Figure 11, in <u>E</u>. <u>coli</u> transformed with the recombinant plasmid containing the fusion gene for <u>galkncox</u>II, a 50 kd protein was detected by immunoblotting with antibodies to beef heart cytochrome oxidase. This is the expected size for a fusion protein composed of 26 kd from galk and 25 kd from COXII. A shorter COXII-containing band

Figure 10. A fluorogram showing the production in E. coli of pDROXII-encoded proteins, as detected by the maxicell The ³⁵S-methionine lysates were analyzed by method. electrophoresis on 18% SDS-urea polyacrylamide gels. Lane 1, MCL22 without plasmid; lane 2, MCL22/pDR540; lane 3, MCL22/pDR540, plus IPTG; lane 4, MCL22/pDROXII; lane 5, MCL22/pDROXII, plus IPTG; lane 6, purified rat liver cytochrome oxidase lacking subunit III. GalK denotes galactokinase; BLA denotes ß-lactamase; and COX denotes cytochrome oxidase subunits. Since the strain MCL22 is not a $\underline{lacI^Q}$ host and the \underline{tac} promoter is not repressed in MCL22, thus the addition of inducer IPTG did not affect the expression as shown in this Figure. The B-lactamase bands in pDR540 are weaker than in pDROXII, this may due to the high level of synthesis of galactokinase in pDR540 and used up the 35 S-methionine in the medium.



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was also detected by the antibody. Compared to galactokinase expression from the control plasmid, the fusion protein was present at a level about 100-fold less than that of galactokinase.

Expression of the ncoxII Gene as a cro-B-gal-COXII Fusion Protein. The ncoxII gene was fused to the 3'-end of the cro-lacZ gene, which has a few nucleotides of the cro gene of phage lambda preceding the 5'-end of lacZ. The protein produced from this cro-lacZ-ncoxII fusion is very stable and forms inclusion bodies in the host cells. As seen in Figure 12, a band appears in the recombinant plasmid lane with molecular weight of 141 kd, which equals the sum of the molecular weights of the cro-ß-gal (115 kd) and COXII (26 kd) peptides. When not fused to the <u>ncox</u>II gene, the cro-lacZ gene product accumulated to about 30% of the total protein. When the <u>ncox</u>II gene was fused to 3'end of the cro-lacZ gene, the cro-ß-gal-COXII product accumulated to only about 0.5% of the cro- β -gal level; there was also some cro-ß-gal without COXII peptide made. Thus the presence of the ncoxII gene somehow inhibits expression of the fusion protein. Some degradation of the COXII portion of the fusion protein may also be occurring.

<u>Endolysin-C Digestion</u>. Endolysin-C hydrolyzes peptides and esters specifically at the carboxyl end of lysine. Figure 11. Immunoblot detection of galk-COXII fusion protein in <u>E</u>. <u>coli</u> N4830-1. Aliquots of cells were boiled, then were loaded onto and electrophoresed through a 12.5% SDS gel, and immunoblotted. Lane 1, 2, 3, 4 are as indicated in the figure; lane 5 represents purified rat liver cytochrome oxidase with the antigenic subunits indicated. FP represents the galk-COXII fusion protein. COXII, IV, and V are subunits of cytochrome oxidase that react with the polyclonal antibody used.



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- COXII



Figure 12. SDS-PAGE analysis of <u>E</u>. <u>coli</u> N4830-1 cells transformed with pEXOXII. Cells were boiled and analyzed on a 7.5% SDS-gel stained with Coomassie blue. FP represents the cro- β -gal-COXII fusion protein; β -gal' represents cro- β gal; the locations of other molecular weight markers are indicated in the figure.

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There are five lysine residues in the COXII sequence and preliminary results show that endolysin-C efficiently digests COXII, resulting in three peptides of 15, 12.5 and 6.5 kd. The endolysin-C-digested cro-ß-gal-COXII mixture was analyzed on an 18.5% urea-SDS gel, immunoblotted by antibodies to both ß-gal and beef heart cytochrome oxidase. The digestion reaction was carried out for 24 h with gentle shaking and a reproducible digestion pattern was seen by SDS-gel analysis. A peptide with molecular weight of 15 kd that showed a very strong reaction with the antibody to beef heart cytochrome oxidase but not with anti-ß-gal was seen. A 15 kd peptide (residues 99-227) is predicted from the sequence of subunit II and the specificity of endolysin-C. This peptide is known to contain the most antigenic region of the subunit (Mariottini et al., 1986). It was purified by preparative polyacrylamide gel electrophoresis and used for sequencing analysis (Figure 13).

Partial Sequencing of COXII Peptide. To further establish that the COXII synthesized in <u>E</u>. <u>coli</u> is the correct peptide, amino acid sequence information on the 15 kd endolysin-C fragment of COXII was obtained. The first five amino acid residues were found to be N-Thr-Met-Gly-His-Gln-C, corresponding to residues 99-103 of the encoded gene product. Met-100 corresponds to one of the residues that required conversion of the original codon, AUA, by site-

Figure 13. SDS-PAGE analysis of purified 15 kd COXII fragment, produced by endolysin-C digestion of cro-ß-gal-COXII fusion protein and detected by coomassie blue staining. Lane 1, molecular weight markers; lane 2, the 15 kd COXII fragment.

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$$Mr(x|O^{-3})$$

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directed mutagenesis to AUG.

Immunoblotting Assay of Antibody Specificity. To determine the specificity of the affinity-purified antibodies of the cro-ß-gal-COXII fusion protein, cro-ß-gal, cro-ß-gal-COXII, and holo cytochrome oxidase were run on a 15% SDS gel and transferred to nitrocellulose paper as described under Experimental Procedures. As shown in Figure 14, the anti-cro-ß-gal reacted with the cro-ß-gal and cro-ßgal-COXII fusion proteins. The affinity-purified anti-COXII reacted with cro-ß-gal-COXII fusion protein and COXII, but not with any other subunits of the enzyme, and as expected shows only very weak cross reaction with the ß-gal peptide.

Inhibition of Cytochrome Oxidase Activity by Anti-COXII IgG. Addition of anti-COXII IgG to a rat liver cytochrome oxidase preparation resulted in a dose-dependent reduction of cytochrome oxidase activity (Figure 15). Almost 90% of the cytochrome oxidase activity of 14 pmole of enzyme was inhibited by the addition of 50 ug of the affinity-purified IgG. As seen in Figure 15, the activity of the enzyme was not significantly inhibited by the addition of the same amount of control IgG or of anti-B-gal IgG.

Figure 14. Immunoblot assay of antibody specificity. (A) Coomassie blue-stained 15% SDS gel. Lane M, molecular weight standards; lane 1, cytochrome oxidase; lane 2, purified cro-ß-gal-COXII fusion protein (FP); lane 3, cro-ßgal (ß-gal') control. (B) Immunoblot of (A), with anti-croß-gal IgG as primary antibody, lanes 1, 2, 3, as in (A). (C) Immunoblot of (A), with anti-COXII IgG as primary antibody, lanes 1, 2, 3, as in (A).



Figure 15. Inhibition of purified rat liver cytochrome oxidase activity by monospecific COXII antibody. Rat liver cytochrome oxidase (14 pmole) was incubated with various amounts of antibodies in PBS containing 0.05% laurylmaltoside at room temperature for 1 h. Steady-state kinetic measurements were performed in 50 mM potassium phosphate, pH 6.5, in the presence of 0.05% laurylmaltoside, 0.5 mM TMPD, 2.5 mM ascorbate, cytochrome c concentration range of 0.01-60 uM. Rates of oxygen consumption were measured polarographically as described by Ferguson-Miller <u>et al</u>. (1976, 1978): black circles represent COXII specific antibody; open circles represent cro-ß-gal antibody; open boxes represent the control IgG.



DISCUSSION

Chapter 1 of this dissertation describes the conversion of a cloned <u>cox</u>II gene of rat liver mitochondrial DNA into the "nuclear" form (<u>ncox</u>II) using site-directed mutagenesis. In this Chapter, the results of <u>ncox</u>II gene expression in <u>E</u>. <u>coli</u> are reported, using different expression vectors in which the promoters and ribosome-binding sequences were varied. A relatively low level of expression was obtained with the <u>ncox</u>II gene alone, but much higher levels were achieved with fusion of <u>ncox</u>II to <u>E</u>. <u>coli</u> genes.

Using the vector pDR540, COXII was expressed without fusion to any other protein. The COXII peptide was sufficiently stable to be detected by the maxicell technique as a radioactive band in the same position as a subunit II standard, and by cross-reaction with antibodies to beef heart cytochrome oxidase. However, COXII was made at only about 1% of the amount of galactokinase produced by the control plasmid under the same conditions. When <u>ncox</u>II was cloned in front of the <u>galk</u> gene in the pPLEX vector, a similar low level of expression was found. When fused to the C-terminal of a truncated <u>galk</u> sequence, the level of expression was improved, though the fusion protein was still produced in much smaller amount than <u>galk</u> alone. In experiments with pEXOXII, the level of expression of the

fusion protein cro-ß-gal-COXII was higher than the galk-COXII fusion (in pPLEX), amounting to about 0.5% of the total cell protein. With galk-COXII and cro-ß-gal-COXII fusion proteins, insoluble inclusion bodies were formed. Other expression vectors such as pKK223-3, pPL-lambda, pCQV2, and pUC18 were also tried, with all these vectors, a nonfusion COXII or a very short fusion at the N-terminal was expected, but no detectable level of COXII was found (see table 2).

Gene expression in E. coli (or any other system) is a trial and error procedure, especially in the case of a mitochondrially encoded polypeptide. The poor expression of the <u>ncox</u>II gene may due to numerous factors, such as the structure of the ribosome binding site, inefficient transcription, instability of the COXII mRNA, or differences in codon usage between nuclear and mitochondrial genes. When the <u>ncoxII</u> gene was fused to the <u>galk</u> or <u>cro-lacZ</u> gene, the level of fusion protein made in each case was far less than for galk or cro-lacZ alone; this suggests that inefficient ribosome binding or initiation of transcription are not the primary causes. It remains a possibility that the low level of expression might be due to the structure of the gene itself; perhaps the presence in the mitochondrial gene of codons that are rarely used in E. coli may be a factor that contributes to the weak expression of the ncoxII gene. Pedersen (1984) proposed that poorly expressed genes

TABLE	2
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Summary of	expression	data for rat	liver <u>ncox</u> II in	<u>E. coli</u>
plasmid construct	vector	promoter	<u>E. coli</u> host	level of OXII
	pKK233.2	Trc	JM105 BB-1	*
pDROXII	pDR540	Tac	JM103, MCL22	0.05%
pUCOXII	pUC18		JM103, JM105	* 0.19
pPLEXOXII	pEX1	PI Pr	N4830-1 N4830-1	28

*There was no detectable COXII generated from these constructs.

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in E. coli have reduced elongation rates and contain more rare codons. When the codon content of the ncoxII gene is compared with that of <u>E</u>. <u>coli</u> genes, distinct differences are found: the <u>E</u>. <u>coli</u> system uses completely different codons for five amino acids (leu, arg, pro, gln, and thr) (De Boer and Kastelein, 1986). Studies on the content and codon recognition of tRNAs show that the tRNAs which correspond to CUA (leu) and CGG/CGA (arg) are present at low levels in E. coli (Kiesewetter et al., 1987), while these are the most frequently used tRNAs in mitochondria. Observations that suggest a slower rate of translation and premature termination include: in the maxicell expression experiment with pDROXII, comparison of the expression of the recombinant plasmid with the control plasmid showed more labeled peptides in the lower molecular weight region of the recombinant lanes than in the control lanes (Figure 10); in the pPLEXOXII experiment, immunoblotting showed a short COXII-containing band in the recombinant lane but not in the control lanes (Figure 11); in the case of pEXOXII, a substantial amount of cro-ß-galk protein without the COXII part was present in the pEXOXII lane (Figure 12). To test whether rare codons are responsible for this behavior, the three rare codons for arginine were changed into the highly used CGC by site-directed mutagenesis (see Chapter 2). (At this point, it was unrealistic to change all CUA codons for leucine). However, changing three minor codons did not

increase the expression to a detectable level in the pDR540 vector system (data not shown). This may not be surprising, since about 40% of the amino acids in the <u>ncox</u>II gene are encoded by rare codons, so that changing three arginine codons may not be sufficient to make a major improvement in expression.

More recent studies show that mitochondrial initiation factors are required for the proper recognition and melting of secondary structure in the 5'-terminal region of mitochondrial mRNAs, as a prerequisite for initiation of protein synthesis in mammalian mitochondria. Further studies also show that initiation factors and initiator tRNA from <u>E</u>. <u>coli</u> neither promote binding of mitochondrial mRNA to its ribosome nor facilitate the melting of the 5' end proximal mRNA stem structure. The factors involved in initiation of mitochondrial protein synthesis appear to be specific for the mitochondrial system (Denslow <u>et al</u>., 1989; Liao and Spremulli, 1989). Therefore, in our case, the poor expression of the <u>ncox</u>II gene in <u>E</u>. <u>coli</u> might due to the lack of those initiation factors.

To firmly establish the identity of the putative COXII peptide synthesized in <u>E</u>. <u>coli</u>, a partial amino acid sequence was obtained. To do this, the purified $cro-\beta$ -gal-COXII fusion protein was digested with endolysin-C. There are about 20 lysine residues in the $cro-\beta$ -gal and the products of the digestion include fragments that are both

larger and smaller than the digested COXII peptides, but none in the same size range of digested COXII peptides. When nonfusion COXII (produced in <u>in vitro</u> translation system) was digested with endolysin-C, three main bands were obtained, of 15 kd, 12.5 kd, and 6.5 kd. The 15 kd peptide, which reacts with antibodies specific for COXII, was isolated and successfully sequenced. Sequencing in this way is not trivial; blockage of N-termini during purification by poorly understood mechanisms can seriously interfere with the reaction. In our case this was overcome by isolating relatively large amounts of the 15 kd fragment (>30 ug). Even though some fraction of the peptide may be blocked during the preparation stage, an adequate amount was still present which could be sequenced. The first five amino acids from the N-terminus of the 15 kd fragment exactly aligned with the deduced amino acid sequence of COXII from position 99-103. This fragment contains the proposed cytochrome \underline{c} and copper binding sites and is less hydrophobic than the N-terminal (1-98) fragment; it may prove to be very useful for future studies.

Although a denatured fusion protein is not the desired product, it is useful for making antibodies to COXII. The high molecular weight cro-B-gal-COXII fusion protein is denatured and proved to be a very good antigen, eliciting a strong, specific polyclonal response. The large amount of fusion protein available made it possible to build an

affinity column for further purification of the COXII antibody. The polyclonal monospecific COXII antibody provides a potential tool for detection and purification of the native-like COXII peptide that is produced in other systems (see below).

CHAPTER 4

EXPRESSION OF <u>NCOX</u>II OF RAT LIVER IN A RABBIT RETICULOCYTE LYSATE <u>IN VITRO</u> TRANSLATION SYSTEM

INTRODUCTION

To understand the function of a complex multisubunit enzyme such as cytochrome c oxidase, it would be useful to be able to study its subunits independently and in various combinations. This approach has been successfully applied to other membrane proteins including Torpedo californica acetylcholine receptor (Fujita et al., 1986) and rat liver ATP synthetase (Garboczi et al., 1988). To this end, the mitochondrial coxII gene of rat liver cytochrome oxidase has been converted into a "nuclear" equivalent (ncoxII) by sitedirected mutagenesis. This gene has been expressed in \underline{E} . coli, but only denatured proteins were obtained. То understand how COXII binds its prosthetic group(s) and plays a role in the electron transfer activities of the complete enzyme, an expression system is required that will produce COXII in a nondenatured form and in sufficient quantities to permit isolation, reconstitution into membranes, and characterization of copper and substrate binding.

This Chapter describes the synthesis of the COXII peptide by <u>in vitro</u> translation in a rabbit reticulocyte lysate. The COXII made in this system has the same molecular weight as that of authentic COXII, and can be immunoprecipitated by COXII-specific antibodies. Production of COXII is found to be stimulated by the addition of either microsomal membranes or artificial lipid vesicles. The COXII subunit exists in a native-like form, is inserted into membranes, and can be extracted by a mild nonionic detergent, laurylmaltoside. All these results indicate that COXII can be synthesized and isolated in a nondenatured form in the absence of other subunits of cytochrome oxidase.

EXPERIMENTAL PROCEDURES

Materials. Plasmid pSP65 (Melton et al., 1984), SP6 RNA polymerase, Dnase I, RNasin, rabbit reticulocyte lysates, and microsomal membranes were purchased from Promega. Poly(A) polymerase and mRNA cap analog G(5')ppp(5')G were from Pharmacia P-L Biochemical. Protein-A-agarose was from Sigma. [³⁵S] Methionine (1200 Ci/mmol) was obtained from New England Nuclear. ¹⁴C-labeled protein molecular weight standards were from Bethesda Research Laboratories. Laurylmaltoside was synthesized according to Rosevear et al. (1980). Asolectin was from Associated Concentrates, Woodside, New York. Beef heart cytochrome oxidase antibody was prepared by J. Hochman (Interx, Lawrence, Kansas). Beef heart cytochrome oxidase subunit II monoclonal antibody was prepared and characterized by T. Taha and S. Ferguson-Miller (manuscript in preparation). Sources of restriction enzymes and other reagents were as described previously.

Construction of the pSPOXII Plasmid, and RNA Synthesis. The <u>ncox</u>II gene containing universal codons was excised from the plasmid pDROXII, and was cloned into pSP65 at the <u>Bam</u>HI site (Figure 16). The recombinant plasmid (PSPOXII) with the insert in correct orientation was selected by restriction mapping, and prepared as described by Maniatis

Figure 16. Construction of plasmid pSPOXII. The <u>ncox</u>II gene was obtained by digestion of pDROXII with <u>Bam</u>HI. It was cloned into the pSP65 transcription vector at the <u>Bam</u>HI site. The recombinant pSPOXII with the <u>ncox</u>II gene in the correct orientation was linearized by digestion with <u>Hind</u>III within the polylinker region, then was used to produce the COXII RNA by <u>in vitro</u> transcription with <u>SP6</u> RNA polymerase.



et al. (1982). The plasmid was linearized by <u>Hind</u>III digestion and used for <u>in vitro</u> transcription according to the supplier's protocol. The transcription reaction was extracted with phenol and chloroform followed by precipitation with 0.1 volumes of 3 M sodium acetate and 2.5 volumes of ethanol. The RNA was adenylated by using poly(A) polymerase as described by Gething <u>et al</u>. (1980) and was further purified using a Sephadex G-50 spin column (Maniatis <u>et al</u>., 1982). The length of the mRNA was checked by agarose gel electrophoresis, and its concentration determined spectrophotometrically.

Preparation of Lipid Vesicles. Lipid vesicles were prepared as described by Casey <u>et al</u>. (1979). Briefly, a mixture of 40 mg/ml of asolectin in 2% potassium cholate, 100 Mm HEPES, PH 7.2, was sonicated to clarity with a point sonicator (using microtip at a dial setting between 3 and 5) with the sample on ice and under argon. The time used to sonicate was about 1 min/ml of suspension (at 30 sec on and 30 sec off intervals) or until clear. The solution was centrifuged at 7,000xg for 15 min to remove titanium particles, dialyzed against 100 mM HEPES for 4 h, and then against 10 mM HEPES, 40 mM KCl, 45 mM sucrose overnight with one change of the same buffer. The dialyzed lipid vesicle solution was centrifuged in a microfuge at 12,000xg for 15 min and the pellet containing larger vesicles was saved and
resuspended in 20 mM Tris, PH 7.5 to a final concentration of approximately 200 mg/ml. The lipid vesicles were aliquoted and stored at -70° C. All the procedures were done at 4° C.

In Vitro Translation. In vitro translation of COXII RNA in rabbit reticulocyte lysates was performed in the presence or absence of canine pancreatic microsomal membranes according to the supplier's instructions In vitro translation reactions were also (Promega). performed in the presence of artificial vesicles at various concentration: 10 mg/ml, 15 mg/ml, and 20 mg/ml, with other components remaining constant as above. In all translation reactions, [³⁵S] methionine was used to label the synthesized peptides. Aliquots of reaction mixtures were directly analyzed for protein by SDS-PAGE according to Laemmli (1970) or by SDS-urea gels as described by Kadenbach at al. (1983); gels were fluorographed as described by Banner et al. (1974). To fractionate the microsomal membranes and lipid vesicles from the translation mixtures, about 50 ul of each reaction was centrifuged in an airfuge at 120,000xg for 1 h. The pellet was washed with PBS and both pellet and supernatant were run on SDS-PAGE as above with modified SDS-sample buffer (62.5 mM Tris-Hcl, pH 6.8, 10% glycerol, 8% SDS, and 5% ß-mercaptoethanol).

Extraction and Immunoprecipitation of COXII. Membraneassociated COXII was solubilized with 0.5 ml of phosphate buffered saline (PBS) containing 2% laurylmaltoside and proteinase inhibitors (0.1 mM PMSF, 10 ug/ml aproteinin) followed by centrifugation for 15 min at 12,000xg at 4°C. The supernatant was saved. Five ul (5 mg/ml) of beef heart cytochrome oxidase antibodies or the COXII monoclonal antibody was added to the supernatant and incubated at $4^{\circ}C$ overnight. About 40 ul of protein A-agarose slurry (preincubated with 5% BSA in PBS) was added to the above mixture and incubated for 2 h at room temperature. The immunoabsorbed proteins were eluted from protein A-agarose with SDS sample buffer (62.5 mM Tris-Hcl, PH 6.8, 10% glycerol, 3% SDS, 5% ß-mercaptoethanol) and analyzed by SDS-PAGE.

Analysis of COXII-Membrane Preparation by Trypsin and Proteinase K Digestion. COXII synthesized by <u>in vitro</u> translation was analyzed by digestion with trypsin or proteinase K. Lipid vesicles or microsomal membranes containing the COXII were collected from 50 ul of translation reaction by centrifuging in the Airfuge as above. For trypsin digestion, the pellet was dissolved in 10 ul of PBS containing 2% laurylmaltoside, and 1 ug of trypsin was added. The digestion reaction was incubated at 4°C for 90 min, stopped by heating at 95°C for 5 min, and

analyzed by 18.5% urea-SDS-PAGE. For proteinase K digestion, the pellet was dissolved in 100 ul of PBS without laurylmaltoside; 10 ul of this COXII-membrane solution was mixed with 1 ul of a solution of proteinase K dissolved in water (0.1 mg/ml) and samples were incubated at 4°C for 60 min. Proteinase K digestions were also performed in the presence of 0.5% sodium deoxycholate. After digestion, 40 ul of sample buffer was added to each tube. Following incubation in a boiling water bath for 2 min, aliquots were immediately loaded onto an 18% urea-SDS-gel and electrophoresed.

RESULTS

In Vitro Translation in the Presence of Microsomal Membranes. Figure 17 shows the products obtained from translation of COXII mRNA derived from PSPOXII in a rabbit reticulocyte lysate system. A peptide migrating to the same position as the authentic COXII appears in Figure 17A, lane 4, which reflects a reaction containing COXII RNA at 10 ug/ml; this band is not present in the control lanes 1, 2 and 3. When COXII RNA is translated in the presence of canine pancreatic microsomal membranes, protein synthesis is stimulated significantly. A peptide with a larger apparent molecular weight is seen in addition to the COXII (Figure 17A, lane 5). Treatment of the mixture with endopeptidase F (Tarentino et al., 1985) leads to conversion of the new band into a band that migrates with COXII, hence the new peptide is a glycosylated form of COXII (data not shown). Both of the bands appear in the membrane fraction after centrifugation (Figure 17B) and both can be immunoprecipitated by COXII-specific antibodies (Figure 18). Beef heart cytochrome oxidase polyclonal antibodies precipitate both forms of COXII, glycosylated and nonglycosylated. The COXII monoclonal antibody precipitates mainly the nonglycosylated COXII. A weak band that migrates intermediate between the glycosylated and nonglycosylated forms of COXII is seen in Figure 18; it is the partially

Figure 17. <u>In vitro</u> translation of COXII RNA in rabbit reticulocyte lysates. Panel A is a fluorograph of a 12.5% SDS gel: lane 1, lysate only; lane 2, lysate plus microsomal membranes; lane 3, lysate plus brome mosaic virus RNA; lane 4, lysate plus COXII MRNA (10 ug/ml); lane 5, lysate plus COXII RNA (10 ug/ml) plus 2 ul of microsomal membranes. Panel B is a fluorograph of a 18.5% urea SDS gel showing fractionation of the <u>in vitro</u> translation mixture shown in lane 5, panel A. OXII' represents the glycosylated form of OXII (see text). A third band above OXII' is presumably a further glycosylated form that formed through some other mechanism.



Figure 18. Immunoprecipitation of <u>in vitro</u> synthesized COXII. Translation reactions were performed in the presence (lane 2) or absence (lane 1) of microsomal membranes. Panel A, immunoprecipitation using beef heart cytochrome oxidase antibodies; panel B, immunoprecipitation using beef heart COXII monoclonal antibody. Bands smaller than the 18.4 kd are prematured COXII peptides.



glycosylated form, and can be precipitated by both antibodies. Since the monoclonal antibody is specific to the C-terminal of the authentic COXII (Taha and Ferguson-Miller, manuscript in preparation), glycosylation at both sites (NAsn-His-Ser²⁰⁵, NAsn-Trp-Ser²²³) of the C-terminal apparently inhibits binding of the COXII to its antibodies. These results indicate that COXII undergoes core glycosylation in the endoplasmic reticulum, following insertion into the membranes.

In Vitro Translation in the Presence of Artificial Lipid Vesicles. In vitro translation of COXII RNA in a rabbit reticulocyte lysate was also strongly stimulated by the addition of artificial lipid vesicles (Figure 19A). The maximum stimulation by the addition of artificial lipid vesicles was achieved at 15 mg/ml (Figure 19B). In this system only the nonglycosylated form was produced and it sedimented with the lipid vesicles upon centrifugation (Figure 19C). This finding indicates that in the presence of lipid vesicles COXII is also inserted into the membranes but it is not glycosylated presumably because the necessary enzymes are lacking.

<u>Proteinase K Digestion</u>. Additional evidence that COXII synthesized <u>in vitro</u> is inserted into membranes derives from studies using proteinase K. If COXII is inserted in the Figure 19. A fluorogram of an 18.5% urea-SDS-polyacrylamide gel showing <u>in vitro</u> translation of COXII RNA in the presence of artificial vesicles. Panel A, lane 1, lysate plus lipid vesicles (15 mg/ml); lane 2 lysate plus COXII RNA (10 ug/ml); lane 3, lysate plus COXII RNA (10 ug/ml) plus lipid vesicles (15 mg/ml). Panel B, <u>in vitro</u> translation in the presence of different amounts of lipid vesicles: lane 1, 10 mg/ml; lane 2, 15 mg/ml; lane 3, 20 mg/ml. Panel C, fractionation of the <u>in vitro</u> translation mixture in the presence of lipid vesicles: lane 1, pellet; lane 2, supernatant.





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predicted manner (Holm et al., 1987) (see Figure 3), residues 27-82 that form the hairpin across the membrane should be protected and a residual peptide of about 7 kd should be detected in SDS-PAGE. Indeed, a peptide with molecular weight of about 7 kd is protected when membraneassociated COXII is digested with proteinase K. This band disappears when membranes are solubilized in the presence of sodium deoxycholate at a final concentration of 0.5% prior to addition of the protease (Figure 20). The faint 7 kd band visible in the presence of lipid vesicles plus detergent is likely due to incomplete solubilization due to the high lipid concentration. Thus, these experiments provide support for the idea that the COXII synthesized in <u>vitro</u> is not only in a nondenatured from but also is inserted into membranes in the expected manner (Holm et al., 1987).

<u>Trypsin Digestion</u>. As shown in Figure 21, trypsin digestion of the newly made COXII leads to two major bands, 15 kd and 6.5 kd, as well as a minor band of 12.5 kd. This digestion pattern matches that predicted for rat liver subunit II and that obtained with the beef heart subunit II peptide (Taha and Ferguson-Miller, manuscript in preparation). These results provide further evidence that the COXII synthesized <u>in vitro</u> is identical to COXII of rat liver cytochrome oxidase.

Figure 20. Proteinase K digestion of membrane-associated COXII. The left two lanes represent SDS-PAGE following translation in the presence of lipid vesicles, the right two lanes show the results from translation in the presence of microsomal membranes. Digestions in the absence (or presence) of sodium deoxycholate indicated with -D (or +D).



Figure 21. Trypsin digestion of <u>in vitro</u> synthesized COXII. Lipid vesicles containing the COXII peptide were pelletted by centrifugation, digested with trypsin, and analyzed by 18.5% urea-SDS-PAGE. Lane 1, molecular weight markers; lane 2, undigested; lane 3, digested.





DISCUSSION

The above experiments demonstrate that subunit II of rat liver cytochrome oxidase can be synthesized in a rabbit reticulocyte lysate in vitro translation system. The peptide made in vitro is identical with the authentic COXII based on the following: first, the synthesized peptide has the same mobility as that of the authentic COXII peptide on SDS-PAGE; second, the peptide can be immunoprecipitated by beef heart cytochrome oxidase antibodies; third, the trypsin digestion pattern of this peptide is the same as that of the authentic COXII; and finally, the COXII peptide made in vitro is inserted into membranes in a manner consistent with predictions from other data as demonstrated by proteinase K digestion and extractability with the nonionic detergent laurylmaltoside. Therefore, the evidence that this peptide represents the authentic COXII is based on size, immunological cross-reactivity, membrane insertion, and peptide mapping.

These results have significance at several levels. It is important for future work in this area to show that a native-like, membrane-associated COXII can be synthesized in the absence of other subunits of the oxidase. Second, the subunit seems to be properly inserted into the membrane as predicted by Holm <u>et al</u>. (1987). Third, production of the COXII peptide is markedly stimulated in the presence of

added membranes, and we also find that artificial membranes can replace microsomal membranes in our <u>in vitro</u> translation system. Finally, in the rabbit reticulocyte lysate translation system, more short peptides were made in the absence of microsomal membranes or artificial lipid vesicles. Thus, the function of the membranes in the translation system may be to provide a hydrophobic environment into which COXII can be integrated (and protected from proteolytic degradation) as it is synthesized. If it is indeed true that membranes are necessary for high level COXII accumulation, the low level of expression of <u>ncox</u>II in <u>E</u>. <u>coli</u> may be due to poor insertion of the peptide into membrane structures, and its subsequent susceptibility to proteases in the cell milieu.

Canine pancreatic microsomal membranes (Shields and Blobel, 1977), mitochondrial membranes (Zhuang <u>et al</u>., 1989), and <u>Xenopus</u> oocyte membranes (Ohlsson <u>et al</u>., 1981) have been used in studies of <u>in vitro</u> translation of membrane proteins. The finding that artificial lipid vesicles prepared from purified soybean phospholipid can replace natural membranes for stimulating the synthesis of COXII peptide is of great interest and provides a number of advantages. Artificial lipid vesicles are easy to make and are much less expensive than the commercial canine pancreatic microsomal membranes. They lack the enzyme activity that catalyzes glycosylations, and they are free of

other proteins so that they provide an easy route for purification of the inserted peptide. The use of artificial lipid membranes may also be valuable in studies of translation of other transmembrane proteins <u>in vitro</u> (Ohlsson <u>et al.</u>, 1981; Katz <u>et al.</u>, 1977).

In vitro synthesis of COXII and its insertion into vesicles provides a unique way of studying COXII in the absence of other subunits of mammalian or bacterial cytochrome oxidase. The rabbit reticulocyte lysate plus artificial lipid vesicles provides a system that is free of endogenous oxidase. Incorporation of whole cytochrome oxidase into lipid vesicles has been routinely used in studies of its kinetics properties (Carroll and Racker, 1977). The insertion of COXII itself into vesicles provides a potentially unique way to investigate its relationship with other subunits from rat liver cytochrome oxidase or from a bacterial cytochrome oxidase such as <u>Rhodobacter</u> <u>sphaeroides</u> (Hosler <u>et al.</u>, 1989).

CHAPTER 5

EXPRESSION OF RAT LIVER CYTOCHROME OXIDASE SUBUNIT II IN <u>XENOPUS</u> <u>LAEVIS</u> OOCYTES

INTRODUCTION

<u>Xenopus</u> <u>laevis</u> oocytes are very active in protein synthesis, and are known to translate very efficiently injected mRNA from a variety of sources (Gurdon et al., 1971). The <u>Xenopus</u> oocyte system has several advantages over cell-free translation. Oocytes can translate injected mRNA with an efficiency about 30 times higher than that of the rabbit reticulocyte lysate in vitro translation system. Furthermore, the synthesized proteins are often directed into the intracellular membranes, in many cases ultimately appearing in the oocyte cytoplasmic membranes. Finally, biologically-active, foreign proteins synthesized in oocytes can acquire their native activity even if this involves complex subunit interactions (Colman, 1984). For example, functional membrane proteins made following injection of mRNA into Xenopus oocytes include the sodium channel from the electric organ of the electric eel, Electrophorus <u>electricus</u> (Noda <u>et al.</u>, 1986); α -subunit of acetylcholine

receptor of <u>Torpedo californica</u> (Mishina <u>et al</u>., 1984); bovine rhodopsin (Khorana <u>et al</u>., 1988), and human ßadrenergic receptors (Kobilka <u>et al</u>., 1987).

This chapter reports on the expression of the $\underline{ncox}II$ gene in X. <u>laevis</u> oocytes. The COXII produced in oocytes has the same properties as that synthesized in rabbit reticulocyte lysates, but is made at a much higher level of expression. X. <u>laevis</u> oocytes proved very efficient in synthesizing COXII, with a yield of about 5-10 ng peptide/cell under optimal conditions. This will provide a valuable source of protein with which to begin a meaningful assessment of the ability of subunit II to interact with its prosthetic groups (heme <u>a</u> and copper) and with ligands such as cytochrome <u>c</u>.

EXPERIMENTAL PROCEDURES

Materials. Xenopus laevis females were purchased from Nasco, Fort Atkinson, WI. The injection apparatus used was as described by Contreras et al. (1981); a Drummond Micropipette (10 ul) was used for injection. Injection needles were made from special glasses (from Drummond), using an electrode puller (Contreras et al., 1981) to taper the tips, which were broken off to about 10 microns diameter with a pair of fine forceps. The tip size was calibrated under a microscope. Sutures and scalpels were from Ethicon, Tran³⁵S-label was supplied by ICN Biomedical. Inc. Collagenase (type 1A), sodium penicillin, streptomycin sulfate and aprotinin were from Sigma. Fetal calf serum was from Gibco, and was dialyzed extensively against modified Barth's saline (MBS) (Khorana et al., 1988) to exchange salts prior to use. Preparation of RNA, of laurylmaltoside, and of beef heart cytochrome oxidase antibody, as well as SDS-polyacrylamide gel electrophoresis and fluorography procedures were previously described in Chapter 4.

<u>Oocyte Injection</u>. Mature <u>Xenopus laevis</u> females were anesthetized by immersing in ice-water for 30 min. An incision was made in the lower part of abdomen, and a length of ovary was removed and washed with sterile Barth's solution (Gundon, 1968). Individual oocytes were obtained

by digestion of the ovary with collagenase at 2 mg/ml in a buffer containing 82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 10 mM HEPES, pH 7.6 (Wallace <u>et al</u>., 1973), for 1-2 h with one change at room temperature. The oocytes were washed with MBS (Khorana <u>et al</u>., 1988) supplemented with 5% fetal calf serum and incubated at 4°C overnight. The next morning healthy oocytes at stage V-VI were selected under a dissecting microscope and used for injection with 50 nl of RNA solution. The RNA was dissolved in water with concentration from 0.02 mg/ml to 0.8 mg/ml. Control oocytes were injected with water. Injected oocytes were incubated in MBS supplemented with 5% fetal calf serum for 24-72 h at 18°C. Tran³⁵S-label was added to the oocyte incubation medium to 0.1 mCi/ml 10 h after injection.

Immunoprecipitation of Expressed Peptide. To immunoprecipitate the expressed COXII, four injected oocytes were pooled and homogenized with 0.5 ml of PBS buffer containing 2% laurylmaltoside, 0.1 mM PMSF, 10 ug/ml aprotinin. Homogenates were incubated at room temperature for 15 min, followed by centrifugation at 12,000xg at 4°C for 15 min. The supernatant was saved and 5 ul beef heart cytochrome oxidase antibody solution (5 mg/ml in potassium phosphate saline) was added and incubated at 4°C overnight with gentle shaking. About 40 ul of protein A-agarose slurry (that had been preincubated with extracts of uninjected oocytes) was added to the above mixture and incubated for 2 h at room temperature. The protein-Aagarose beads were transferred into a polypropylene column and washed with an excess of buffer containing 20 mM sodium phosphate, pH 7.4, 0.1 M NaCl, 1 mM EDTA, 1% BSA, 1% nonidet P-40, and 0.1 mM PMSF, and then with PBS buffer. The immunoprecipitated peptide bound to the beads was eluted with SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol 3% SDS, 5% ß-mercaptoethanol) and electrophoresed on a 12.5% SDS-polyacrylamide gel.

<u>Oocyte Membrane Preparation</u>. All manipulations were performed at 4°C. Oocyte cytoplasmic membranes were prepared by the method of Kobilka <u>et al</u>. (1987). About 4,000 oocytes that had been injected with 20 ng of RNA/oocyte were homogenized in 20 ml of PBS containing 30% sucrose, 0.1 mM PMSF, with a Dounce homogenizer. The homogenate was centrifuged at 3,000xg for 10 min to remove cell debris and pigment granules. The supernatant was centrifuged at 10,000xg for 15 min, and the resulting supernatant was centrifuged at 200,000xg for 2 h. The pellet from this high speed spin was the membrane preparation, and was stored at -70° C.

Extraction of the COXII Peptide with Laurylmaltoside. Oocyte membranes prepared from 4,000 injected oocytes were

solubilized in 10 ml PBS containing 2% laurylmaltoside, 5% sucrose, 20 mM potassium phosphate, pH 7.4, and 0.1 mM PMSF. After stirring at 4°C for 30 min, the detergent-solubilized membrane solution was centrifuged at 200,000xg for 30 min. Aliquots of supernatant and pellet were analyzed on a 12.5% SDS-polyacrylamide gel. The supernatant from this spin contains the solubilized membrane components.

FPLC Purification of COXII. The solubilized membrane extract was filtered through a 0.45 um millipore filter before it was loaded onto a mono-Q 5/5 column, which had been equilibrated with buffer A (5% sucrose, 20 mM potassium phosphate, pH 7.4, 4 mM laurylmaltoside). The column was washed with buffer A until the absorbance at 280 nm reached zero, then was eluted with a linear gradient of KCl formed from 0-100% buffer B (1 M KCl in buffer A) at a flow rate of 1 ml/min. Since the COXII was labeled with ³⁵S-methionine, detection of the peptide was done by fluorography of an SDSpolyacrylamide gel. Aliquots of different fractions were precipitated by the addition of 10% trichloroacetic acid to 5% final concentration and analyzed by SDS-PAGE. Fractions containing COXII were pooled, diluted, and reapplied to the FPLC mono Q 5/5 column at 150 mM KCl, washed with a continuous gradient of KCl formed from 0-100% buffer B (1 M KCl in buffer A), containing a two-step salt gradient, with a flow rate of 1 ml/min. The first step starts with a hold at 200 mM salt concentration for 5 min and gradually goes to

340 mM salt concentration during a 15 min time period; the second step starts with hold at 340 mM salt concentration for 5 min and gradually goes to 500 mM salt concentration in 15 min. Fractions were collected as above.

RESULTS AND DISCUSSION

Xenopus Oocyte Injection and Immunoprecipitation of Expressed COXII. Injection of RNA derived from the pSPOXII construct into Xenopus oocytes led to the synthesis of full length COXII. Oocytes that were injected with COXII RNA show two major immunoprecipitation bands that migrate on an SDS gel to the same positions as glycosylated and nonglycosylated COXII; both bands are absent in the control oocytes. As shown in Figure 22, there is no detectable difference in gel mobility between COXII made in oocytes and the peptides made in rabbit reticulocyte lysates in the presence of microsomal membranes. This indicates that most of the COXII peptide undergoes core glycosylation in the endoplasmic reticulum of oocytes and is inserted into oocyte membranes. The maximum expression of COXII was achieved at about 20 ng RNA/oocyte, injection of more than 40 ng RNA/oocyte led to decreased expression of the peptide. The highest level of expression was achieved about 48 h after injection (Figure 23) and longer time incubation also leads to an apparent decrease in the level of expression. Under optimal conditions, the yield of COXII was 5 to 10 ng peptide/oocyte.

<u>Oocyte Membrane Preparation and Extraction of COXII</u> with Laurylmaltoside. Oocytes injected with COXII RNA (20 Figure 22. A fluorogram of a 12.5% SDS-polyacrylamide gel showing the expression of COXII RNA in <u>X</u>. <u>laevis</u> oocytes. The synthesized COXII was immunoprecipitated by using beef heart cytochrome oxidase polyclonal antibodies. Lane 1, molecular weight markers; lane 2, <u>in vitro</u> translation in rabbit reticulocyte lysate of COXII mRNA in the presence of microsomal membranes. Lane 3-7 represent data from injected oocytes: lane 3, 20 ng RNA/oocyte; lane 4, 10 ng RNA/oocyte; lane 5, 5 ng RNA/oocyte; lane 6, 1 ng RNA/oocyte; lane 7, control oocytes injected with water. OXII' represents the glycosylated form of OXII (cytochrome <u>c</u> oxidase subunit II).



Figure 23. A fluorogram of a 12.5% SDS-polyacrylamide gel showing the expression of COXII at different times after injection into X. <u>laevis</u> oocytes. The expressed COXII was immunoprecipitated by using beef heart cytochrome oxidase polyclonal antibody. Lane 1, molecular weight markers; lane 2, oocytes incubated for 24 h after injection; lane 3, oocytes incubated for 48 h after injection; lane 4, oocytes injected for 72 h after injection; lane 5, oocytes injected with water and incubated for 72 h.

97.4--

68**-**

43-

29-

•••

_OXII'

18.4-

ng RNA/oocyte) were labeled with [³⁵S] methionine, and the membrane and supernatant fractions were prepared and analyzed by SDS-PAGE. The results from the fractionation study demonstrate that COXII synthesized in oocytes is in a membrane-associated form (Figure 24). When the membranes were extracted with a mild nonionic detergent, laurylmaltoside, all the COXII was solubilized to the membrane extract fraction (Figure 25).

FPLC Purification of COXII. FPLC anion-exchange chromatography has been used for cytochrome oxidase purification from wheat, rat liver, and beef heart. Since COXII made in oocytes is in a nondenatured membraneassociated form and contains a large number of negative charges, it might be expected to chromatograph on a mono-Q column in a manner similar to holo cytochrome oxidase. Figure 26 shows that this is indeed the case. All the COXII and other membrane components are bound to the column, as seen from the fact that no detectable amount of COXII and COXII' are in the flow-through from the column. The ^{35}S labeled COXII and COXII' elute as peaks around 300 mM salt concentration, with an elution profile comparable to that of holo cytochrome oxidase, which is released around 340 mM KCl (data not shown). It is known that the binding of the holo enzyme to the column is via the COXII subunit. After two runs on a FPLC mono-Q column, the COXII has been purified

Figure 24. Fractionation of the oocyte membranes after synthesis of COXII from injected RNA. ³⁵S-protein content of oocyte membranes and cytosol fractions were analyzed on a 12.5% SDS-polyacrylamide gel. The gel was fluorographed as described in the Experimental Procedures. Lane 1, complete oocyte membranes; lane 2, oocyte cytosol.



Figure 25. A fluorogram of a 12.5% SDS-polyacrylamide gel showing laurylmaltoside extraction of the oocyte membranes. Lane 1, supernatant of laurylmaltoside solubilized membranes (membrane extracts); lane 2, pellet of laurylmaltoside solubilized membranes.


Figure 26. Fluorographic detection of ³⁵S-COXII in a 12.5% SDS-polyacrylamide gel. Fractions collected from the mono-Q column were precipitated by trichloroacetic acid and analyzed by SDS-PAGE. The corresponding salt concentration is shown at the bottom. The OXII' represents the glycosylated OXII from oocytes; lanes 4-29 correspond to fraction numbers from the column.



significantly, but it is far from homogeneity. It was not possible to purify COXII by only using FPLC, due to the loss of sample and denaturation of COXII which occurs during the course of purification. At this point, the design of a monoclonal antibody affinity column may be the most efficient next step in the purification process (Khorana <u>et</u> al., 1988).

In conclusion, then, injection of COXII RNA into <u>Xenopus</u> oocytes results in the synthesis of substantial quantities (about 10 ng peptide/oocyte) of a COXII peptide that is in a nondenatured and membrane-associated form. The advantage of the oocyte expression system is that it contains an excess of heme a and copper, which should allow for isolation of the expressed peptide and assay for the heme \underline{a} and copper associated with this subunit. Although there is an endogenous <u>Xenopus</u> cytochrome oxidase, the rat liver COXII is inserted into the cytoplasmic membranes, which are easily separated from the mitochondria. The oocyte expression system should also be suitable for the synthesis of COXI of rat liver, following conversion of its gene from mitochondrial to nuclear form. Purification of COXII by FPLC is possible only to a limited extent. Antibody affinity columns may ultimately lead to successful isolation of this subunit.

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CONCLUSIONS

The results described in this thesis may be summarized as follows.

- (1) The gene for subunit II of rat liver cytochrome oxidase has been converted into the "nuclear" form by site-directed mutagenesis.
- (2) The gene has been expressed as a denatured protein in <u>E</u>. <u>coli</u>. Despite the use of a wide variety of vectors, it has not been possible to obtain a nondenatured form in <u>E</u>. <u>coli</u>.
- (3) The converted subunit II gene has been expressed in a native or native-like form by <u>in vitro</u> translation in rabbit reticulocyte lysates, and by injection of COXII mRNA into <u>Xenopus</u> oocytes. In both these systems, the COXII peptide is inserted into membranes and can be extracted with the mild nonionic detergent laurylmaltoside.
- (4) An unexpected discovery from this work is that artificial lipid vesicles are equally as good as natural membranes in stimulating production of COXII in reticulocyte lysates.

All these results indicate that it is possible to express an individual subunit of cytochrome oxidase in native form and in quantities suitable for further experimentation. This is the first time to my knowledge that cytochrome oxidase has been studied by this approach, which opens a new route to investigate the thirteen different subunits of mammalian cytochrome oxidase individually. An important and doable future experiment will be to obtain enough subunit II to determine if it contains copper in the correct stoichiometry and with the spectral characteristics indicative of a truly native form.

Another direction to be pursued is the conversion of the gene for subunit I of rat liver cytochrome oxidase to its nuclear form. Coexpression of subunit I and II of rat liver cytochrome oxidase either by in vitro translation with reticulocyte lysates or in X. laevis oocytes will enable us to determine if they will associate and show any aspect of cytochrome oxidase activity, since they are predicted to be minimal functional unit of the enzyme. The ultimate goal will be coexpression of various combinations of both mitochondrial and nuclear genes in a suitable high yield expression system, in order to determine the structural basis of the functional and regulatory properties of the enzyme. The most promising expression system to be investigated in the future are the R. sphaeroides from which the native cytochrome <u>aa</u>, has been deleted or the insect cell/baculovirus expression system.

The insect cell/baculovirus system (Smith <u>et al</u>., 1983) has been used to synthesize many recombinant proteins as

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biologically active fusion proteins which include several amino acid residues of the polyhedron protein. More recently, virus vectors have been used to express nonfusion proteins to high levels (up to 1% of the total soluble protein) (Funk <u>et al</u>., 1989). This system may be able to direct the expression of subunit II of rat liver cytochrome oxidase to high levels as well as coexpression of subunit I and II.

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