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THE STRUCTURE OF CYTOCHROME *b5* AND ITS FUNCTION IN Δ12 OLEATE DESATURATION IN THE MICROSOMES OF DEVELOPING SAFFLOWER SEEDS

By

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ABSTRACT

THE STRUCTURE OF CYTOCHROME *b5* AND ITS FUNCTION IN Δ12 OLEATE DESATURATION IN THE MICROSOMES OF DEVELOPING SAFFLOWER SEEDS

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Intermediate electron donors for microsomal membrane-bound fatty acid desaturases of higher plants have not been previously identified. However, the involvement of cytochrome *b5* as an electron donor to Δ-9 and Δ-6 mammalian desaturation systems has been well documented. These studies revealed an electron shuttling system involving NADH-cytochrome *b5* reductase, cytochrome *b5*, and a desaturase.

To investigate the role of cytochrome *b5* in microsomal fatty acid desaturation in higher plants, the cytoplasmic domain of microsomal cytochrome *b5* was purified from cauliflower florets, and murine polyclonal antibodies were prepared. A classic test of the activity of cytochrome *b5* in microsomes is the NADH-dependent reduction of exogenous cytochrome *c*. Purified immune IgG from mouse ascites fluid inhibited the NADH-dependent reduction of cytochrome *c* by 62% in microsomes from developing Safflower seeds, suggesting that the IgG sterically hindered electron transfer through cytochrome *b5* to the exogenous cytochrome *c*.

The Δ-12 desaturase assay is complicated because the substrate for desaturation is thought to be phosphatidylcholine but the substrate provided is ^{14}C-oleoyl-CoA. Thus, the overall assay measures both acyltransferase and desaturase activities at the very least. The IgG had no effect on acyltransfer from ^{14}C-oleoyl-CoA to phosphatidylcholine and other phospholipids. However, the IgG blocked the desaturation of ^{14}C-oleic acid to ^{14}C-linoleic acid in all phospholipids by 93%, suggesting that cytochrome *b5* is the electron donor for Δ-12 desaturase.

IgG quenched with cytochrome *b5* showed a linear decrease in inhibitory effect with increased cytochrome *b5*, while excess purified cytochrome *b5* alone had little effect on the rate of Δ-12 desaturation. Therefore, IgG which specifically binds cytochrome *b5* inhibits Δ-12 desaturation.

Partial amino acid sequence was obtained from the purified cauliflower cytochrome *b5*. To examine the structure of plant cytochrome *b5* in more detail, the gene encoding the protein was cloned and sequenced. Four λUNI-ZAP cDNA clones encoded the same 134 amino acid protein.

GAUDEAMUS IGITUR...

SEMPER SINT IN FLORE !

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ACC	acetyl-CoA carboxylase
ACP	acyl carrier protein
BrPC	1-palmitoyl-2-dibromostearoyl phosphatidylcholine
CHAPS	(3-[3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate)
CoA	coenzyme A
DAF	days after florets break from bud
DAG	diacylglycerol
DDG	digalactosyldiacylglycerol
ΔN	positioned N carbons from the ester on the fatty acid chain
DTT	dithiothreitol
ER	endoplasmic reticulum
FAS	fatty acid synthesis
FPLC	Fast Protein Liquid Chromatography
G-3-P	glycerol-3-phosphate
IgG	Immunoglobulin G
KAS	3-ketoacyl-ACP synthase
LPS	lipopolysaccharide
Lyso-PX	a phospholipid with headgroup X and only one acyl chain
MDG	monogalactosyldiacylglycerol
μ	ionic strength
NMR	nuclear magnetic resonance
OM	outer mitochondrial membrane
PA	phosphatidic acid
PBS	phosphate buffered saline

PC	phosphatidylcholine
pCMB	p-chloromercuribenzoate
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
pI	isoelectric point
PM	plasma membrane
PS	phosphatidtylserine
SQD	sulfoquinovosyldiacylglycerol
SUV	small unilamellar vesicles
X:O	a chain of X carbons containing O double bonds

CHAPTER ONE
INTRODUCTION

Applications of Research on Lipids of Higher Plants

A basic understanding of lipid biosynthesis and regulation in higher plants will provide building blocks for the future of North American agriculture. Major agricultural issues currently being addressed by plant lipid researchers are the molecular basis for cold-sensitivity and heat tolerance, the involvement of plant communication signals in the collective protection of crops, and the diversification of farm productivity. In addition, control of lipid composition via genetic engineering may be advantageous in increasing energy efficiency within the plant and improving the ability of plants to cope with environmental stresses such as pollution and water deficit.

The research presented in this dissertation represents a first step in the ability to genetically engineer lipid biosynthesis in crop plants. Enzyme purification and immunoinhibition were used to identify cytochrome *b5* as the electron donor to the endoplasmic reticulum Δ12 desaturation activity in the oilseed crop plant, *Carthamus tinctorius*, better known as safflower. In the second phase of the dissertation, cDNA clones for cytochrome *b5* were identified and sequenced. Antisense constructs of the

cDNA sequence of cytochrome *b5* can now be used in experimental plants to test the possibility that regulation of cytochrome *b5* can affect the overall lipid biosynthesis of a plant. In addition, the cDNA can be used to overproduce plant cytochrome *b5* in *Escherichia coli* for further studies involving the protein.

Higher Plant Lipid Biosynthesis

A number of extensive reviews have been written regarding lipid biosynthesis in plants[1,2,3,4,5]. Aspects of lipid biosynthesis pertinent to the understanding of the Δ12 desaturase studied in this dissertation will be presented here.

Fatty Acid Synthesis. Fatty acid synthesis (FAS) in higher plants is a type II or non associated process like that found in most bacteria. It occurs primarily in the plastids[6] and possibly in the mitochondria[7]. In the first step of fatty acid synthesis, acetyl-Coenzyme A (acetyl-CoA) is activated to malonyl-CoA by acetyl-CoA carboxylase (ACC). Malonyl-CoA is then transacylated to malonyl-acyl carrier protein (malonyl-ACP). Malonyl-ACP is condensed with another molecule of acetyl-CoA to form acetoacetyl-ACP, releasing CO_2. The condensation is performed by 3-ketoacyl-ACP synthase-III (KAS III). Acetoacetyl-ACP is then reduced by 3-ketoacyl-ACP reductase to 3-hydroxybutyryl-ACP, which is then dehydrated to trans-2-acyl-ACP by 3-hydroxacyl-ACP dehydratase. Finally, one of the two forms of enoyl-ACP reductase reduces the enoyl to a saturated acyl chain and thus completes one cycle of fatty acid synthesis with the net addition of two carbons to the original chain length. This cycle

continues until the chain length reaches 16 or 18 carbons. Palmitoyl-ACP (16:0) and stearoyl-ACP (18:0) are the primary products of fatty acid synthesis.

Fatty Acid Translocation. Once palmitoyl-ACP and stearoyl-ACP have been made in the FAS cycle, stearoyl-ACP may be desaturated to oleoyl-ACP by a soluble stearoyl-ACP desaturase, also known as Δ9-desaturase[8]. Palmitoyl and oleoyl fatty acids either remain in the chloroplast where they are used for lipid synthesis by the prokaryotic pathway or they are transported from the chloroplast to serve as substrates for lipid biosynthesis in the endoplasmic reticulum[9]. It is still unclear how fatty acids migrate in the plant cell, although data on *in vitro* lipid transfer in plants has been accumulated[10,11,12]. The transport of acyl-ACPs from the chloroplast probably involves the formation of free fatty acids by acyl-ACP hydrolases[13], also called thioesterases[14,15]. These fatty acids are then re-esterified to CoA by an acyl-CoA synthetase found on the outer membrane of the chloroplast envelope[16]. Much work has been done on the components of lipid translocation in animal systems. For a recent review on animal lipid translocation, please see van Meer[17].

Lipid Synthesis. As stated above, the lipid biosynthesis pathway branches between the chloroplast and the endoplasmic reticulum (ER) after fatty acid synthesis. In each branch, parallel reactions occur: fatty acids are esterified to glycerol-3-phosphate (G-3-P) to form phosphatidic acid (PA) and a series of reactions then converts PA to the other glycerol lipids. An additional minor site of lipid biosynthesis is the mitochondrion in which the formation of PA, phosphatidylglycerol (PG), and

cardiolipin has been reported[18,19]. The amount of lipid consigned to each branch is dependent on the type of plant. In general, plants which have a PA phosphatase to produce diacylglycerol (DAG) in the chloroplast are called 16:3 plants[20,21]. Plants in which the chloroplast DAG is predominantly imported from the endoplasmic reticulum are called 18:3 plants. The DAG derived from the ER never contains 16 carbon fatty acids at the sn-2 position, while DAG formed in the chloroplast has exclusively 16 carbon fatty acids at the sn-2 position. In large part, the composition of DAG is due to the specificity of acyltransferases[22,23].

In the chloroplast, DAG is converted to PG[24], monodigalactosyldiacylglycerol (MDG)[25,26,27], digalactosyldiacylglycerol (DDG)[25], and sulfoquinovosyldiacylglycerol (SQD)[28]. Phosphatidylcholine (PC) in the chloroplast is only on the cytosolic side of the outer membrane of the chloroplast envelope and presumably represents lipid traffic from the ER[29].

In the ER, lipid biosynthesis forms PG[30], phosphatidylethanolamine (PE)[30], phosphatidylinositol (PI)[30,31], PC[30,32,33,34,35,36], and phosphatidylserine (PS)[30]. PS contains unique long chain fatty acids[37] and may be a carrier of fatty acids to the epidermis for wax deposition[38].

Lipid Modifications. Once formed, a lipid molecule is not static. Headgroups can exchange and fatty acids can be further modified by desaturation and other reactions which will not be discussed here, such as hydroxylation, epoxidation, and elongation.

In higher plants, desaturation of lipid acyl chains takes place in both the chloroplast and the endoplasmic reticulum[39] and occurs toward the methyl end of the acyl chain[40]. This is in contrast to the animal desaturases which introduce double bonds toward the ester end of the acyl chain and implies that the plant desaturases orient their substrates in a unique way in the active site of the enzyme. This unique orientation of desaturations appears to be the basis of the requirement for plant fatty acids in animal diets[40]. The first double bond is introduced into 18-carbon fatty acids by the plastid enzyme stearoyl-ACP Δ9 desaturase[8]. This enzyme, which is the only soluble desaturase known, requires reduced ferredoxin as an electron donor. The gene for this protein has been cloned and the protein has been crystallized for further biochemical study[41]. Information about the desaturation reactions located in the chloroplast was elucidated by studies of both labelled intact chloroplasts [42] and *Arabidopsis thaliana* mutants because it had not been possible to directly measure enzymatic activity following chloroplast rupture[43]. Limited biochemical work has been done on the membrane bound desaturases of the chloroplast although a correlation has been made between the *fadD* phenotype and a deficiency in a 90kD protein[44]. A *trans* double bond is introduced at the Δ3 position of 16:0 on PG only[45]. All other double bonds are introduced in the *cis* conformation. The 16:0 of MGD and DGD is desaturated at the Δ9 position[9,46]. The second and third double bonds of all chloroplast lipids are introduced at Δ12 and Δ15 positions respectively[47,48]. Recently, by lysing spinach chloroplasts in CHAPS detergent and adding fatty acid substrates and cofactors, Schmidt and Heinz retained partial activity of the Δ12 and Δ15 chloroplast membrane bound desaturases[49]. The activity was dependent on added

ferredoxin, and in the dark, also required NAD(P)H. Antibodies against ferredoxin:NADP oxidoreductase inhibited the activity in the dark suggesting that the components of the electron transfer system for desaturation in chloroplasts are ferredoxin:NADP oxidoreductase, ferredoxin, and a desaturase.

In the ER, desaturation of 18:1 to 18:2 occurs at the Δ12 position and desaturation of 18:2 to 18:3 occurs at the Δ15 position[50]. Desaturation on PC[51,52] and PE[53] has been documented, and it is possible that other phospholipids also serve as desaturase substrates in the ER. Since occasionally, "insignificant" amounts of desaturation products on CoA have been noted[51], desaturation may also use acyl-CoA as a substrate. However, acyl chains can exchange between PC and CoA[54]. Therefore, an assay for desaturation which blocks this back-exchange to CoA would have to be developed in order to state definitively that acyl-CoA is a desaturase substrate. Metabolite channeling between acyltransferases and desaturases has been suggested as an explanation of why, during desaturase assays, labelled phospholipids do not appear to mix with the bulk unlabelled phospholipids of the ER[51].

However, definitive substrate specificities can be discovered only with purified enzymes in hand. This dissertation presents the purification of a protein involved in desaturation in the ER of higher plants. The decision to purify cytochrome *b5* and determine its role in desaturation was based on the known functions of this protein in mammalian systems.

Functions of Mammalian Cytochrome *b5*

Interactions with NADH-cytochrome *b5* reductase. NADH-cytochrome *b5* reductase, a flavoprotein electron transferase, donates electrons to another membrane bound redox protein, cytochrome *b5*, as well as to soluble compounds such as ferricyanide. Strittmatter *et al.* showed that the interaction between cytochrome *b5* and NADH-cytochrome *b5* reductase depended on "translational diffusion" of the proteins within synthetic dimyristoyl lecithin liposomes[55]. They took advantage of phase transition temperatures, at which a change in membrane fluidity occurs, to show that increasing the membrane fluidity increased the rate of NADH-cytochrome *c* reduction through cytochrome *b5* while the rates of ferricyanide reduction remained steady. Since the ratio of cytochrome *b5* to NADH-cytochrome *b5* reductase in rabbit liver microsomes[56] is 10:1 and since the level of cytochrome *b5* in microsomal membranes is in the picomole per mg total protein range, it is likely that "translational diffusion" of the reductase is important in *in vivo* reduction of cytochrome *b5*.

Once having met within the membrane, NADH-cytochrome *b5* reductase and cytochrome *b5* maintain their contact through "charge pairs" in order to establish a stable junction for electron transfer[57]. The identity of these "charge pairs" has been established by introducing the two proteins into synthetic phospholipid vesicles and then crosslinking them[57,58]. The 1-ethyl-3-(2-dimethylaminopropyl) carbodiimide hydrochloride used in crosslinking forms amide bonds between carboxyl groups of cytochrome *b5* and lysyl residues on the reductase[58]. The cytochrome *b5* carboxyl

groups are important in electron acceptance from the reductase and electron transfer to soluble and membrane bound electron acceptors. The "charge pairs" found in the complex were between the cytochrome *b5* heme propionate's carboxyl group and serine 162 of the reductase, between glutamic acid 52 and/or glutamic acid 60 of the cytochrome and lysine 41 of the reductase, and between glutamic acid 47 and/or glutamic acid 48 of the cytochrome and lysine 125 of the reductase. The involvement of lysine 163 in the complex was also possible and the three lysyl groups of the reductase interacted with carboxyl groups surrounding the heme edge of the cytochrome[57]. Rogers and Strittmatter[59] failed to find a complex of kinetic significance between the reductase and cytochrome *b5*, suggesting a rapid interaction followed by dispersion. In the crosslinking experiments, purified cytochrome *b5* was amidated before introduction into the small unilamellar vesicles (SUVs) to avoid crosslinks between its own lysyl and carboxyl groups[57,58].

The precaution of amidating cytochrome *b5* before crosslinking to the reductase points out that in theory cytochrome *b5* could transfer electrons to other cytochrome *b5* molecules in a membrane. It has been suggested by Spatz and Strittmatter[56] that cytochrome *b5* exists as a polymer in membranes *in vivo*. Thus, rates of electron transfer to a final electron acceptor such as a desaturase could be decreased by runoff to other cytochrome *b5* molecules. Dixon *et al.*[60] investigated the self-exchange of electrons between molecules of trypsin solubilized bovine liver cytochrome *b5*, using ^{1}H-NMR techniques. The rate of cytochrome *b5* self-exchange was 2.6×10^3 $M^{-1}s^{-1}$ at pH 7, ionic strength (μ) = 0.1 M sodium phosphate, and 25°C. An increase

to μ = 1.5 M sodium phosphate increased the self-exchange rate to 4.5 x 10^4 $M^{-1}s^{-1}$. Since cytochrome *b5* levels in microsomal membranes are in the picomole per mg total protein range and since access for self-exchange would be limited by the constraints of the membrane and other membrane bound proteins, the amount of self-exchange occurring *in vivo* would be minuscule. Indeed, in experiments where deuteroheme cytochrome *b5* was added to membranes containing NADH-cytochrome *b5* reductase and cytochrome *b5*, the deuteroheme was reduced directly from the reductase rather than through electron exchange with native cytochrome *b5* molecules[59]. Presumably if cytochrome *b5* polymers formed, they would contain both deuteroheme and native forms of cytochrome *b5* and intra-polymer electron exchange would have equalized the reduction rates for the two forms.

In addition to the crosslinking experiments discussed above, investigations of the interactions between cytochrome *b5* and the NADH-cytochrome *b5* reductase have been carried out using site directed mutagenesis. Both Yubisui *et al.*[61] and Shirabe *et al.*[62] used site-directed mutagenesis of human NADH-cytochrome *b5* reductase, at serine 127 and four cysteines respectively, to investigate its interaction with cytochrome *b5*. Neither of these experiments resulted in a change in the interaction with cytochrome *b5*. Thus, to date, the only residues identified as important in interactions between cytochrome *b5* and NADH-cytochrome *b5* reductase are the carboxyl/lysyl charge partners found by Strittmatter *et al.*[57].

Interactions with Cytochrome *c*. As noted above the carboxyl groups of cytochrome *b5* which interact with the lysines of NADH-cytochrome *b5* reductase also interact in turn with the acceptors of electrons from cytochrome *b5*. The classic test for the presence of NADH-cytochrome *b5* reductase and cytochrome *b5* in a membrane is the NADH-driven reduction of exogenously added cytochrome *c*. Using ^{13}C-NMR and ^{1}H-NMR, Burch *et al.*[63] found that the interaction between these proteins appeared to be coordinated by regions of complementary charge on the surface of the molecules rather than specific pairs of residues. Burch *et al.*[63] termed this regional interaction the "rolling ball model." It is important to keep in mind that these interactions were between soluble cytochromes. Although membrane constraints did not affect the reducing ability of bound cytochrome *c* in a recent study by Cheddar *et al.*[64], the membrane may affect the ability of cytochrome *c* to approach cytochrome *b5* in the classical NADH reduction studies mentioned above. Like Burch *et al.*[63], Rodgers *et al.*[65] also found salt linkages between the carboxylic acid residues of cytochrome *b5* and the lysyl residues of cytochrome *c*. Their experiments showed that hydrogen bonding as well as salt bridges were important in the complex formation. A 1:1 complex of cytochrome *b5* and cytochrome *c* was most stable at a pH between the two isoelectric points of the cytochromes, *ie.* pH 7-8, at which 83% of the cytochrome *b5* is complexed with cytochrome *c*[66]. Mauk *et al.*[66] suggested that the pH dependence is due to proton release during short range protein-protein interactions in the formation of the 1:1 complex. However, electron transfer between cytochrome *b5* and cytochrome *c* should not be discussed solely in terms of a 1:1 complex. Whitford *et al.*[67] found that increasing amounts of cytochrome *c* allowed a

1:2 ratio of cytochrome *b5* to cytochrome *c* in the complex. There appears to be a single high affinity site for cytochrome *c* on cytochrome *b5* and a secondary low affinity binding site which is only utilized at high concentrations of cytochrome *c*.

Interactions with Cytochrome *P450*. In addition to interactions with cytochrome *c*, cytochrome *b5* also cooperates with cytochrome *P450* (*P450*) in some NADPH-dependent monooxygenase reactions. In 1985, Chiang *et al.*[68] found that antibodies against cytochrome *b5* inhibited both the NADH-cytochrome *c* reductase and NADPH-7-ethoxycoumarin-O-deethylase. Addition of cytochrome *b5* equimolar to *P450* increased the activity in three reconstituted *P450* monooxygenase systems. Kuwahara and Omura[69] found that the requirement for cytochrome *b5* in *P450* reactions depended on which *P450* was involved and the identity of the substrate. Noshiro *et al.*[70] studied the inhibition of four *P450* monooxygenase reactions using a series of antibodies to NADPH cytochrome *c* reductase, NADH cytochrome *b5* reductase, cytochrome *b5*, and *P450* to decipher the order of electron flow in cytochrome *b5*/ *P450* interactions. The results of these studies showed that electrons from NADH must pass through NADPH-cytochrome *c* reductase to reduce the *P450*. There are separate pathways for the first and second electron, one goes through NADPH-cytochrome *c* reductase and the second can reduce *P450* via NADPH-cytochrome *c* reductase or NADH-cytochrome *b5* reductase. Thus NADH and NADPH have a synergistic effect. This branched pathway scenario supports previously obtained results by Hildebrant and Estabrook[71], which showed that cytochrome *b5* acted at a step after the primary reduction of *P450*, since there was

no lag time between cytochrome *b5* reoxidation and product formation. NADH increased the rate limited NADPH reduction of *P450*. Since NADH alone reduces *P450* very slowly, and since a new spectral species was apparent during steady state, the effect of NADH on *P450* reduction was believed to proceed through cytochrome *b5*, possibly in a complex with *P450*, which would account for the unknown spectral species. Canova-Davis *et al.*[72] found that although the effects of cytochrome *b5* on *P450* depended on the protein:protein and protein:lipid ratios in both microsomes and reconstituted liposomes, a cytochrome *b5* in which Mn^{3+} replaces Fe^{3+} disrupts the O-demethylation of methoxyflurane because it can not donate electrons to *P450*. Since this modified cytochrome *b5* is not changed in conformation, this data suggests that the importance of cytochrome *b5* is solely in donating electrons. An interesting study of a dual function *P450* by Shinzawa *et al.*[73] suggests that cytochrome *b5* may also be an agent of conformational change in this *P450*. The maximal activities of both reactions were unaffected by the addition of cytochrome *b5*. However, cytochrome *b5* increased the optimal pH in both reactions and repressed the activities at suboptimal pHs. Removal of cytochrome *b5* decreased the lyase activity and uncoupled it from the hydroxylase activity. A third study suggested that cytochrome *b5* changes both the conformation of the *P450* and its spin state[74]. When the cytochromes were crosslinked, the spin state of *P450* was greater than when free and its monooxygenase activity was increased. Its binding affinity for the substrate, benzphetamine was increased tenfold as compared with that of the free *P450* in the absence of cytochrome *b5*. These authors propose a ternary complex in which the first electron from NADPH-cytochrome *c* reductase is transferred from *P450* to

cytochrome *b5*, creating a high spin ferric *P450* and allowing *P450* to accept a second electron from the NADPH reductase. After binding molecular oxygen, the *P450* recovers the first electron from cytochrome *b5* and catalyzes reactions requiring two electrons.

Interactions with other electron acceptors. Cytochrome *b5* has also been implicated in a number of other electron requiring activities: membrane repair and *P450* detoxification reactions in the liver's cytotoxic response to *Escherichia coli* lipopolysaccharide (LPS)[75], electron transfer in the cis-dehydration between C1 and C2 of 1-O-alkyl-2-acyl-sn-phosphatidylethanolamine during ethanolamine plasmalogen synthesis[76], and methaemoglobin reduction in erythrocytes[77]. Oshino and Sato[78] claim that ρ-cresol stimulates the reoxidation of cytochrome *b5*. Possibly the phenol is oxidized by the desaturase, since the effect on cytochrome *b5* is correlated to the existence of the desaturase in microsomes, since small amounts of stearoyl-CoA inhibit the ρ-cresol effect, and since this ρ-cresol oxidation is inhibited by cyanide. The authors also claim that aniline and hydroxylamine stimulate reoxidation of cytochrome *b5*. The reoxidation by aniline is hard to understand since other published data[70] shows that cytochrome *b5* is not involved in aniline metabolism. If the information of Oshino and Sato[78] is not flawed, the interactions of cytochrome *b5* in mixed oxidase reactions must be more complex than now envisioned. Cytochrome *b5* also reoxidizes in the presence of [1,3-^{14}C]malonyl-CoA (malonyl-CoA) and NADH as malonyl-CoA is incorporated into fatty acids[79]. The reoxidation rate is approximately 10% faster than the incorporation rate at all substrate concentrations

studied. In the absence of malonyl-CoA, the cytochrome *b5* is not reoxidized. The fatty acid, 18:1, accumulates as the major product of this elongation and the accumulation of 18:1 is only partially inhibited by 1 mM KCN. At this level of KCN, desaturation from 18:0 to 18:1 is completely inhibited. Therefore, elongation can occur either before desaturation or after. Further, Keyes *et al.*[80] showed that, while control IgG had no effect, IgG against cytochrome *b5* inhibited 60% of the incorporation of malonyl-CoA into 18:1 under N_2, where desaturation involving cytochrome *b5* and absolutely requiring O_2 would be inhibited. Thus, cytochrome *b5*, in addition to its other functions, is involved directly in elongation. Takeshita *et al.*[81] also noted the involvement of cytochrome *b5* in palmitoyl-CoA elongation. They found evidence for a branched pathway of electron transfer in the elongation. Mercuric chloride and *ρ*-chloromercuriphenylsulfonate both blocked elongation through blocking NADH-cytochrome *b5* reductase. However, these inhibitors did not block NADPH-dependent elongation. An antibody against cytochrome *b5* reductase also blocked NADH-driven elongation. Trypsin digestion of microsomes lowered the elongation rates supported by either NADPH or NADH and the addition of detergent solubilized cytochrome *b5* allowed a recovery of elongation activity. The authors suggested that cytochrome *b5* can be reduced either from NADH-cytochrome *b5* reductase or from NADPH-cytochrome *P450* reductase before transferring electrons on to an elongase. Lastly, Reddy *et al.*[82] described the involvement of cytochrome *b5* in rat liver cholesterol biosynthesis at the level of Δ7 sterol desaturase. The desaturase requires O_2 and NADH or ascorbic acid. It is inhibited by KCN, sulfhydryl blocking agents, and antibodies against rat cytochrome *b5*, but not by

carbon monoxide (CO).

Electron transfer to acyl chain desaturases. In 1966, Oshino *et al.*[83] described the Δ9 desaturase in rat liver microsomes. This desaturase was driven preferentially by NADH with lower activities using NADPH or ascorbic acid. With all reducing agents, the desaturase was KCN sensitive and free iron appeared to be needed. The desaturase was not inhibited by the *P450* inhibitors, ethyl isocyanide and CO. Phenobarbital feeding of rats did not increase the desaturase activity although it did increase the level of *P450* in microsomes. Thus some other electron donor was involved. Later, Strittmatter *et al.*[84] proved this electron donor to be cytochrome *b5* by purifying the Δ9 desaturase in detergent, exchanging the detergent for PC, and reconstituting the Δ9 desaturase activity by adding cytochrome *b5* and NADH-cytochrome *b5* reductase to the Δ9 desaturase-PC vesicles. The activity also required NADH, stearoyl-CoA and O_2 and could not be reconstituted using the soluble fragment of cytochrome *b5*. Lee *et al.*[85] inhibited Δ6 desaturase with antibodies against rat liver cytochrome *b5*. Δ6 desaturase introduces a double bond between carbons 6 and 7 in three different acyl chains: (Δ9)-18:1, (Δ9,12)-18:2, and (Δ9,12,15)-18:3. They monitored the inhibition of Δ6 desaturation by measuring the radiolabelled products after separation by argentation thin layer chromatography. The Δ6 desaturation of (Δ9)-18:1 is similar to the Δ12 desaturase studied in this dissertation in introducing a second double bond on an 18 carbon acyl chain. Okayasu *et al.*[86] also used antibodies against rat liver cytochrome *b5* to show the involvement of cytochrome *b5* in the Δ6 desaturation of 18:2 to γ18:3 in rat liver microsomes.

However, the IgG used by Okayasu *et al.*[86] was not as inhibitory as that used by Lee *et al.*[85]. For further review of desaturation in animal systems see Holloway[87].

Since cytochrome *b5* was shown to be an electron donor to plasmalogen, sterol, and acyl chain desaturases in animals, it seemed reasonable to test its involvement in a plant endoplasmic reticulum desaturase, which probably would be more like an animal desaturase than the chloroplast desaturases would be.

Mammalian Cytochrome *b5*

Having looked at the functions of cytochrome *b5*, let us now look at the protein itself. Cytochrome *b5* is a protein in the range of 16 kDa bound to the membrane by approximately 40 hydrophobic amino acids at the carboxyl-terminus. The protein is defined by its oxidized versus reduced spectrum characteristics: a Soret band at 424nm, a ß-band at 525nm, and an α-band at 556nm. The protein can be solubilized with detergent and purified to homogeneity in animal systems.

Primary Structure. The primary structure of rat liver cytochrome *b5* was determined by Ozols *et al.*[88]. The hexosamine content was less than 0.1 mol/mol protein, suggesting the absence of oligosaccharides on the cytochrome. The primary structure of cytochrome *b5* from rabbit, human, cow, chicken, monkey (*Aloutta fusca*), and pig are highly conserved, exhibiting 100% homology in the heme binding region[89,90].

There are two forms of cytochrome *b5* in mammals and chicken, a soluble erythrocyte form and a membrane bound form. Abe *et al.*[91] found that the 97 amino acids of the soluble erythrocyte forms from human, pig, and cow were identical to the first 96 amino acids of the membrane bound forms from each of these animals. In the cases of pig and human, the 97th amino acid of the erythrocyte form is serine and proline, respectively, while the 97th residue in the membrane bound form in both cases is threonine. These researchers suggested that the two forms of cytochrome *b5* might be translated from two closely related mRNAs. Slaughter *et al.*[92] solubilized membrane bound cytochrome *b5* from cow liver and obtained two products, a fragment of 95 amino acids and a fragment of 107 amino acids. The 95 amino acid fragment corresponded to the two erythrocyte forms in cow. From this data, Slaughter *et al.*[92] suggested that erythroid proteases solubilize microsomal cytochrome *b5* from the membranes during erythrocyte maturation to form the soluble erythrocyte protein. Recently, Zhang and Somerville[93] found that cDNAs from chicken erythrocytes and liver had identical DNA sequences encoding cytochrome *b5* proteins each with the membrane binding hydrophobic carboxy tail.There appeared to be only one gene encoding these cDNAs. This data implies that if chicken erythrocytes have a soluble cytochrome *b5*, it is a post-translational modification of the membrane bound form.

Secondary and Tertiary Structure. Extensive characterization of the secondary and tertiary structure of calf liver cytochrome *b5* has been carried out by Mathews and colleagues[94,95]. Figure 1.1 gives an artist's rendition of the protein. Sixty percent of

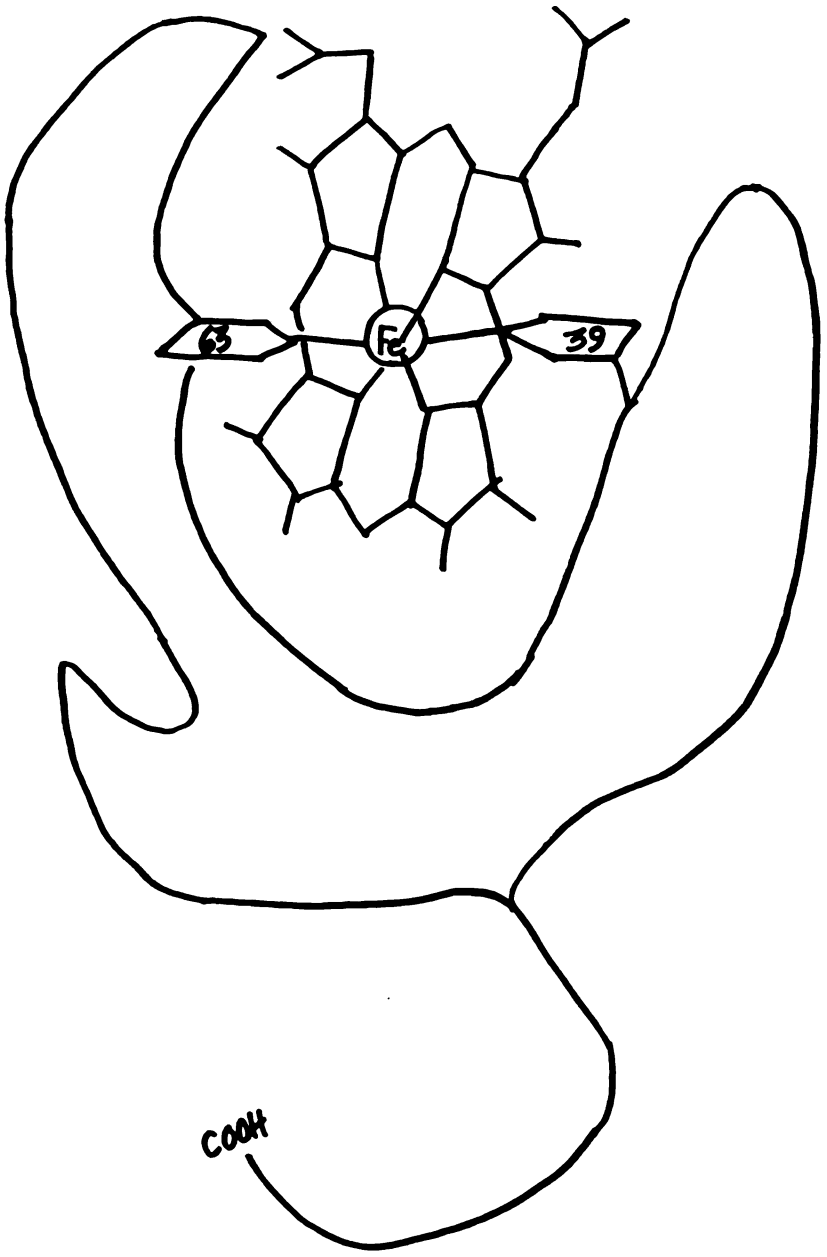

FIGURE 1.1 Depiction of the mammalian cytochrome *b5* molecule based on studies of the crystallized protein.

the amino acid residues are involved in secondary structures. The protein contains six α-helical regions, five ß-pleated sheet structures, and six ß-bends.

The calf liver cytochrome *b5* is 37Å in height and 31Å in diameter[94] and, from work on rabbit cytochrome *b5*[96], the protein is composed of two domains, a hydrophilic region containing the heme and a hydrophobic region anchoring the protein into the membrane. Four α-helices in the top half of the protein lie parallel to the cylindrical axis while the remaining two α-helices reside in the bottom of the protein, one very near the carboxy-terminus[94]. A ß-pleated sheet structure separates the nonpolar heme region from a second hydrophobic core or tube of hydrophobic residues, also referred to as the hydrophobic groove, in the bottom portion of the protein. The heme region extends approximately 3/5th of the length from the top (cytosolic side) down. The heme region is a nonpolar milieu for electron transfer. Histidines 39 and 63 bind the heme non-covalently and also interact in π-π stabilizations between histidine 39 and leucine 46 and between histidine 63 and phenylalanine 58[95]. A cluster of acidic amino acids at the tops of the four, parallel α-helices forms a ring of negative charge which excludes the heme, except for its propionate groups, from interaction with the solvent[94]. The vinyl groups of the non-covalently bound porphyrin IX heme are embedded deeply within the heme pocket, while at least one propionate is bound to the surface of the protein[95]. The heme does not react with cyanide, CO, or O_2 and oxidation/reduction probably does not occur by direct contact with the iron atom. A displacement of side chains during interaction with the reductase may result in reduction through a propionate[94].

In addition to the clustering of acidic residues at the top of the protein, basic residues tend to cluster at the bottom of the molecule and a strip of neutral amino acids from the top of the protein down the side between helices II and V, called the hydrophobic patch, includes the hydrophobic groove. There are also six salt bridges on the surface of the protein usually involving glutamic acid residues[94].

The above information on secondary and tertiary structures has been gathered from work on crystallized protein. However, the conformation of the protein in either crystal or solution form is analogous[97,98]. In fact, cytochrome *b5* can be reduced in the crystal form without breakup of the crystal[98].

Much work has been done on heme binding in cytochrome *b5*. One report by Senjo *et al.*[99] claims that a glutathione S-transferase type enzyme is required for heme insertion in the soluble rat liver cytochrome *b5*. However, 50μmol of holoprotein was formed in 3 min without the enzyme in these experiments. Depending on turnover rates for cytochrome *b5*, this nonenzymatic insertion may supply *in vivo* needs. In addition, Strittmatter *et al.*[100] routinely used nonenzymatic heme insertion *in vitro* to study apocytochrome *b5* from lipase solubilized calf liver cytochrome *b5* and reported reinsertion of the heme into apocytochrome in less than five seconds. This reconstituted protein could be reduced by cytochrome *b5* reductase. Cytochrome *b5* solubilized from rat liver and devoid of heme does not fully unfold under physiological pH and temperature[101]. The core of the protein apparently remains stable *in vivo* while awaiting heme insertion. Konopka[102] *et al.* also suggested that the

conformation of the protein is highly stable and does not depend on inserted heme. The replacement/displacement of heme after diethylpyrocarbonate modification in apocytochrome post-treated with hydroxylamine, in trypsin solubilized cytochrome, and in detergent solubilized cytochrome exchanged into phosphatidylcholine was less than 15%, implying a similarity in the conformation of these forms of cytochrome. Only detergent solubilized cytochrome in aqueous solution exists as an octamer and appears to be in a different conformation having a displacement of 30% after the same level of diethylpyrocarbonate modification.

In early studies of amino acids involved in heme binding, Strittmatter *et al.* [100] modified lipase solubilized apocytochrome by iodination and acetylation and monitored uptake of molar equivalents of heme versus uptake in the unmodified apocytochrome. They hypothesized that histidines were involved in binding the noncovalent heme. Using site directed mutagenesis, Funk *et al.*[103] tested the hypothesis that serine 64 stabilizes the propionate-7 of the heme by H-bonding. The oxidized protein was not destabilized by the lack of one H-bonding site for the propionate.

Another area of intense work on cytochrome *b5* is the binding of the protein to membranes. Fleming *et al.* [104] made use of the natural fluorescence of tryptophan 109 when they added the C-terminal nonpolar portion of cow cytochrome *b5* to bilayers of lipids with trinitrophenyl- or dansyl-labelled headgroups, which quench the fluorescence. The quenching is weaker when the tryptophan is farther away from the

headgroup. Tryptophan 109 was located approximately 22Å below the headgroup region or in the outer half of the bilayer. However, it was not clear whether the end of the protein looped back to the outside of the vesicles. Takagaki *et al.*[105], studying cytochrome *b5* in asymmetrical bilayer vesicles with photoactivatable ^{14}C-phosphatidylcholine (PC) on the outer surface and photoactivatable ^{3}H-PC on the inner surface, suggested that, when it was tightly bound, the protein spanned the bilayer with the last amino acids in the interior of the vesicle. When the protein was loosely bound, the nonpolar anchoring portion had a broader distribution of location in the bilayer. The last amino acid (aspartic acid 133) was looped to the outer side of the membrane, suggesting that the anchor portion of cytochrome *b5* was looped into the outer half of the bilayer, somewhat like a fish hook. Tryptophan fluorescence is enhanced in vesicles of 1-palmitoyl-2-oleoyl-phosphatidylcholine, but it is quenched in vesicles of 1-palmitoyl-2-dibromostearoyl-phosphatidylcholine (BrPC)[106]. When detergent solubilized cytochrome *b5* was loaded into small unilamellar BrPC vesicles, the greatest quenching occurred with 6,7-dibromostearoyl. This finding indicated that tryptophans 108, 109, and 112 were all located approximately 7Å below the surface of the lipid. The same localization was estimated using asymmetrical bilayers and was probably not an artifact of membrane perturbation since BrPC can incorporate into fibroblast culture cells with no effect[107]. A more comprehensive study by Tennyson *et al.*[108] showed that tryptophan 108 was 7Å below the surface of large or small vesicles whether it was tightly bound or loosely bound. Tennyson *et al.*[108] also noted that the lowered quantum yield of fluorescence for cytochrome *b5* in the loose binding conformation could be due to the "looped-back" position of the peptide chain

in the membrane.

Biosynthesis and localization. Cytochrome *b5* protein is translated from free ribosomes. Rachubinski *et al.*[109] translated mRNAs derived from isolated free ribosomes and immunoprecipitated a 17,500 Mr protein using anti-cytochrome *b5* serum. The hydrophobic carboxyl tail was short enough to remain in the ribosome until the end of protein synthesis when the protein apparently bound to the nearest membrane. Similar immunoprecipitation experiments by Okada *et al.*[110] showed that the NADH:cytochrome *b5* oxidoreductase from rat livers was also made on free ribosomes, that neither the reductase nor the cytochrome was a glycoprotein, and that neither protein underwent cleavage either co- or post-translationally. Both proteins were post-translationally inserted into membranes. The turnover rate of ^{14}C-leucine labelled cytochrome *b5* in *in vivo* microsomes is 117 to 123 days[111].

Blobel and colleagues[112] found that no signal peptide was required for cytochrome *b5* insertion into membranes and referred to an "insertion sequence" in the carboxy-terminus sequence as being important for "unassisted and opportunistic insertion" into membranes. Bendzko *et al.*[113] suggested that the insertion sequence was not a conserved domain but rather a "topogenic determinant" such as an α-helical or globular structure with internal H-bonding in the hydrophobic carboxy-terminus that was important for insertion. Regardless of the exact mechanism for binding, it was obvious from work by Strittmatter *et al.*[114] that the 40 amino acids of the carboxy-terminus were involved. Carboxypeptidase removal of six residues from the carboxy

terminus resulted in loss of tight binding although loose binding ability remained[115]. Chemical modification to deactivate anion charge in this region or deletion of 18 residues from the carboxy-terminus also resulted in loss of tight binding, but loose binding was cited as allowing the protein to interact functionally with both the reductase and stearoyl-CoA desaturase. Dailey and Strittmatter[115] suggested that *in vivo* the carboxy-terminus could be sequestered from carboxypeptidase Y by ionic interactions between glutamic acid 132 , asparagine 133, and the head groups at the membrane surface. Carboxypeptidase Y cut the loose binding cytochrome *b5*[116].

As discussed above, cytochrome *b5* inserts into membranes post-translationally in an apparently random fashion. Thus, cytochrome *b5* has been described in many membranes of the cell. Remacle *et al.*[117] used ferritin labelled hybrid anti-cytochrome *b5*/ anti-ferritin antibodies to localize cytochrome *b5* in isolated membranes visualized under electron microscopy. Smooth and rough endoplasmic reticulum (ER) contained large amounts of cytochrome *b5*[117], while Golgi, plasmamembrane, and peroxisomes were labelled to a much lower extent[118]. All of these membranes had undergone various degrees of purification, and it seems that thin sectioning of cells might result in less membrane breakage and better labelling of the Golgi and plasma membrane (PM). Ito *et al.*[119] purified three peaks of b-type cytochrome from the outer membrane of the mitochondria (OM). These cytochrome peaks have reduction spectra very similar to that of the peaks of cytochrome *b5* from the ER and they cross react immunologically in the following manner. Peak 1 of the OM preparation and peak 1 of the ER crossreact with an antibody raised against a mitochondrial

cytochrome peak. Peaks 2 and 3 of both OM and ER preparations crossreact with the antibody raised against the ER cytochrome *b5*. Based on the immunological data and amino acid composition, Ito *et al.*[119] suggested that there are two cytochromes, one from the ER membrane and the other from the mitochondria. The purification of three peaks appears to be due to the presence of more than one membrane type in the membrane preparations. However, the two immunologically distinct cytochromes may be in both membranes *in vivo* or the transfer of cytochrome *b5* between disrupted membranes[116] may occur during the preparation process.

Cytochrome *b5* and cytochrome *b5* reductase in endogenous membranes do not transfer to the Golgi or liposomes[120]. However, both proteins in liposomes transfer to other vesicles even at 0°C by a mechanism that does not involve vesicle fusion. Greenhut *et al.*[121] found that cytochrome *b5* preferentially bound small vesicles of approximately 212Å which were highly curved and might therefore imitate disrupted membranes. Greenhut *et al.* hypothesized that curved membrane regions might be important distributing factors for vesicle traffic in cells. Christiansen *et al.*[122] cited catalase and ornithine carbamyltransferase as instances in which a soluble membrane destabilization factor was important for insertion of proteins made on free polysomes. In their experiments, ^{3}H-cytochrome *b5* in micelles of 1-^{14}C-palmitoyllysophosphatidylcholine (lyso-PC) transferred to smooth or rough ER vesicles while maintaining a constant ratio of ^{14}C to ^{3}H in both the micelles and the ER vesicles. This constant ratio implies that cytochrome *b5* moves with an escort of lipid, which would destabilize the target vesicle. In these studies it was shown that

both acyltransferase to form ^{14}C-PC and degradation to ^{14}C-palmitate occurred in the vesicles. Thus the lipid from the micelles was indeed transferring with the cytochrome *b5*, which inserted in a tight binding conformation.

For further reading on the characteristics of b-type cytochromes in non-plant systems, please see reviews by von Jagow and Sebald[123], Hagihara *et al.*[124], and Cramer *et al.*[125].

Since the understanding of lipid biosynthesis in plants requires the purification of the enzymes involved and since cytochrome *b5* was a good candidate for involvement due to its functions in mammalian systems, this dissertation research was undertaken. The following chapters describe the purification to homogeneity of a protein involved in desaturation in the ER of higher plants (Chapter Two), the raising of antibodies against this protein and immunoinhibition studies to determine the function of the protein (Chapter Three), and molecular genetic analysis of the coding sequence (Chapter Four).

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CHAPTER TWO
CYTOCHROME b5 PURIFICATION FROM CAULIFLOWER MICROSOMES

Cytochrome *b5* of Higher Plants

The plant cytochrome *b5*, like its animal counterpart, appears to be located in multiple membranes. Two classic reviews of cytochromes in plants have localized a b-type cytochrome resembling cytochrome *b5* in microsomes, which consist mostly of ER membranes. Hendry *et al.*[1] used spectrophotometric methods to survey the cytochrome content in microsomes from imbibed mung beans and found light regulated levels of cytochromes *P450*, *P420*, *b5*, *b560.5*, and *b562.5*. The levels of cytochromes *b5*, *b560.5*, and *b562.5* were higher in light versus dark, and the levels of cytochromes *P450* and *P420* in the light were lower with the ratio of *P450/P420* higher in the light. Bendall and Hill[2] established the location of a b-type cytochrome with a room temperature reduction α-band at 555 nm in the microsomes of *Arum maculatum*. In a later paper, Rich and Bendall[3] showed that although the cytochrome complement of plant microsomes varied from species to species, a cytochrome *b5*, which was not reducible by ascorbate, was present in all plant microsomes investigated. The cytochrome *b5* of cauliflower microsomes described by Rich and Bendall[3] had a Soret band of 425 nm, a ß-band of 526 nm, and an α-band of 556 nm.

Luster and Donaldson[4] reported NADH:cytochrome *c* reductase activity characteristic of the presence of NADH: cytochrome *b5* reductase and cytochrome *b5* on the outer side of glyoxysome membranes. Hicks and Donaldson[5] described a cytochrome in castor bean glyoxysomal membranes with a room temperature reduction Soret band at 424 nm, a ß-band at 526 nm, and an α-band at 555 nm. It is assumed that NADH from ß-oxidation and NADPH from isocitrate oxidation is re-oxidized by a membrane redox system involving cytochrome *b5*[6] since these nucleotides cannot pass through the membrane[7].

The localization of cytochrome *b5* in the PM has also been suggested. In two phase polymer purified PM preparations from cauliflower, zucchini, bean, and mung bean, Asard *et al.*[8] identified cytochrome *c* reductase activity at an average rate of about 40 nmol/min/mg total protein and also found low amounts of cytochrome *b5*. However, they were concerned that this presence of a cytochrome *b5* redox system might be due to ER contamination. Goldsmith *et al.*[9] found a cytochrome in the PM of corn coleoptiles with a 2°C blue light minus dark reduction Soret band at approximately 426 nm. Leong *et al.*[10] also described a cytochrome in the PM of etiolated corn coleoptiles which had a 4°C light minus dark reduction spectrum with a Soret band at 427 nm, a ß-band at 528 nm, and an α-band at 557 nm.

In 1985, Bonnerot *et al.*[11] partially purified cytochrome *b5* from potato tubers using a CHAPS detergent solubilization technique. To increase the amount of cytochrome *b5* in the tubers, they treated cut slices with Rindite, which induces germination,

before starting the purification. With this procedure, Bonnerot *et al.* obtained 670 μg of cytochrome *b5* from 15 kg of tubers. This represented a partial purification of 352-fold with 10.4% yield. The protein was 16,700 Mr and had a low temperature reduction spectrum similar to the cytochrome *b5* from animals and yeast with a Soret band at 422 nm, a ß-band at 525 nm, and an α-band at 552 nm with a shoulder at 558 nm.

Madyastha *et al.*[12] partially purified cytochrome *b5* from the microsomes of whole etiolated *Catharanthus roseus* seedlings obtaining a 63-fold purification and a 2% yield. This protein was 16,500 Mr and had a room temperature reduction spectrum with a Soret band at 424 nm, a ß-band at 525 nm, and an α-band at 555 nm with a shoulder at 559 nm. Examination of the purification table shows a purification of 0.3 mg cytochrome *b5* per mg protein or 30%.

Using Triton-X 100 solubilization, Jollie *et al.*[13] obtained a 173-fold purification with a 9.3% yield for a 16,400 Mr cytochrome from the microsomes of etiolated pea stems. An examination of the purification table reveals a purification of 75%. This cytochrome was identified as cytochrome *b5* on the basis of a Soret band of 424 nm and its reduction by an NADH-cytochrome *b5* reductase purified in the same paper. The identity of the reductase was based on its turnover number in reduction of potassium ferricyanide. The cytochrome reacted on a western blot with antibodies to the hydrophilic portion of rat cytochrome *b5*. However, the first 21 N-terminal amino acids of this cytochrome are not homologous to the N-terminals of cytochrome *b5*

from other organisms including the cytochrome *b5* purified in this dissertation. A further discussion of the amino acid sequences is presented in chapter four.

The experiments presented in this chapter concern the purification to homogeneity of cytochrome *b5* from the microsomes of cauliflower florets using trypsin solubilization[14].

Experimental Procedures

Materials. Cauliflower heads were purchased at the local market. Safflower S400 seed was obtained from Seedtech Inc. (Woodland, CA). Safflower plants were grown in a glasshouse with supplemental illumination and were hand pollinated. All chemicals used were reagent grade.

Preparation of microsomes. All procedures were carried out at 4°C. In a typical preparation, stems and leaves were removed from seven heads of cauliflower (ca. 3.5 kg each). The florets were cut into one-inch pieces and blended to a paste in a Waring blender with 1.5 liters of fresh grinding buffer (0.3 M mannitol, 10 mM HEPES (pH 7.2), 1 mM EDTA, 0.05% cysteine-HCl, 1 μM leupeptin, 1 μM pepstatin (from a 1 mM stock in methanol), 0.2 mM phenylmethylsulfonyl fluoride (PMSF) (from a 100 mM ethanol stock)). The paste was then filtered through three layers of cheesecloth, and the filtrate was readjusted to pH 7.2 with KOH. The filtrate was centrifuged at 14,700 g_{av} for 45 minutes. The supernatant was adjusted

to 50 mM $MgCl_2$ and stirred for 10 minutes, then centrifuged at 14,700 g_{av} for 20 minutes. The gelatinous pellets were resuspended in 0.3 M mannitol, 10 mM potassium phosphate (pH 7.2), 5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 1 μM leupeptin, 1 μM pepstatin. The concentration of cytochrome *b5* was estimated from the α-band of oxidized minus reduced spectra (556 nm - 570 nm) using an extinction coefficient of 20 cm^{-1} mM^{-1} (Hendry *et al.*[1], Klingenberg[15]) in all purification steps.

Purification of Cytochrome *b5.* The resuspended microsomes were washed according to Omura et al.[16] except that the microsomes were initially pelleted at 105,000 g_{av} for 1 h in a Beckmann 60Ti rotor. After resuspension in 0.1 M potassium phosphate (pH 7.2) to a concentration of approximately 10 mg/ml, the microsomes were treated with 520 units/ml of Sigma type IX porcine pancreas trypsin at 0° C for 7 h[16]. Immediately after the trypsin digest, the microsomes were pelleted at 105,000 g_{av} for 1 h in a Beckmann 60Ti rotor and the supernatant was loaded onto a 90 ml DEAE-Sephacel column equilibrated in 0.1 M potassium phosphate (pH 8.3) (buffer A). The column was then washed with 180 ml of buffer A and developed with an 800 ml linear gradient from 0.05 M to 0.5 M KCl in buffer A at 30 ml/h.

The fractions from the DEAE column containing cytochrome *b5* were combined and concentrated to approximately 1 ml in an Amicon concentrator with an YM5 filter and applied to a 200 ml (2 cm^2 x 100 cm) G50-Sephadex column equilibrated in buffer B (0.1 M sodium phosphate,pH 8.3). The column was eluted at 16 ml/h.

The cytochrome *b5* fractions recovered from the G50-Sephadex column were filtered through a 0.2 μ filter and applied to a 5 ml Mono Q FPLC column (Pharmacia) equilibrated in buffer B. The column was developed with buffer B at 1 ml/min as follows: 0 to 5 min, buffer B; 5 to 45 min a linear gradient from 0 to 0.5 M NaCl; 45 to 49 min, a linear gradient to 1.0 M NaCl; 49 to 52 min, 1.0 M NaCl.

Measurement of Cytochrome *b5* in Safflower seed microsomes. A stock of Safflower S400 seed microsomes (6 mg total protein) frozen at -20° C was defrosted and treated with 520 u/ml of Sigma type IX porcine pancreas trypsin for 7 hrs at 0° C. The amount of cytochrome *b5* per total microsomal protein was estimated by oxidized versus dithionite reduction spectrum as described above assuming that 80% of the cytochrome *b5* had been solubilized.

Other methods. Electrophoresis in SDS-polyacrylamide (15-25% gradient) gels was performed as described[17]. Protein content was measured with a dye-binding assay[18]. Total protein was extracted from microsomes for gel electrophoresis with an equal volume of phenol buffered with 10 mM Tris-HCl pH 7.8, 0.1 mM EDTA, and the protein was precipitated from the phenol phase with 5 volumes 0.1 M ammonium acetate in methanol overnight at -20° C. The precipitate was pelleted for 10 min in a microfuge at 23° C, washed with -20° C acetone, dried, and resuspended in Laemmli cracking dye.

Results and Discussion

The oxidized minus reduced spectra of cauliflower microsomes and of cytochrome *b5* purified from cauliflower microsomes are shown in Figure 2.1. The amount of cytochrome *b5* present in cauliflower microsomes was estimated from the difference in absorbance between oxidized and dithionite reduced samples at 556 and 570 nm using an extinction coefficient of 20 cm^{-1} mM^{-1} [1]. Using this coefficient, an average value of 174 pmol/mg microsomal protein was obtained for cauliflower microsomes. This is comparable to previous estimates of 100 to 300 pmol/mg in microsomal membranes from safflower, pea, and potato[19,13,11]. Measurements of the cytochrome *b5* content in microsomes of developing safflower S400 seeds (fourteen days after flowering) gave a much lower average value of 11.2 pmol/mg (Fig 2.2). This is consistent with previous estimates which placed an upper limit of 25 pmol/mg protein on the amount of cytochrome *b5* in microsomes from developing safflower seeds[20]. By comparison, the concentration of cytochrome *b5* in microsomal membranes from rat liver was reported to be 630 pmol/mg total protein[21].Cauliflower florets were chosen as the starting material for purification of cytochrome *b5* because this tissue is readily available in large quantities, lacks chlorophyll or other chromophores which mask the reduction spectrum of cytochromes in crude preparations, and is closely related to several oilseeds of agronomic importance, such as safflower and *Brassica napus*. For the purpose of producing enough protein to prepare antibodies, it was advantageous to exploit the methods used previously to solubilize the protein from animal membranes by treatment with trypsin[16]. Trypsin releases cytochrome *b5* from

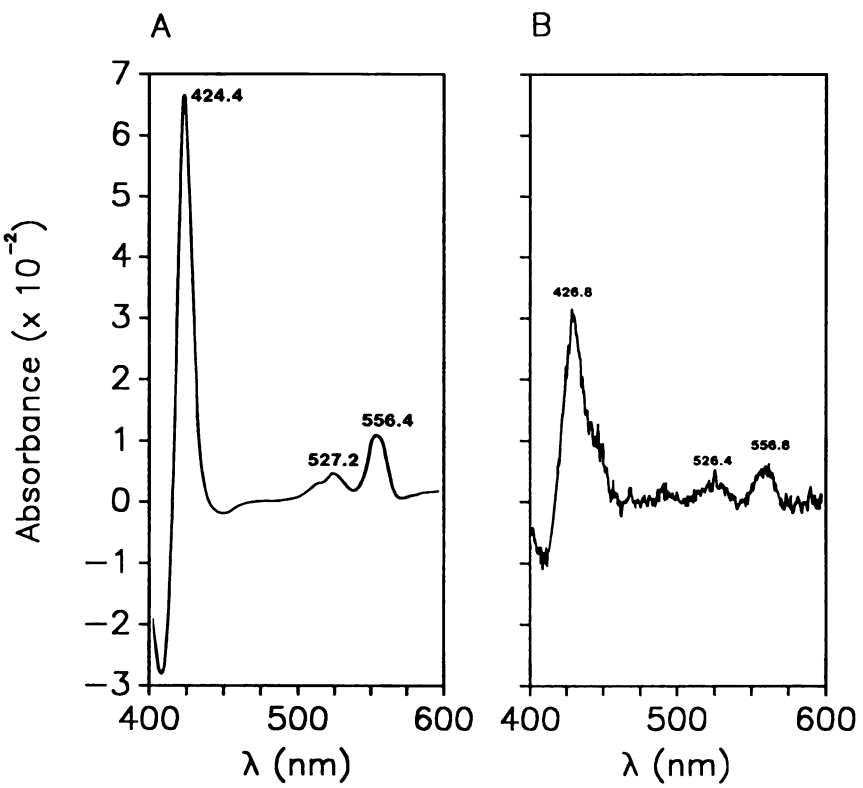

FIGURE 2.1 Oxidized minus dithionite reduced spectra of cytochrome *b5* generated by dual-beam spectrophotometry. Thirty μl of a 15 mg/ml sodium dithionite solution in dH_2O was added to the reduction cuvette and an equal volume of H_2O to the reference, air oxidization, cuvette. (A) FPLC MonoQ purified trypsin-solubilized cytochrome *b5* from cauliflower microsomes (5 μg/ml);(B) intact cauliflower microsomes (1.4 mg/ml total microsomal protein).

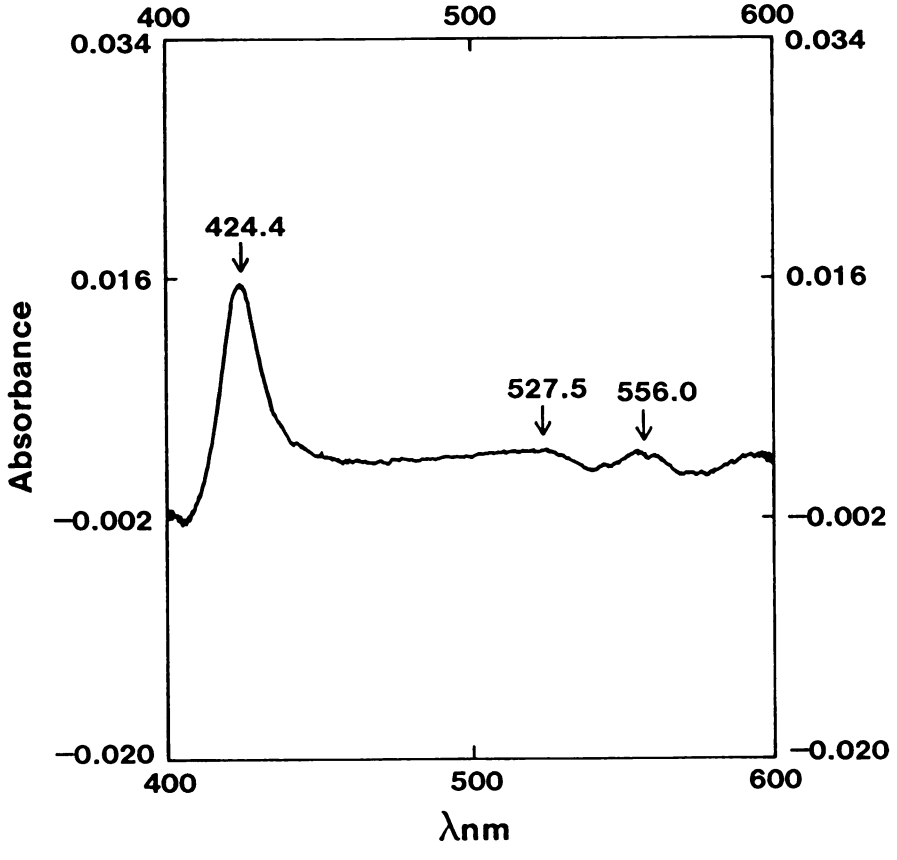

FIGURE 2.2 Oxidized minus dithionite reduced spectrum of cytochrome *b5* trypsin solubilized from 6 mg total microsomal protein. Microsomes were made from safflower S400 seeds 14 days after flowering.

the membrane by cleaving the hydrophobic carboxyl-terminal membrane anchor from the heme-containing hydrophilic domain of the cytochrome[22].

Trypsin treatment released as much as 80% of the cytochrome *b5* from cauliflower microsomal membranes and resulted in a 3.5-fold purification (Table 2.1). The difference spectrum of the solubilized cytochrome was shifted slightly, relative to the membrane bound form (Figure 2.1). Chromatography of the solubilized cytochrome on DEAE-Sephacel resolved two peaks containing the characteristic chromophore (Figure 2.3), and resulted in an 11-fold purification. The presence of two peaks suggests that trypsin treatment results in differentially truncated forms of the protein or that there are isoforms of cytochrome *b5* within the microsomal membranes. The two peaks were combined and applied to a G50-Sephadex column. The cytochrome eluted as one peak with an apparent molecular weight of 17,000 (Fig 2.4 and Fig 2.5). The step afforded a 4-fold purification. The cytochrome *b5*-containing fraction was applied to an FPLC Mono Q column from which the cytochrome eluted as two peaks designated peak-I and peak-II (Figure 2.6).

Peak-II gave a single band with an apparent molecular weight of 12,200 on SDS-PAGE (Figure 2.7) and was considered homogeneous at this stage. The overall purification was approximately 800-fold. The Peak-I fraction from the mono Q column gave a single band on SDS-PAGE with an apparent molecular weight of 16,100 (Figure 2.7). Figure 2.7 also shows total phenol extracted microsomal protein from developing safflower seeds.

TABLE 2.1 Summary of purification procedures for cytochrome *b5* from cauliflower florets

Step	cyt *b5* (μg)	Total protein (mg)	Yield (%)	Purification (-fold)
Washed microsomes	1706	1264	100	1
Trypsin digest	734	154	48.7	3.7
DEAE Sephacel	576	10.4	33.7	42.6
G50 Sephadex	459	2.2	26.9	160
Mono-Q Peak-I	75	0.07	4.4	769
Mono-Q Peak-II	151	0.15	8.9	775

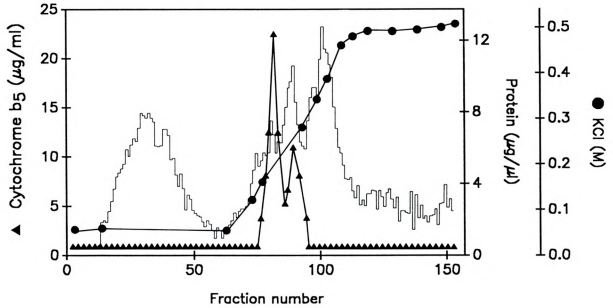

FIGURE 2.3 DEAE-Sephacel column chromatography of trypsin-solubilized cytochrome *b5* from cauliflower microsomes.

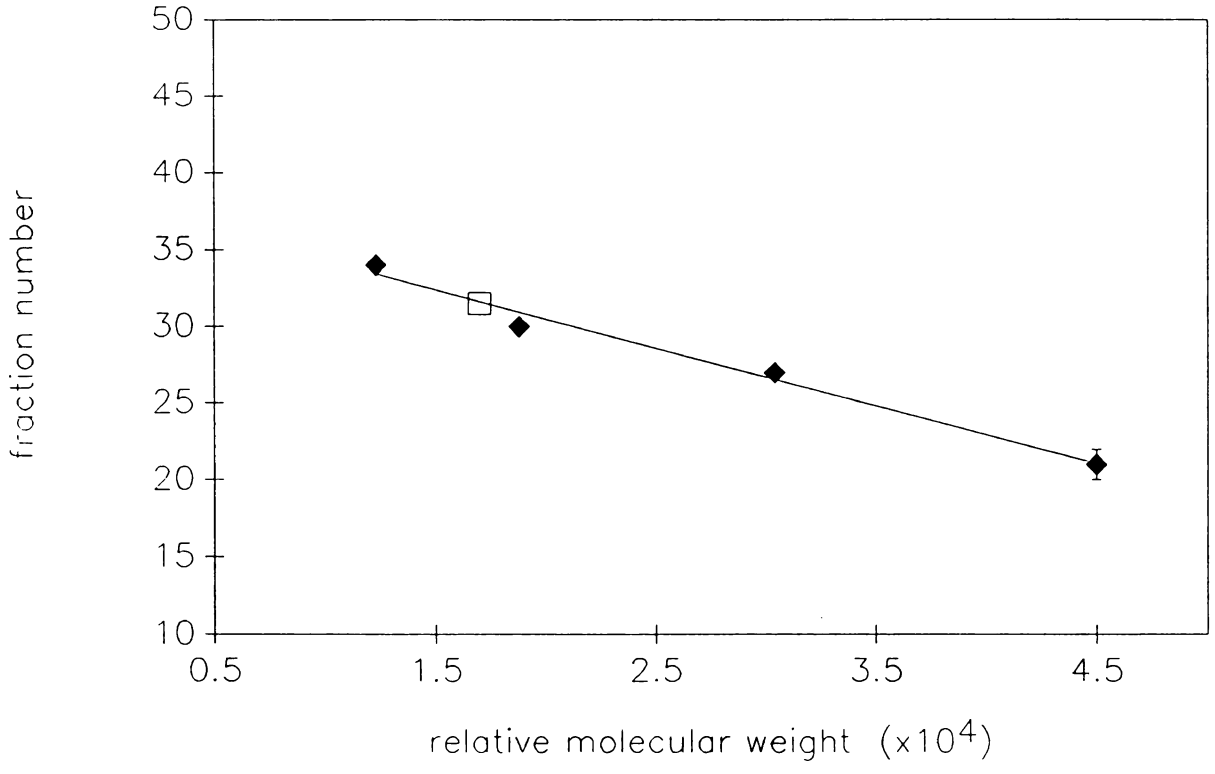

FIGURE 2.4 Molecular weight estimate for FPLC MonoQ Peak I purified trypsin-solubilized cauliflower cytochrome *b5* based on elution from G-50 sephadex relative to standards. □, cytochrome *b5* (17,000 Mr); ♦, cytochrome *c* (12,000), myoglobin (18,800), carbonic anhydrase (30,400), ovalbumin (43,000). Cytochrome *b5* was run separately from the mixed standards. N=3±SE

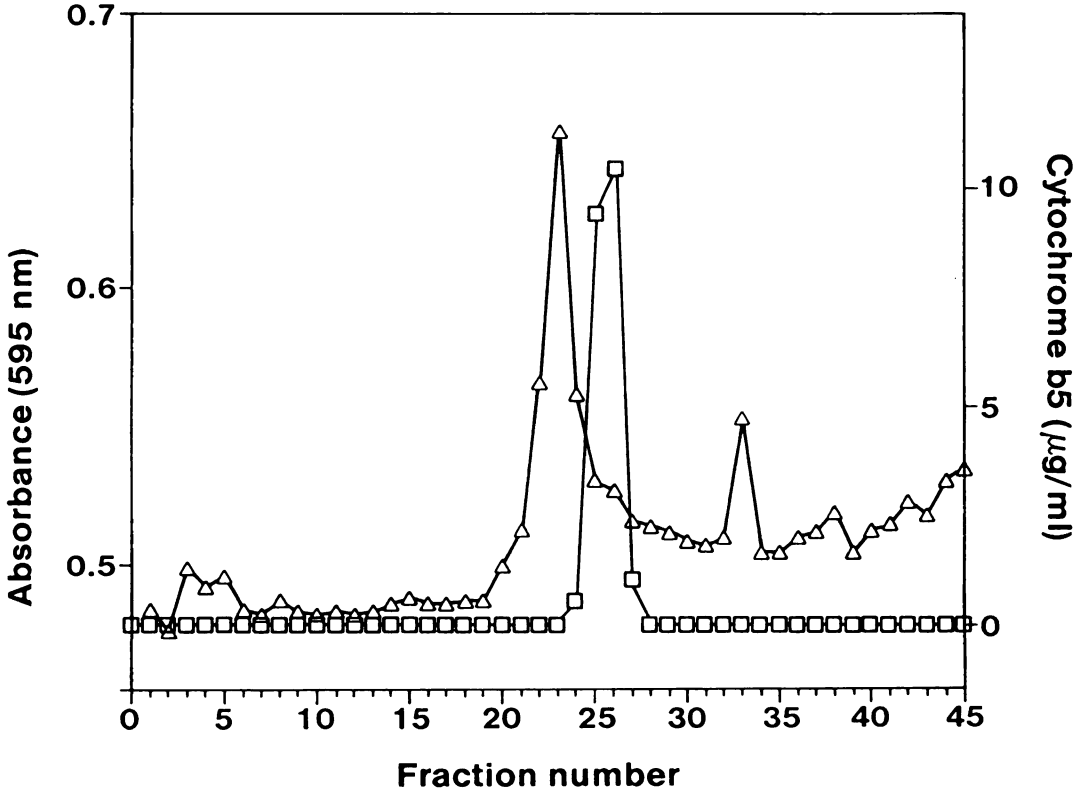

FIGURE 2.5 G-50 Sephadex column chromatography of trypsin-solubilized cytochrome *b5* from cauliflower microsomes. □, cytochrome *b5*; △, Absorbance 595 nm.

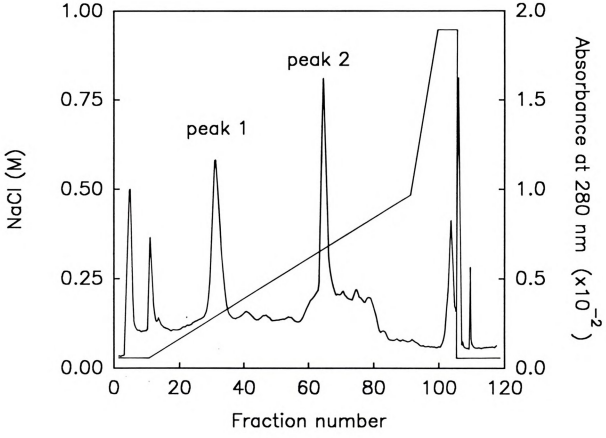

FIGURE 2.6 FPLC MonoQ column chromatography of trypsin-solubilized cytochrome *b5* from cauliflower microsomes. Peaks I and II contained cytochrome *b5*.

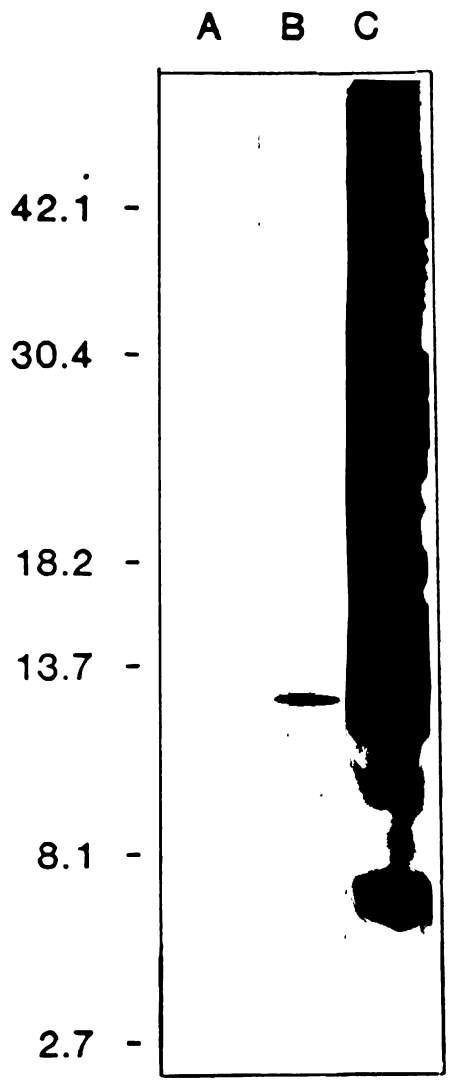

FIGURE 2.7 15 to 25% Laemmli gradient SDS-PAGE Coomasie stained. (A) FLPC MonoQ purified trypsin-solubilized cytochrome *b5* from cauliflower (5μg total protein Peak I); (B) FPLC MonoQ purified trypsin-solubilized cytochrome *b5* from cauliflower (5μg total protein Peak II); (C) 700μg total microsomal protein phenol extracted from safflower S400 seed microsomes.

Studies of cytochrome *c* reduction and Δ12 desaturase using a polyclonal antibody preparation against Peak-I are discussed in chapter three.

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CHAPTER THREE
FUNCTION OF CYTOCHROME b5
IMMUNOINHIBITION STUDIES IN SAFFLOWER MICROSOMES

Microsomal Δ12 Desaturation in Photosynthetic Organisms

Δ12 desaturase activity has been studied in soybean cell culture microsomes[1], potato tuber microsomes[2], pea microsomes[3,4,5,6,7,8,9], microsomes and leaves of sunflower, safflower, and spinach[9], castor leaves, lettuce leaves, and *Chlorella vulgaris*[10,11], and safflower seed microsomes[12,13,14,15,16,17,18]. Activity of the microsomal Δ12 desaturase can be measured by incubating microsomal membranes with [^{14}C]oleoyl-CoA, NAD(P)H, and O_2 and measuring the accumulation of [^{14}C]linoleate in microsomal lipid extracts[5,15]. *In vivo* studies using whole leaves have been performed by misting the leaves with ^{14}C-oleic acid ammonium salt[9], by immersing the stem in ^{14}C-oleate[10], or by adding ^{14}C-oleate to chopped leaf tissue[10]. Attempts to solubilize the Δ12 desaturase for more precise studies have resulted in loss of activity[7]. Thus, direct evidence for the nature of substrates and cofactors is lacking.

As noted in chapter one, the Δ9 desaturase from vertebrates utilizes stearoyl-CoA as a substrate[19]. By contrast, an 18:1 acyl chain at the sn-2 position of phosphatidylcholine (PC) appears to be a substrate for the higher plant microsomal

Δ12 desaturase[4,5,6,7,15,18]. The possibility that PC is a precursor for a substrate such as an acyl chain which has been transferred to the active site of the desaturase has not been ruled out. In pulse chase experiments with pea microsomes, 98% of the Δ12 desaturase activity was at the sn-2 position, while in spinach and safflower, approximately 70% of the ^{14}C-oleate substrate for the Δ12 desaturase activity was at the sn-2 position[9]. In potato tuber microsomes, the apparent preference of Δ12 desaturase for the sn-2 position may have been due to an sn-2 preference of the oleoyl-CoA:PC acyltransferase[2]. Slack *et al.* observed desaturation at both sn-1 and sn-2 positions of PC and saw a 33.7% higher rate of desaturation when ^{14}C-oleoyl-CoA was the substrate rather than ^{14}C-oleoyl-PC[15]. This finding has been interpreted as the result of lipid "channeling" from the acyltransferase to the desaturase[5,11,15]. However, the slower utilization of ^{14}C-oleoyl-PC may be due to its lower solubility in these assays as compared to that of ^{14}C-oleoyl-CoA. Murphy *et al.*[5] showed that the rate of Δ12 desaturation is linear with increasing amounts of either ^{14}C-oleoyl-CoA or ^{14}C-oleate esterified to PC, but the rate increases faster with increasing ^{14}C-oleate esterified to PC. Murphy's evidence suggests strongly that oleoyl-CoA is esterified to PC before being desaturated. However, it does not rule out other substrates or the possibility of PC being a precursor to an enzyme-bound substrate such as an acyl chain. In all of these experiments, desaturation occurs where it is monitored by ^{14}C. In addition, other phospholipids may serve as substrates. Lyso-PC, lyso-phosphatidylethanolamine, and lyso-phosphatidylglycerol enhanced the Δ12 desaturase activity from ^{14}C-oleoyl-CoA in N10 safflower seed microsomes[16]. These lyso-phospholipids may enhance the monitored rate of Δ12 desaturation by driving

acyltransfer onto themselves to provide more radiolabelled lipid substrate. However, lyso-phosphatidylserine(lyso-PS) also enhanced Δ12 desaturation in these microsomes, but there was no evidence for a lyso-PS:oleoyl-CoA acyltransferase in these microsomes[16]. Thus, the lysophospholipids may act as repositories for the removal of ^{14}C-linoleate from PC after desaturation. This back transfer could allow the continuous cycling of more ^{14}C-oleate onto PC for desaturation. Safflower S400 seed microsomes, the material used in this dissertation research, esterify ^{14}C-oleoyl-CoA to all lyso-phospholipids tested: lyso-PC, lyso-PE, and lyso-PA[16]. Thus, the possibility exists that all of these lipids might be substrates in the Δ12 desaturase assays presented in this chapter.

The action of oleoyl-CoA itself as a substrate has not been decisively ruled out due to the acyl exchange between oleoyl-CoA and PC[3,20]. In soybean cell suspension cultures, the product of the Δ12 desaturase was recovered as 18:2-CoA, leading the authors to believe that 18:1-CoA itself might act as a substrate[1]. Acyltransferase activity transferred only approximately 10% of the 18:1-CoA to PC, PE, PI, PG, PA, and neutral lipids over 60 min, and this 18:1 was not desaturated or hydroxylated. The rate for microsomal Δ12 desaturation resulting in 18:2-CoA was 117 pmol/mg/min. Thus, the microsomes from cell suspension cultures were active in desaturation. Therefore, the undifferentiated cell suspension cultures, apparently deficient in acyltransferase activity, unmask the use of 18:1-CoA as a substrate for Δ12 desaturase. Definitive statements about the substrate specificity of the enzyme cannot be made until it is purified and reconstituted.

The higher plant Δ12 desaturase used NADH or NADPH[10] and absolutely required O[8]. The activity is enhanced by added ascorbic acid[3] and by catalase[3,4], which presumably assists in the O_2 requiring step, and is developmentally regulated producing increased levels of 18:2 in safflower seeds at 14 to 18 days after flowering(DAF, *ie.* when the first florets break out of the bud)[12,18]. This is the developmental stage when safflower seeds undergo rapid increases in both seed weight and oil content[13]. In *Chlorella vulgaris*, the activity is also enhanced by growth on poor media such as phosphate buffers[10]. Activity appears to be inhibited by the absence of light[10] and by dithiothreitol (DTT)[8], by N_2[8], and dithiobis(nitrobenzoate)[1]. Higher plant Δ12 desaturation appears to be inhibited specifically at the desaturase protein by α,α'-dipyridyl and o-phenanthroline[1,18], which form complexes with ferrous ions but do not inhibit reduction of cytochrome *b5*[18], and by concentrations of H_2O_2 above 2.5 mM[3,4], which does not cause oxidation of lipid or NADH and does not inhibit acyltransferase nor the reduction of cytochrome *c* via NADH:cytochrome *b5* reductase and cytochrome *b5*. The thiol inhibitor, p-chloromercuribenzoate (pCMB)[18], as well as N-ethylmaleimide[1] inhibit Δ12 desaturase activity at the NADH-cytochrome *b5* reductase. The cytochrome *P450* inhibitor, CO, has no effect on the activity[18].

Prior to the publication of data in this dissertation, the nature of the intermediate electron donor between NADH and the Δ12 desaturase was unknown and the possibility of cytochrome *b5* as an intermediate was uncertain because the protein was reportedly present in very low amounts and because the addition of rat liver

cytochrome *b5* did not increase the Δ12 desaturase activity[16]. As discussed in chapter one, antibodies against the cytoplasmic domain of vertebrate cytochrome *b5* inhibited the activity of enzymes utilizing cytochrome *b5* as an intermediate electron donor. The inhibition is assumed to be steric in nature, interfering with the free access of proteins to each other during electron transfer. In the experiments described here, antibodies against cytochrome *b5* solubilized from cauliflower florets were used in immunoinhibition studies to examine the role of cytochrome *b5* in Δ12 desaturation in safflower seed microsomes. The results of these studies provide evidence that cytochrome *b5* is the electron donor for the Δ12 desaturase in higher plant microsomes. These results were subsequently confirmed by another group which used these antibodies and antibodies to bovine cytochrome *b5* in a similar safflower seed microsomal Δ12 desaturase system[17]. Concurrent to this work, it has been shown that addition of oleoyl-CoA to NADH-reduced microsomal membranes resulted in cyanide-sensitive partial oxidation of cytochrome *b5*, also suggesting that cytochrome *b5* is the intermediate electron donor in the desaturation of lipid linked oleic acid[18].

Experimental Procedures

Materials. Safflower S400 seed was obtained from Seedtech Inc. (Woodland, CA). Safflower plants were grown in a glasshouse with supplemental illumination and were hand pollinated. All chemicals used were reagent grade. Horse heart cytochrome *c* (Type IV) and non-immune mouse serum were obtained from Sigma (St. Louis, MO).

Goat anti-mouse alkaline phosphatase was purchased from Kirkegaard and Perry. [^{14}C]-oleate was obtained from CEA-France.

Production of antibodies. All column chromatography and procedures involving microsomes were done at 4° C except where noted. Purified cytochrome *b5* (140μg of Peak I) was injected intraperitoneally into a female Balb/c mouse every two weeks for 22 weeks. The initial injection was in Freund's complete adjuvant, and subsequent injections were in Freund's incomplete adjuvant. Serum was obtained by bleeding from the eye. Ascites fluid was obtained after seven injections.

Serum or ascites fluid was diluted with an equal volume of 10 mM potassium phosphate, pH 7.2, 150 mM NaCl (azide free PBS), filtered through a 0.2μm filter and applied to a 5 ml protein-G Sepharose column (Pharmacia). The column was washed with 25 ml of 20 mM potassium phosphate (pH 7.2), then the IgG was eluted with 100 mM glycine-HCL (pH 2.7). The pH of the 2 ml fractions was immediately adjusted to pH 6 with 50 μl of 100 mM Tris base. The IgG-containing fractions were combined and concentrated in a Centricon-10 unit (Amicon) and resuspended in azide free PBS to a concentration of 76 mg/ml. The IgG fraction from nonimmune mouse serum was prepared in an identical fashion.

Cytochrome *c* reduction assays. Commercially available (Sigma, St. Louis, MO) horse heart cytochrome *c* was further purified by applying a solution in 50 mM Tris-HCl (pH 8.0) to an FPLC Mono Q column and eluting with the same buffer. The

cytochrome *c* eluted with the void volume, whereas contaminating cytochrome *c* reductase activity was retained by the column. Microsomes were prepared from fresh developing safflower S400 seeds 6 to 8 days after flowering (*ie.* when the florets burst from the buds) by homogenizing 0.5 g of tissue with a Polytron homogenizer (Brinkman) at intermediate speed for 30 to 40 seconds in 30 ml of 100 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1 mM $MgCl_2$, 0.33 M sorbitol, 10 mM cysteine-HCl, and 0.1% (w/v) BSA. The homogenate was filtered through one layer of miracloth and centrifuged at 7,800 g_{av} for 10 minutes. The supernatant fraction was retained and brought to a final concentration of 50 mM $MgCl_2$. The mixture was then centrifuged at 11,200 g_{av} for 10 minutes. The pellet was resuspended in 0.1 M potassium phosphate (pH 7.5), 10 mM EDTA, and 0.002 % (v/v) Triton X-100 and filtered through one layer of Miracloth to maximize homogeneity.

The cytochrome *c* reduction assay was adapted from that of Rogers and Strittmatter[21]. In a typical assay, 54µg of microsomal protein were incubated with immune or nonimmune serum in an 80µl volume for 2 hrs on ice. After incubation, purified cytochrome *c* was added to a final concentration of 5 µM and the volume adjusted to 1 ml in 0.1 M potassium phosphate (pH 7.5). The contents of duplicate tubes were mixed briefly and placed into two cuvettes. The reaction was initiated by the addition to one cuvette of NADH to a final concentration of 0.1 mM. The reduction was monitored at 550 nm continuously over three minutes by dual beam spectrophotometry on a Perkin-Elmer Lamba 7 UV/VIS spectrophotometer. Rates were determined from initial velocity tangents to the curve of change in absorbance

at 550 nm over time.

Δ12 Desaturase assays. ^{14}C-oleoyl-CoA was prepared according to Taylor *et al.*[22] Microsomes were prepared fresh from developing safflower S400 seeds 14 to 18 days after flowering as described[20] and were resuspended in 0.1 M potassium phosphate (pH 7.2). Microsomes were incubated with the appropriate IgG for 2 hrs on ice. Assays were performed as described[14], except that the total reaction volume was 250 μl containing 0.25 mg total microsomal protein, 9nmol ^{14}C-oleoyl-CoA, and final concentrations of 7mM potassium phosphate buffer pH 7.5 and 3 mM NADH. The reactions were incubated at 30°C for 30 min with shaking at 80 rpm. Lipid was extracted in 4 ml chloroform:methanol (2:1;v/v) with 1 ml 0.9% (w/v) NaCl, and the lower phase was dried under N_2. Fatty acid methyl esters were prepared by resuspending the dried lipid in 1.0 M methanolic-HCL then refluxing for 45 min at 80°C. An equal volume of hexane and 0.9% (w/v) NaCl were added, and methyl esters were extracted into the upper (hexane) phase, which was removed and dried under N_2. The dried methyl-esters were resuspended in hexane and separated by argentation TLC in hexane/diethyl ether (80/20) on silica Si250-PA TLC plates impregnated with 15% (w/v) $AgNO_3$ and rhodamine b. The plates were prepared by soaking commercially available plates (Baker) in 15% (w/v) $AgNO_3$, 0.5 mg/ml rhodamine b in acetonitrile for 10 min, air drying for 30 min, and baking at 120°C for 30 min directly before use. All procedures were performed under reduced illumination to protect the plates from discoloration by light. The regions of the plate containing 18:2 methyl esters as determined by co-migration with a standard were

scraped into scintillation fluid and incubated overnight before determination of radioactivity by scintillation counting. Efficiency of counting was controlled with a ^{14}C standard under the same conditions.

Measurement of incorporation of oleic acid into microsomal lipid was performed identically to the desaturase assays except that chloroform extracted lipid was separated in chloroform/methanol/ammonium hydroxide (65/25/1) on silica TLC plates activated at 120° C for 30 min. Incorporation was measured with a Bioscan TLC radiochromatogram scanner.

To determine the specificity of the ascites IgG inhibition, the active IgG was quenched by incubation with excess purified cytochrome *b5* overnight at 4° C. Quenched IgG was then incubated with microsomes in desaturase assays as above. The effect of excess soluble cytochrome *b5* on the desaturase activity was measured in similar reactions lacking the IgG.

Other methods. Microsomes used as electrophoresis samples were extracted with an equal volume of phenol buffered with 10 mM Tris-HCl pH 7.8, 0.1 mM EDTA and the protein was precipitated from the phenol phase with 5 volumes 0.1 M ammonium acetate in methanol overnight at -20° C. The precipitate was pelleted for 10 min in a microcentrifuge and washed with -20° C acetone before resuspension in Laemmli cracking dye[23]. Electrophoresis in SDS-polyacrylamide (15-25% gradient) gels was performed as described[23]. Western blots[24] were developed with 1/1000 dilutions of

protein-G purified IgG using 5% (w/v) non-fat dry milk as a blocking agent. The blots were developed with goat anti-mouse IgG conjugated to alkaline phosphatase[25]. Protein content was measured with a dye-binding assay[26] or, in the case of IgG preparations, by measuring absorbance at 280 nm[27].

Results and Discussion

Polyclonal antibodies to cytochrome *b5* (Peak I) were raised in a mouse. Although raised against apparently homogenous protein, these antibodies cross-reacted on Western blots with a number of other protein bands in SDS-polyacrylamide gels containing total protein from developing S400 safflower seed microsomal membranes (Figure 3.1, panel C). The lowest molecular weight band had an apparent molecular mass of about 16.5 kDa, which is in agreement with values previously reported for native cytochrome *b5*[28,29]. Another band of approximately 18 kDa could represent an isoform of cytochrome *b5*. However, this does not seem a likely explanation for the cluster of bands of approximately 45 kDa. Since these bands were not seen on Western blots of the preparation of purified cytochrome *b5* used to prepare the antibodies (Figure 3.1, panel A), this cross-reactivity does not appear to be due to contamination of the injected antigen with other polypeptides. The cross-reactivity may be due to conservation of idiotypes among different proteins in the safflower seed microsomes. In support of this concept, comparisons of the amino acid sequence of vertebrate cytochrome *b5* with deduced amino acid sequences of nitrate reductase from *Arabidopsis thaliana*, chicken sulfite oxidase, and yeast flavocytochrome *b2*

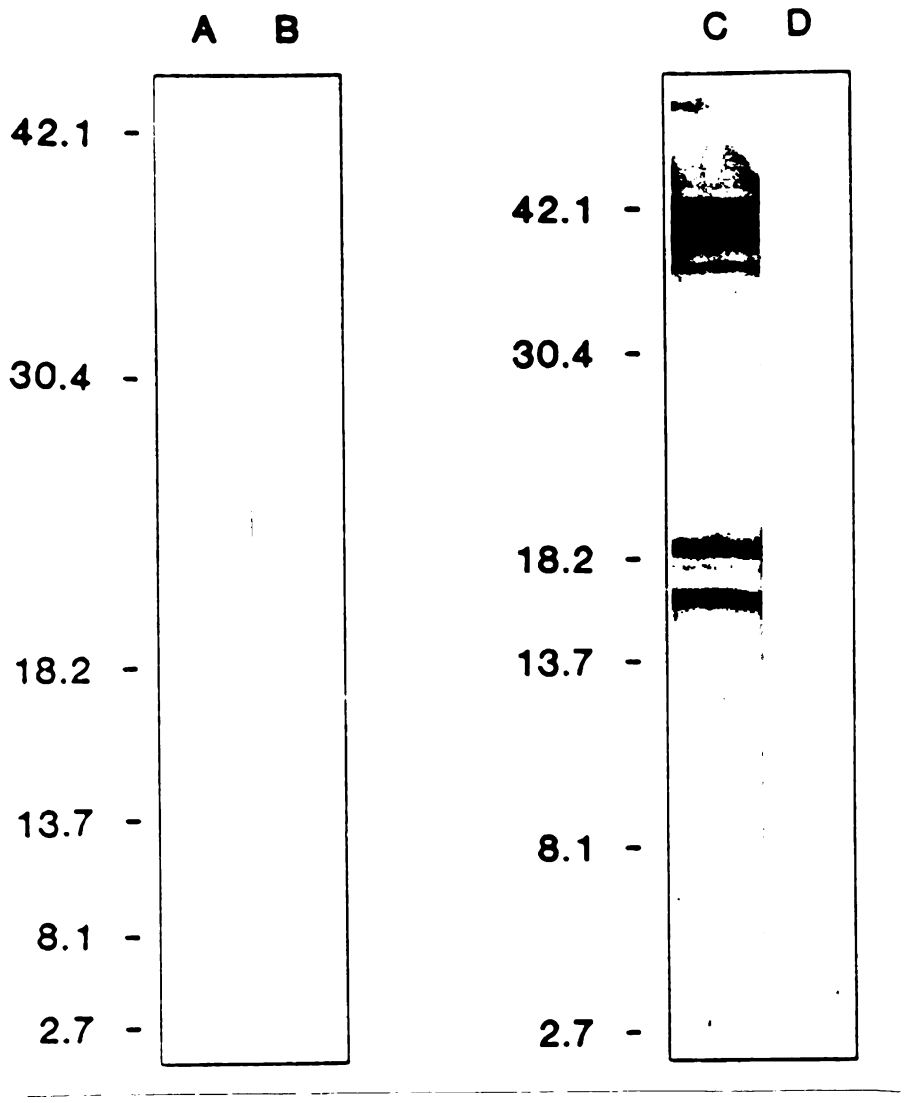

FIGURE 3.1 Western blots developed with FPLC Protein-G purified IgG. (A) 5μg Peak I developed with immune IgG (B) 5μg Peak I developed with non-immune IgG (C) 700 μg phenol extracted total microsomal protein from safflower seed microsomes developed with immune IgG (D) 700μg phenol extracted total microsomal protein from safflower S400 seeds developed with non-immune IgG.

revealed regions of sequence homology in all three proteins[30].

Microsomal membranes catalyze NADH-dependent reduction of soluble cytochrome *c*. Part of this activity is due to electron flow from NADH to NADH-cytochrome *b5* reductase to cytochrome *b5* and then to the exogenously added cytochrome *c*[31]. Thus, a criterion for an effective antibody against cytochrome *b5* is that it should decrease the rate of NADH-dependent cytochrome *c* reduction by microsomes. An example of the absorbance 550 nm curves used to obtain the data presented here is shown in Figure 3.2. The rate of cytochrome *c* reduction by microsomes from developing S400 safflower seeds was 117 ± 20 nmol/min/mg protein. Antibodies raised against cauliflower cytochrome *b5* specifically blocked electron transfer from NADH to exogenous cytochrome *c* by up to 62% in safflower microsomes (Figure 3.3). Thus, the immune IgG effectively blocked either reduction of cytochrome *b5* or electron transfer from cytochrome *b5*. The inhibition is assumed to be due to steric inhibition of the protein-protein contact required for electron transfer. The antibodies do not interact with cytochrome *c* on western blots (Figure 3.4) and the microsome-independent reduction of impure cytochrome *c* is not blocked by these antibodies. NADPH-cytochrome *c* reductase can also utilize NADH to reduce cytochrome *c* to a low degree in a reaction which does not involve cytochrome *b5*[32]. Thus, complete sequestration of cytochrome *b5* by antibodies would not be expected to result in complete inhibition of NADH-dependent cytochrome *c* reduction because of this second system.

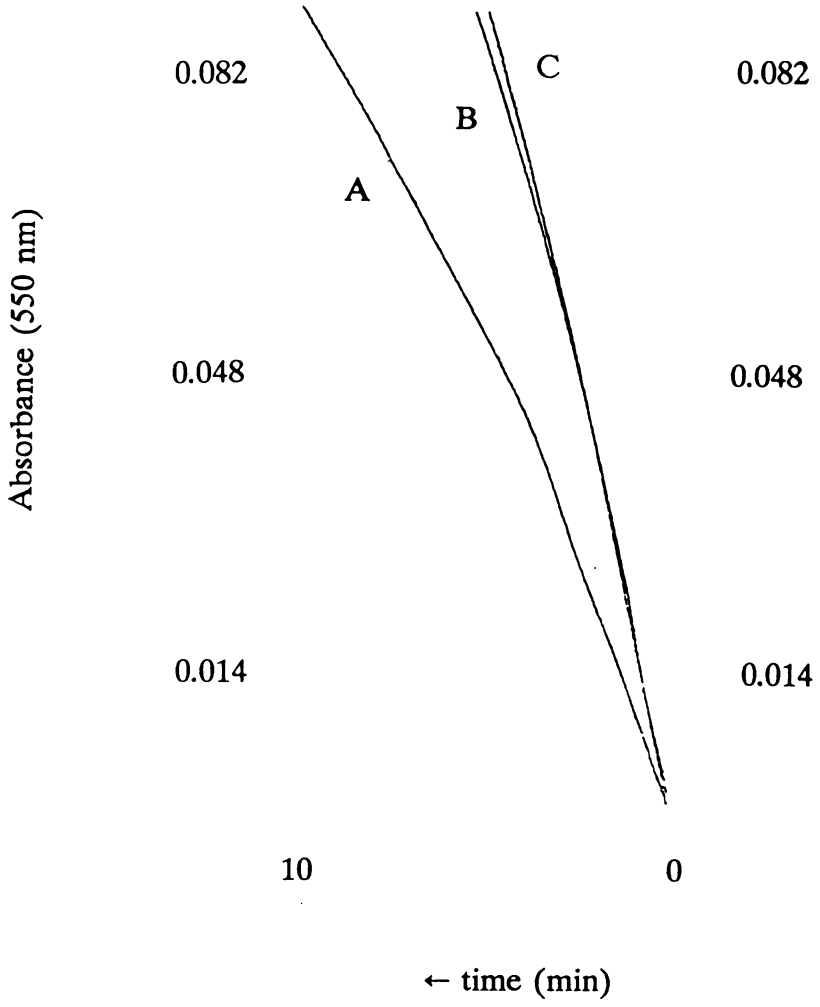

FIGURE 3.2 An example of absorbance 550 nm curves showing reduction of cytochrome *c* over time by safflower microsomes preincubated with (A)immune IgG, (B) nonimmune IgG, and (C) PBS without IgG. Tangents to the initial velocity were taken to determine rates.

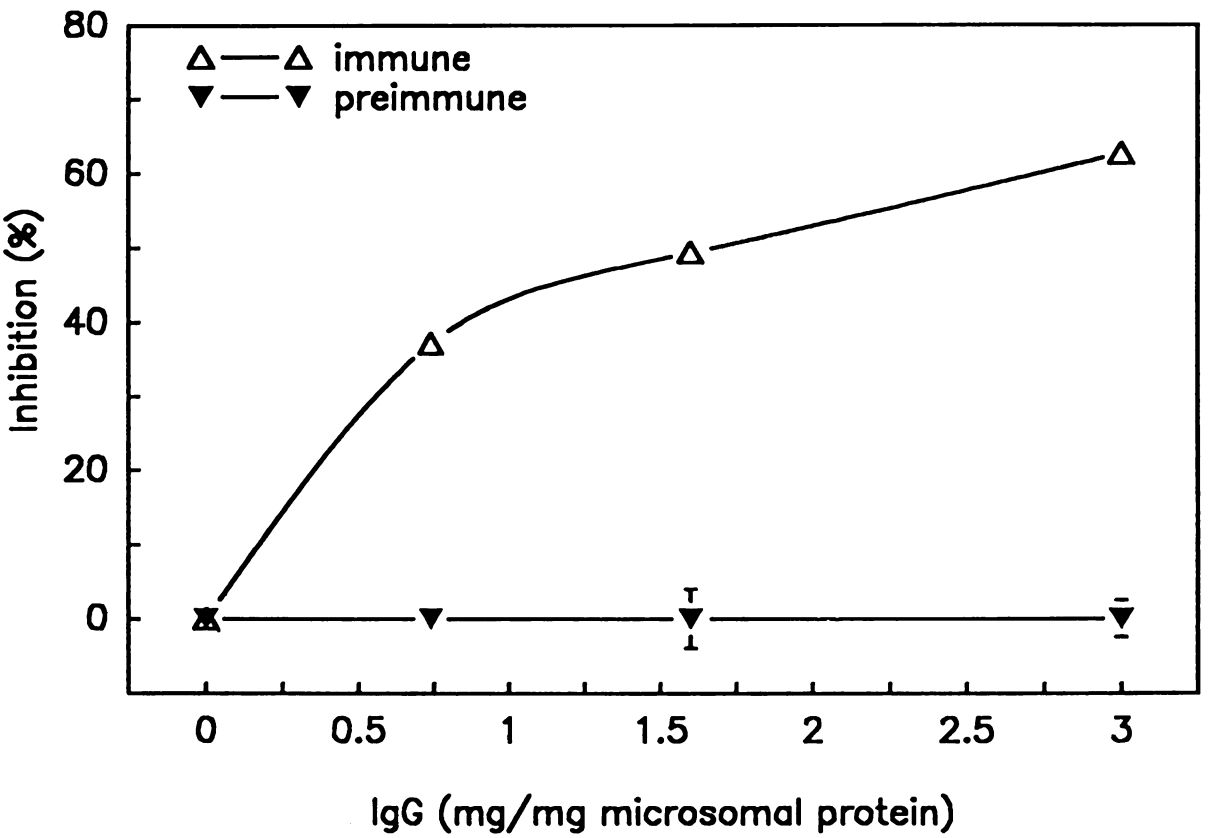

FIGURE 3.3 Effect of IgG on NADH-dependent cytochrome *c* reduction by safflower seed microsomal membranes. n=3 ± SE.

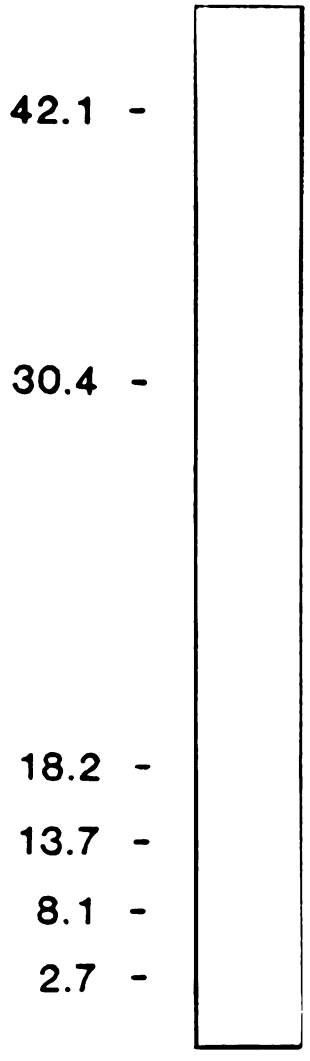

FIGURE 3.4 Western blot of 3 μg cytochrome *c* developed with FPLC Protein-G purified ascites IgG. Transfer of prestained markers showed that protein had transferred from the 12% Laemmli gel to the nitrocellulose.

As discussed in the introduction of this chapter, the assay for the effect of the anti-cytochrome *b5* antibody on desaturase activity is complicated by the fact that at least one substrate for desaturation has been identified as phosphatidylcholine[5,15] but the substrate provided is oleoyl-CoA. Thus, the overall assay measures both acyltransferase activity and desaturase activity at the very least. Approximately 70% of the ^{14}C added to the desaturase assays was recovered as methyl esters from the chloroform soluble lipid fraction. Measurement of extent of incorporation of oleoyl-CoA into phosphatidylcholine or other phospholipids indicated no effect of the antibody on acyltransferase activity (Figure 3.5). The rates of incorporation into PC and other phospholipids with no IgG were 130 ± 7 pmol/mg protein/min and 41 ± 4 pmol/mg protein/min, respectively. With anti-cytochrome *b5* IgG, the rates of incorporation into PC and other phospholipids were 133 ± 23 pmol/mg protein/min and 35 ± 9 pmol/mg protein/min, respectively. With nonimmune IgG, the rates for PC and other phospholipids were 140 ± 16 pmol/mg protein/min and 30 ± 12 pmol/mg protein/min, respectively. These rates are in good agreement with those obtained by Stymne and Appelqvist[14], 105 pmol/mg protein/min for PC and 43.6 pmol/mg protein/min in 30 minute incubations similar to the no IgG incubations above.

Microsomes prepared from developing S400 safflower seeds desaturated [^{14}C]oleate to linoleate at an average rate of 34 ± 3 pmol/mg protein/ min. This rate was comparable to rates of 45 pmol/mg protein/min obtained previously by Stymne and Appelqvist[14]. An example of the argentation TLC separation of methylesters used to

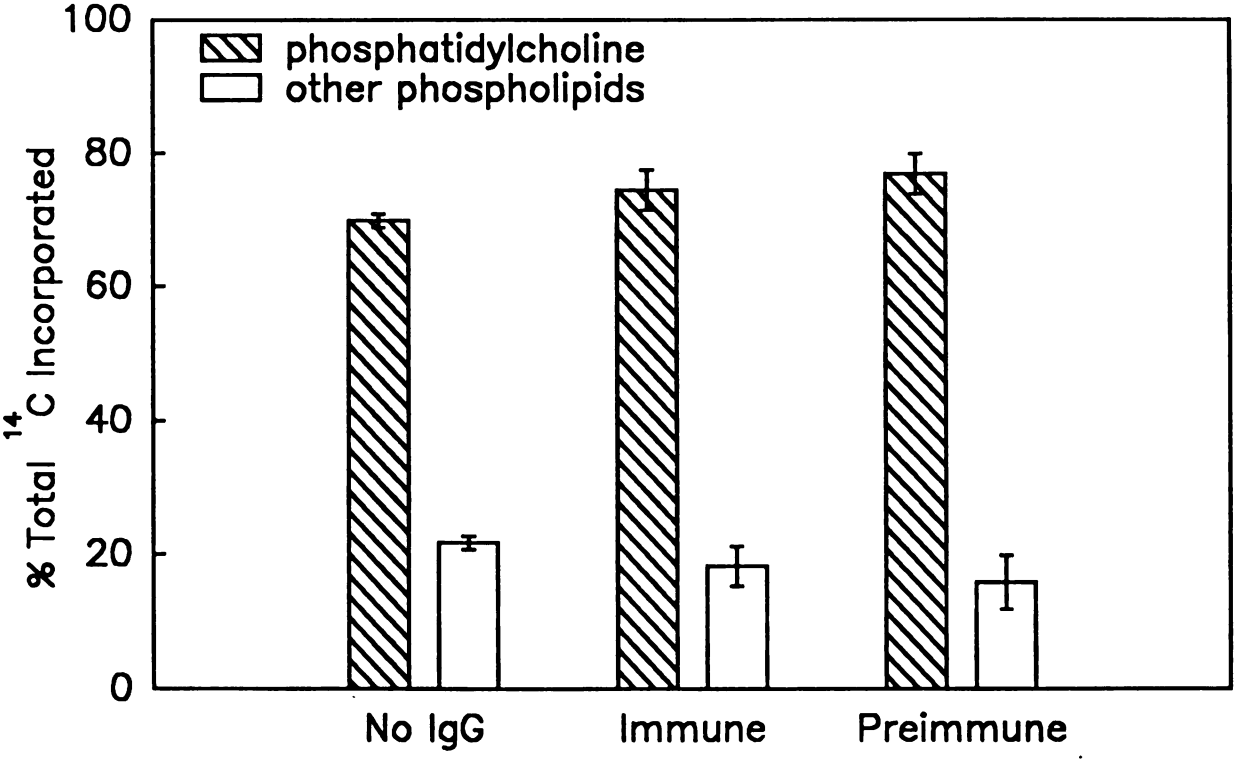

FIGURE 3.5 Effect of IgG on incorporation of [^{14}C]-oleic acid into phospholipids. Safflower microsomes were preincubated with addition of buffer, immune IgG or non-immune IgG (2.7 mg IgG/mg microsomal protein) for 2 h at 4°C, then assayed for desaturase activity under standard conditions. n=4 ± SE.

obtain the data on inhibition is show in Figure 3.6. Preincubation of microsomal membranes with immune IgG for 2 h on ice prior to assay blocked Δ12 desaturation by up to 93% (Figure 3.7). By contrast, the highest level of nonimmune IgG inhibited desaturase activity by only 25%. Although there was a pronounced difference between the effects of immune and nonimmune IgG on desaturase activity, an additional criterion was applied to ensure that the inhibitory effects of the immune IgG were specifically due to the presence of anti-cytochrome *b5* IgG. In this experiment, IgG was preincubated with various amounts of pure cytochrome *b5* before being assayed for the effect on desaturase activity. The inhibitory effect of ascites IgG on desaturation was completely prevented by preincubation of the IgG with purified cytochrome *b5*, while excess purified cytochrome *b5* alone had little effect on the rate of desaturation (Figure 3.8). This result confirms that the inhibition of desaturation is caused by IgG specific to cytochrome *b5*, indicating that cytochrome *b5* is the electron donor for microsomal Δ12 desaturation in higher plants. This observation has several implications. First, since both the intermediate electron carrier and at least one of the substrates (i.e., phosphatidylcholine) for the desaturase are now known it may be possible to reconstitute desaturase activity in detergent-solubilized microsomal membranes, as was done for stearoyl-CoA desaturase from vertebrates[19], by providing substrate, exogenous cytochrome *b5* reductase, and cytochrome *b5*. Second, the availability of the anti-cytochrome *b5* antibodies should permit a similar assessment of the role of cytochrome *b5* in other NAD(P)H-dependent reactions catalyzed by plant endomembranes.

FIGURE 3.6 A representative argentation TLC plate developed in hexane/diethylether (80/20) and photographed under UV to visualize the fluorescing rhodamine b stained fatty acid methylesters. Lane A, an 18:1 methylester standard, lane B, an 18:2 methylester standard, lanes C through I are methylesters prepared from the total chloroform soluble lipid in desaturase assays.

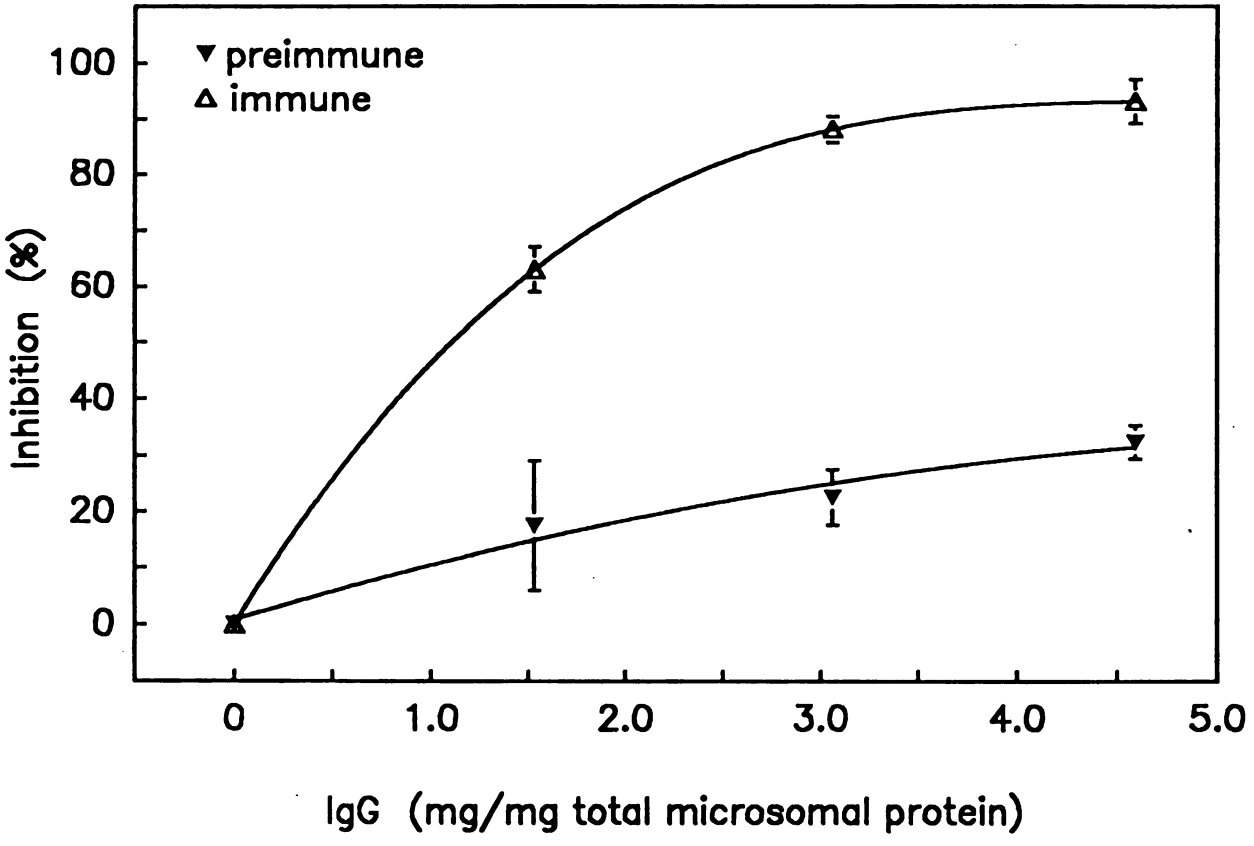

FIGURE 3.7 Inhibition of Δ12 desaturation by immune IgG. Safflower microsomes were preincubated with IgG for 2 h at 4° C, then assayed for desaturase activity. n=3 ± SE.

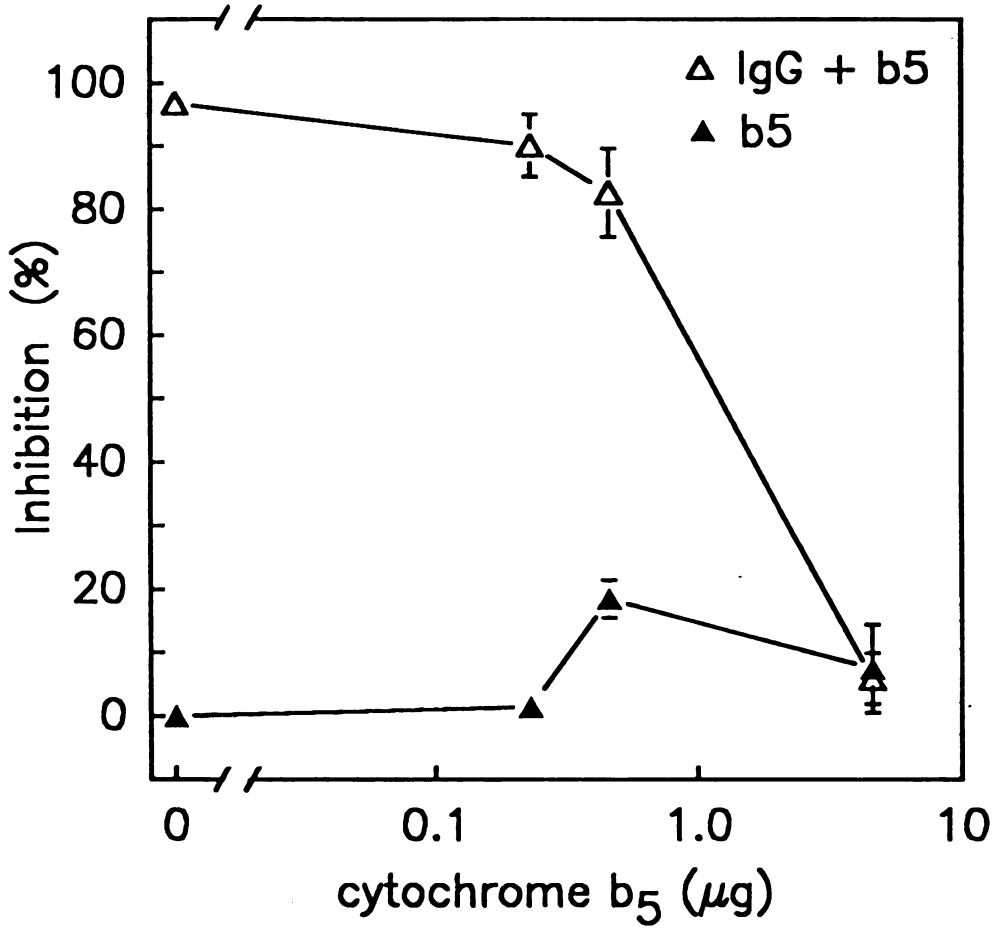

FIGURE 3.8 Effect of soluble cytochrome *b5* on inhibition of Δ12 desaturation by immune IgG. One-milligram aliquots of IgG were incubated overnight with purified cytochrome *b5* at 4° C. Safflower microsomes were then incubated with this quenched IgG or with purified cytochrome *b5* alone for 2 h at 4° C and assayed for desaturase activity. n=3 ± SE.

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CHAPTER FOUR
GENETICS OF CYTOCHROME b5

Mammalian and Avian Cytochrome *b5* Genes

The first molecular genetics involving cytochrome *b5* was done in 1986, when Beck von Bodman *et al.*[1] used the amino acid sequence of rat hepatic cytochrome *b5* to produce a synthetic cytochrome *b5* gene. This synthetic gene featured a 5' *Escherichia coli* ribosome binding site and spacer region which allowed its overexpression in *E. coli*. Overexpression of the whole protein resulted in membrane insertion. The fact that cytochrome b5 inserted into the *E.coli* membrane re-enforces the idea that cytochrome *b5* inserts randomly into the closest membrane by anchoring its hydrophobic C-terminal into the lipid. Overexpression of the soluble portion to 8% of the total *E. coli* protein turned the cell culture red with heme. Studies of the heme binding histidine 63 were carried out using site directed mutagenesis. When histidine 63 was replaced by alanine or methionine, the cytochrome *b5* was produced in its apoprotein form without heme. Upon purification, the methionine 63 apoprotein could be reconstituted with heme, resulting in a high spin, five coordinate iron within the heme. Following the production of this synthetic gene, cDNA sequences were published for the liver cytochrome *b5* protein of rabbit[2], human[3], chicken[4] and cow[5].

The human cDNA was 743bp long with the region from 53 to 457bp encoding a protein of 134 amino acids[3]. The rabbit cDNA was 1006bp with a 402bp open reading frame encoding a 134 amino acid protein[2]. The chicken cDNA contained a 414bp open reading frame which encoded a protein of 138 amino acids. The first six N-terminal amino acids were not accounted for in the original protein and thus appear to be cleaved off the protein during processing to the mature form of 132 amino acids[4]. The chicken cDNA was used as a probe to obtain genomic clones of the chicken cytochrome *b5*, which appears to be encoded by one gene. However, these genomic clones have not been sequenced[6]. The bovine cDNA was 715bp with a 402bp open reading frame encoding a 134 amino acid protein[5].

Higher Plant Cytochrome *b5* genes

This chapter is the first description of any higher plant cytochrome *b5* gene. The sequence data from four independent cDNA clones derived from a cauliflower floret meristematic surface λUNI-ZAP XR library are presented as well as the characteristics of the protein encoded by these cDNAs. The characteristics of the protein are compared with those of cytochrome *b5* proteins deduced from the mammalian and avian cDNAs above. In addition, the *A.thaliana* mutant, *fad2*, deficient in the endoplasmic reticulum Δ12 desaturase was shown to translate a protein of approximately 16.1 kD which was recognized by anti-cauliflower cytochrome *b5* IgG.

Experimental Procedures

Bacterial strains, plasmids, and bacteriophage. Epicurian coli XL1-Blue cells (endA1, hsdR17(r_k-,m_k-), supE44, thi-1, λ-, recA1-, gyrA96, relA1, (lac-),[F', proAB, lac $I^qZ\Delta M15$, Tn10, (tet^r)]) were obtained from Stratagene (La Jolla, CA). λUNI-ZAP XR phage (att, int, xis, c1857(nin 5)), containing the excisable plasmid Bluescript SK- (lac I, amp^r, col E1 ori, lac Z, MCS, F1 (-) ori), were obtained from Stratagene (La Jolla, CA).

Materials. *Arabidopsis thaliana* var. Columbia wild type and a *fad2* mutant line in the Columbia background were grown under constant light at 23°C on Bacto mix soil topped with fine vermiculite and were watered as needed with *Arabidopsis* nutrient solution (5 mM KNO_3, 2.5 mM potassium phosphate, pH 5.5, 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 2.5 mM ferric salt of EDTA (ethylene diamine tetraacetic acid disodium), 70 μM H_3BO_3, 14 μM $MnCl_2$, 0.5 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.2 μM $NaMoO_4$, 10 μM NaCl, 0.01 μM $CoCl_2$). The plants were harvested for use after two weeks of growth. A λUNI-ZAP XR library of meristematic surface of cauliflower floret cDNA was the gift of Dr. June Medford (Penn State). A 0.2 kb Polymerase Chain Reaction (PCR) product encoding amino acids 3 through 30 of the cauliflower cytochrome *b5* amino acid sequence was obtained from Pamela Keck (Monsanto, St. Louis, MO), who used oligonucleotides based on the cauliflower amino acid sequence as PCR primers and *Brassica napus* cDNA as the PCR template. The N-terminal cauliflower cytochrome *b5* amino acid sequence was obtained by Joe Leykam (Macromolecular

Structure Facility, Michigan State University) and was confirmed by Pamela Keck (Monsanto, St. Louis, MO) both using the protein purified in chapter two. Pamela Keck extended the amino acid sequence data by sequencing trypsin fragments of the protein purified in chapter two.

^{32}P-Labelling of probes. The 0.2 kb *Brassica napus* PCR-derived probe was random hexamer labelled with α^{32}P-dCTP by the method of Feinberg and Vogelstein[7]. The labelled probe was purified from unincorporated nucleotides by the G50 spin column technique of Penefsky[8]. The purity of the column eluate was checked by Thin Layer Chromatography (TLC) on Baker Cellulose PEI-F paper developed with 1 M Phosphoric Acid (pH 3.5) for 15 min and autoradiographed for 15 min at 23°C.

Screening of the λUNI-ZAP XR cauliflower cDNA library. Approximately 570,000 plaques were grown on XL1-Blue *E. coli* lawns as described in the Stratagene (La Jolla, CA) λUNI-ZAP XR protocol manual. Plaque lifts to nitrocellulose were performed as per Maniatis[9]. After baking, the nitrocellulose filters were prehybridized in 5 X SSPE (180 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, final pH 7.7), 0.5% nonfat dry milk, 0.5% SDS (sodium dodecylsulfate), 5% dextran sulfate for 1 hr at 65°C with shaking. The 0.2 kb probe was added directly to the prehybridization solution and hybridization proceeded overnight at 65°C with shaking. The nitrocellulose filters were then washed twice in 2 X SSPE, 0.1% SDS at 23°C for 10 min each followed by one wash at 65°C for 10 min, and autoradiographs were developed at -70°C for 3 to 5 hours. Hybridizing plaques were picked into 1 ml SM

buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM $MgSO_4$) and rescreened twice before being chosen for Bluescript excision. Four clones were excised from the λUNI-ZAP XR phage as Bluescript SK- plasmids as per the Stratagene λUNI-ZAP XR protocol manual.

Sequencing the λUNI-ZAP XR derived clones. The four Bluescript plasmids were amplified in XL1-Blue in LB (10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl per liter, pH 7.5) supplemented with 100 μg/ml ampicillin and the DNA was purified from 3 ml cultures using the Promega (Madison, WI) Magic Mini-Prep Kit. Double stranded sequencing[10] was performed using 3 to 6 μg of template DNA and 5 ng of primer and the United States Biochemical Corporation (Cleveland, OH) Sequenase version 2 kit. The dGTP nucleotide mixture was diluted 1:10(v/v) rather than the standard 1:5 dilution to obtain more label in the shorter polymerization products. The initial primers used were M13 universal and reverse primers followed by oligonucleotides based on internal sequence. Sequence reactions were heated at 75° C for 3 min and electrophoresed in 30 x 39 x 0.0005 cm flat gels consisting of 43%(w/w) urea, 18%(v/w) 40:2 acrylamide:bisacrylamide, 1 X TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0) sequencing gels which were run at 30W constant power in 1 X TBE. The gels were fixed overnight in 10% methanol, 10% glacial acetic acid and dried for 1 hr under vacuum. Autoradiographs were developed for 24 hrs at -70° C. Sequence data was analyzed using the eighth version (March 1991) of the Hitachi HIBIO DNASIS DNA Sequence Analysis System and PROSIS Protein Sequence Analysis System.

Analysis of cytochrome *b5* in *fad2*. Leaves were harvested from Columbia wild type and *fad2 Arabidopsis thaliana* plants grown as described above. Microsomes were made from two week old plants by grinding 0.5 g leaf tissue per ml grinding buffer (0.1 M potassium phosphate, pH 7.2, 0.1% bovine serum albumin, 0.33 M sucrose) on ice, filtering the homogenate through two layers of Miracloth, diluting with 5 volumes of ice chilled grinding buffer, and centrifuging at 18,000 g for 20 min in a Sorvall SS34 rotor at 4°C. The supernatant was filtered through two layers of Miracloth and centrifuged at 105,000g in a Beckmann 60Ti fixed angle rotor for 90 min at 4°C. The pellet was resuspended in 0.1 M potassium phosphate, pH 7.2. Total microsomal protein was phenol extracted and electrophoresed on a Laemmli 15 to 25% gradient gel as described in chapter three. Western blotting and development with FPLC Protein G purified immune IgG was performed as described in chapter three.

Results and Discussion

Four independent cDNAs from the meristematic surfaces of cauliflower florets encode the same cytochrome *b5* protein (Figure 4.1). The first clone contained sequence from -95bp to 490bp, the second two clones contained base pairs -27 to 580. All three of these clones ended in a poly A tails 19 base pairs in length and represented as airplanes in Figure 4.1. The fourth clone lacking a poly A tail spanned the region from -21 to 643bp and had a G→T transversion at base pair +15. This transversion may be due to an error in the reverse transcription of the cDNA or it

```
-95              AGTTCTCAAGTAGGTGAAGAGTTACCCCACCAGGTGCT

-57 ATCGCAATTCTGAATCTGAAAGCAGAAAGATCCCCCACCTTTGATTTG

 1            M  A  S  E  K  K  V  L  G  F  E  E  V
-9  AGGAGAGAGATGGCTTCAGAGAAGAAGGTTCTAGGTTTCGAAGAAGTT
                          T
14  S  Q  H  N  K  T  K  D  C  W  L  I  I  S  G  K
40  TCGCAGCACAACAAAACCAAGGATTGTTGGCTTATTATCTCCGGCAAG

30  V  Y  D  V  T  P  F  M  D  D  H  P  G  G  D  E
88  GTCTATGATGTGACCCCTTTCATGGATGATCATCCCGGTGGCGATGAA

46  V  L  L  S  S  T  G  K  D  A  T  N  D  F  E  D
136 GTGTTATTGTCCTCAACAGGGAAAGATGCTACGAATGACTTTGAAGAC

62  V  G  H  S  D  T  A  R  D  M  M  E  K  Y  Y  I
184 GTTGGTCACAGCGACACCGCGAGGGACATGATGGAGAAGTACTACATT

78  G  E  I  D  S  S  T  V  P  A  T  R  T  Y  V  A
232 GGCGAGATCGATTCGTCTACTGTTCCAGCGACAAGGACATACGTTGCA

94  P  V  Q  P  A  Y  N  Q  D  K  T  P  E  F  M  I
280 CCAGTGCAACCCGCGTACAACCAAGACAAGACACCAGAATTCATGATC

110 K  I  L  Q  F  L  V  P  I  L  I  L  G  L  A  L
328 AAGATCCTTCAGTTCCTTGTTCCAATCTTGATCTTGGGTCTTGCTCTC

126 V  V  R  Q  Y  T  K  K  E  *
376 GTCGTCCGTCAGTATACTAAGAAAGAGTAGAAGCAGAGAGAGGCTTGC

424 GGTATTGCTTCCCATTGGATAACTCTTTCTCATATCTCTGTATTTCGA
                          ✈
472 AACCTTGGTTTGGTTCTAGATTGCACACCTCTGTTCTAAAAATTATTC

520 TTGTTAGAACTACTATAAACCAATAATCAATGTGTTGTTAGTGTTTTC
                       ✈
568 GTTGGTGGTATCATCTGGTTTATTTTGTGTGTTGATTGAGATGTGTTC

616 AACTTTGATGATCAAATAAAAGAGTGTT
```

FIGURE 4.1 cDNA sequence data for cauliflower cytochrome *b5* compiled from four cDNAs. One cDNA spans the region from -95 to 490 bp, two cDNAs span the region from -27 to 580 bp. These cDNAs ended in poly A tails marked by airplanes. The fourth cDNA did not have a poly A tail and spanned the region -21 to 643 bp.

could represent the existence of two almost identical genes. The transversion results in a change from lysine to asparagine at amino acid five.

The cytochrome *b5* encoded by all of these clones is 134 amino acids in length and is 100% identical to the amino acid sequence obtained from the purified cauliflower cytochrome *b5* protein (Figure 4.2). The first five amino acids of the cDNA deduced protein do not appear in the amino acid sequence derived from the purified cauliflower protein. The first six N-terminal amino acids encoded by the cDNA are also not observed in the chicken cytochrome *b5* protein sequence[4], and in this case the sixth amino acid is not a substrate for trypsin digestion.

As mentioned in chapter two, the purification of higher plant cytochrome *b5* to homogeneity was reported three times, from *Catharanthus roseus* microsomes[11], from pea microsomes[12] and, in this dissertation research, from cauliflower microsomes[13]. Examination of the purification table for the *C. roseus* cytochrome shows a purification of 30% and no sequence data was obtained. The pea cytochrome was purified to greater than 75% and the first 21 N-terminal amino acids were sequenced by Edman degradation. This pea sequence has no homology to the first 21 N-terminal amino acids of cDNA deduced cytochrome *b5* protein from cow[5] and insignificant homology to the first 21 N-terminal amino acids of the chicken protein as deduced from the chicken cDNA sequence[4] (Figure 4.3A), as determined by the amino acid homology search program, NBRF-PIR, of the Hitachi PROSIS Protein Sequence Analysis System. In the first 21 N-terminal amino acids, the bovine and the chicken

```
                        10         20         30         40
cDNA DEDUCED  MASEKKVLGFEEVSQHNKTKDCWLIISGKVYDVTPFMDDHPGGDEVL
              :::::::::::::::::::::::::::::::::::::::::::::
PROTEIN            KVLGFEEVSQHNKTKDCWLIISGKVYDVTPFMDDHPGGDEVL

                  50         60         70         80         90
                 LSSTGKDATNDFEDVGHSDTARDMMEKYYIGEIDSSTVPATRTYVAP
                                          ::::::::::.::.::
                                          YYIGEIDSSXVPXTR

                    100        110        120        130
                   VQPAYNQDKTPEFMIKILQFLVPILILGLALVVRQYTKKE
```

FIGURE 4.2 Comparison of the amino acid sequence of cauliflower cytochrome *b5* derived from the coding region of the cDNAs and from the actual protein, : represents a perfect match.

A

```
                  10        20
      PEA     ALLQEDEAIDDFDDGALDDDG
               .. ..:: ..    :
   CHICKEN     MVGSSEAGGEAWRGRYYRLEEVQKHNNSQS
                        .:: :::.:::::::.:
      COW           MAEESSKAVKYYTLEEIQKHNNSKS
```

B

```
      PEA     ALLQEDEAIDDFDDGALDDDG
                             10        20        30
CAULIFLOWER             MASEKKVLGFEEVSQHNKTKDCWLIISGKVY
                         :   .   .::: .::.... :.:.  ..:
   CHICKEN     MVGSSEAGGEAWRGRYYRLEEVQKHNNSQSTWIIVHHRIY
                        .:: :::.:::::::.:::.:.:...:
      COW           MAEESSKAVKYYTLEEIQKHNNSKSTWLILHYKVY

              40        50        60        70
DVTPFMDDHPGGDEVLLSSTGKDATNDFEDVGHSDTARDM
:.: :.:.::::.::: . .: :::..:::::::..::..
DITKFLDEHPGGEEVLREQAGGDATENFEDVGHSTDARAL
:.:::::::::::::::::::::::::::::::::::::.:
DLTKFLEEHPGGEEVLREQAGGDATENFEDVGHSTDAREL

              80        90       100       110
MEKYYIGEIDSSTVPATRTYVAPVQPAYNQDKTPEFMIKI
 :.. :::..... :      .  .    . . ..     :
SETFIIGELHPDDRPKLQKPAETLITTVQSNSSSWSNWVI
:.:::::::::::::.:. ::.:..:::..::.: :.:::.:
SKTFIIGELHPDDRSKITKPSESIITTIDSNPSWWTNWLI

          120
LQFLVPILILGLALVVRQYTKKE
  . .  :. :
PAIAAIIVALMYRSYMSE
:::.:..:::.:. : ::
PAISALFVALIYHLYTSEN
```

FIGURE 4.3 Comparison of cytochrome *b5* amino acid sequences from various organisms, : represents a perfect match, . represents a match in amino acid type.

cDNA deduced amino acid sequences are 71.4% similar. By contrast, the cauliflower cytochrome *b5* N-terminal sequence is 45.5% similar to the chicken N-terminal, 40% similar to the cow N-terminal. There is 100% similarity to both the chicken and cow proteins in short regions surrounding the heme binding histidines at positions 39 and 63. Translation of the pea N-terminal could begin at a site 5' on the mRNA to that of the start sites for the other cytochrome *b5* N-terminals. This would result in a longer protein with an N-terminal which does not match the N-terminals of other cytochrome *b5* proteins. Since the whole pea sequence is not available, a definitive statement on whether this sequence represents an actual cytochrome *b5* sequence cannot be made. However, since there is no homology even between the two plant N-terminal sequences from cauliflower and pea and since the pea protein is 75% pure, the possibility arises that the pea sequence might be due to the collection of a contaminating protein rather than the cytochrome *b5* during the preparatory HPLC chromatography prior to amino acid sequencing.

As determined by the Hitachi PROSIS Protein Analysis System, the cauliflower protein has a Chou, Fasman, Rose secondary structure prediction (Figure 4.4) and Rose hydropathy prediction (Figure 4.5) very similar to those of the cow and chicken proteins. Although the amino acid sequence is not highly similar in the carboxy-terminal portion of these proteins, all three of these cytochromes have a hydrophobic carboxy-terminal with helical and sheet structures which allow the carboxy-terminal to anchor the proteins in the membrane. This conservation of form rather than sequence content is described as a "topogenic determinant" by Bendzko *et al.*[14]. The

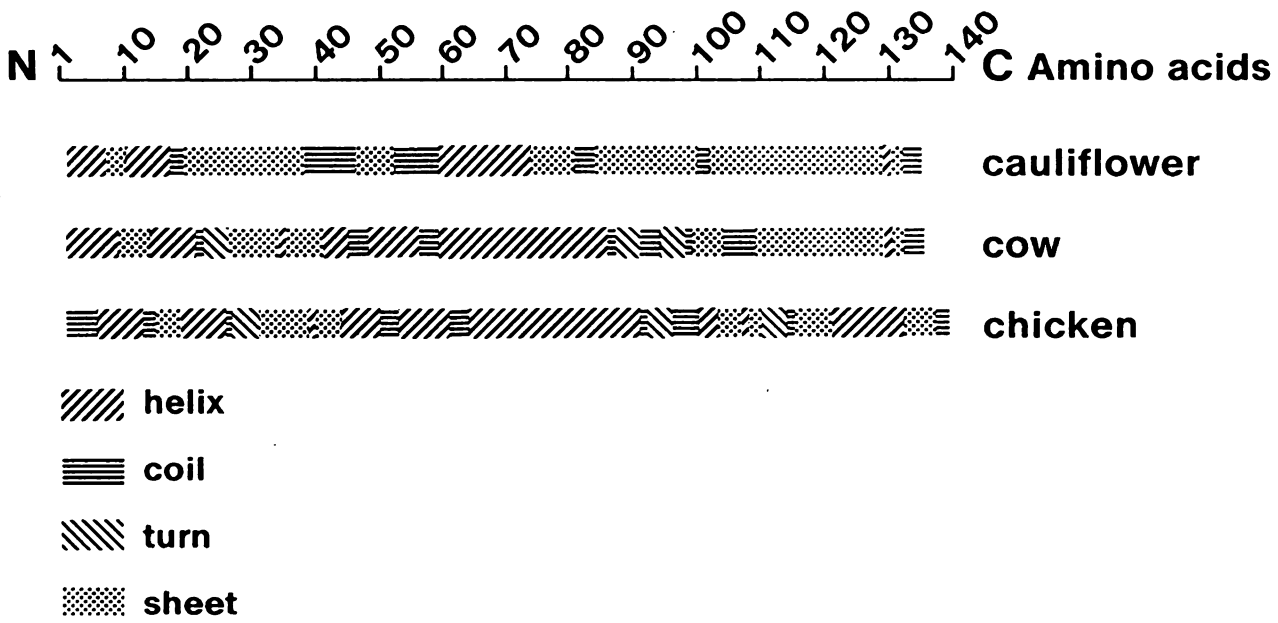

FIGURE 4.4 Chou, Fasman, Rose secondary structure predictions for cytochrome *b5* proteins from cauliflower, chicken, and cow. These predictions are based on the idea that a turn is an area of minimal hydropathy.

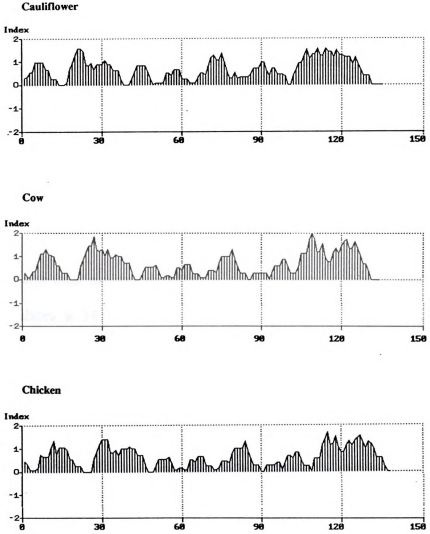

FIGURE 4.5 Rose hydropathy predictions for cytochrome *b5* from cauliflower, chicken, and cow. These predictions are based on the free energy of transfer from an aqueous solution to an organic solution. The hydropathy index of each residue is positive, with the more positive being more hydrophobic.

isoelectric points (pI) of these proteins predicted on the basis of the pKa of charged amino acids and the carboxyl and amino terminal pKas are 4.7, 4.8, and 4.9, for the cauliflower, cow, and chicken proteins, respectively. Harr plots comparing the cauliflower protein with cow and chicken proteins show striking homology when the standard equivalence groups, K=R, D=E, and V=L=I, are used (Figure 4.6A and 4.6B). Based on the obvious similarities in protein character as well as the ß-peak of the cauliflower protein (Figure 2.1) and the amino acid sequence homolgy to that of chicken and cow in regions of importance such as those surrounding the heme binding histidines, the cauliflower protein is the first authentic cytochrome *b5* to be purified to homogeneity and sequenced in higher plants.

The *A. thaliana fad2* mutant deficient in endoplasmic reticulum Δ12 desaturation activity translates a 16.1 kD protein which is recognized by IgG raised against cauliflower cytochrome *b5* (Figure 4.7). The possibility that *fad2* is a point mutation which allows translation of a nonfunctional cytochrome *b5* protein has not been ruled out. However, the rolling ball model of interaction described by Burch *et al.*[15] implies that very few, if any, amino acid residues are absolutely required for a functional cytochrome *b5* protein. Even a mutation in one of the heme coordinating histidines might result in a five coordinate heme which would still transfer electrons[1]. There are 32 possibilities for a nonsense mutation resulting a truncated protein. Thus, the probability of a point mutation resulting in a protein translated to the correct size yet nonfunctional is low. Therefore, the reduced Δ12 desaturation of the *fad2* mutant probably is not due to a lesion in cytochrome *b5*. Until an obvious mutant in

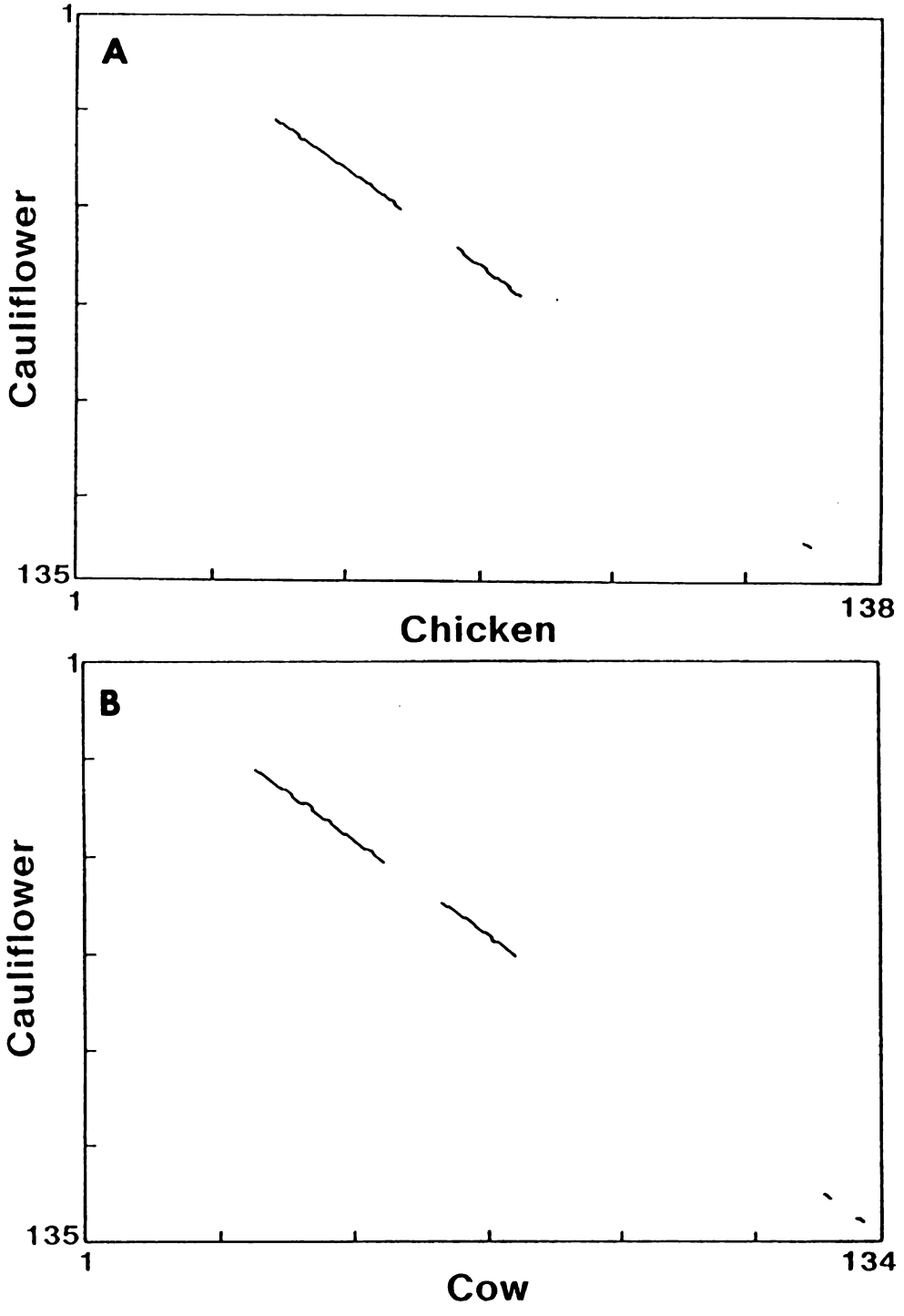

FIGURE 4.6 (A) Harr plot of maximum amino acid homology for cytochrome *b5* from cauliflower and chicken. (B) Harr plot of maximum amino acid homology for cytochrome *b5* from cauliflower and cow. The parameters were V=L=I, K=R, D=E, 6 matches out of 9 amino acids.

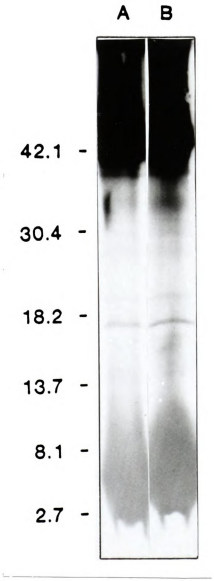

FIGURE 4.7 Western blot of 600μg total phenol extracted microsomal protein from (A) the *fad2* mutant of *A.thaliana* var. Columbia and (B) wildtype var. Columbia.

cytochrome *b5* is found, studies of the function of this protein at the whole plant level are limited to transformation of plants with overexpression and antisense constructs based on the cDNA sequence presented in this chapter.

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CHAPTER FIVE
SUMMARY AND PERSPECTIVES

Biochemical Advances

As mentioned in chapter four, cytochrome *b5* has been purified to 75% from pea microsomes and to 30% from *Catharanthus roseus*. Thus, the purification presented in this dissertation is the first purification to homogeneity of cytochrome *b5* from higher plants. Polyclonal antibodies against this protein were raised in mouse. These are the first antibodies to be generated against a higher plant cytochrome *b5*. Ascites IgG was purified on an FPLC protein G column and its ability to block electron flow through cytochrome *b5* was tested using the reduction of exogenous cytochrome *c* via cytochrome *b5* as an assay. In safflower seed microsomes, immune IgG blocked the reduction of exogenously added cytochrome *c* while nonimmune IgG did not, indicating that the immune IgG blocked electron flow through cytochrome *b5*. This immune IgG also blocked Δ12 desaturase in safflower seed microsomes, but did not block the incorporation of ^{14}C-oleoyl-CoA into phospholipids. Phospholipids such as phosphatidylcholine and phosphatidylethanolamine are known to be substrates for Δ12 desaturase and it is not clear whether ^{14}C-oleoyl-CoA itself can serve as a substrate. Therefore, it was important to show that the ^{14}C-oleoyl-CoA could be incorporated into a known substrate in the presence of the IgG. In addition, when the

immune IgG was quenched with soluble purified cytochrome *b5*, it was no longer inhibitory in the Δ12 desaturase assay. Thus, the inhibition is due to IgG which specifically recognizes a cytochrome *b5* idiotype. Addition of solubilized purified cytochrome *b5* alone had no effect on the Δ12 desaturase activity. These immunoinhibition data strongly suggest that cytochrome *b5* is the electron donor to Δ12 desaturase in safflower seed microsomes.

Amino acid sequence of the cauliflower cytochrome *b5* was obtained for the N-terminal from the protein purified in this dissertation (Joe Leykam, Macromolecular Structure Facility, Michigan State University). This N-terminal sequence data was later confirmed by sequencing of tryptic fragments (Pamela Keck, Monsanto, St. Louis, MO). The total amino acids sequenced includes the first 42 amino acids of the mature N-terminal and a second amino acid fragment stretching from amino acid 69 to amino acid 84.

Molecular Genetic Advances

Data included in this dissertation is the first to describe the genetics of cytochrome *b5* in any plant. Four independent cauliflower cDNAs from a λUNI-ZAP XR library of surface meristematic tissue were identified on the basis of hybridization to a 0.2kb cytochrome *b5* probe. This probe was obtained from Pamela Keck (Monsanto, St. Louis, MO), who used oligonucleotides based on the amino acid sequence as Polymerase Chain Reaction (PCR) primers in PCR reactions with *Brassica napus*

cDNA as the template. The cauliflower cDNAs encoded the cytochrome *b5* protein of 134 amino acids. The cDNA deduced protein is 100% identical to the sequenced portions of the purified cauliflower protein and similar to the cytochrome *b5* from cow and chicken in amino acid sequence and in secondary structure, hydropathy, and isoelectric point (pI) predictions.

The *fad2* mutation of *A.thaliana* does not appear to be deficient in the cytochrome *b5* protein, although the ability of the protein to transfer electrons has not been tested.

Perspectives for further Biochemical Research

The antibodies raised in this dissertation research lay the ground work for further studies of electron transport systems utilizing cytochrome *b5* in higher plants. Recently, Frank van de Loo in the Somerville laboratory used these antibodies and the protocol for immunoinhibition described in chapter three to show cytochrome *b5* involvement in fatty acid hydroxylation in castor bean. Further experiments on the involvement of cytochrome *b5* in fatty acid elongation in plants are under way.

It is currently assumed that cytochrome *b5* inserts randomly into higher plant membranes as it does in animal membranes. Using transmission electron microscopy and gold-labelled samples of these antibodies, localization of cytochrome *b5* in higher plant cells is now possible. It would be interesting to see if certain membranes are

targets for insertion in certain cells. For instance, if, in oil producing seeds, the cytochrome *b5* is more abundant in the endoplasmic reticulum where it is needed for lipid biosynthesis, while in epidermis cells it is more abundant in the plasma membrane where it might be involved in light absorbtion. The possibility that prenylation or fatty acid tagging directs this protein to particular membranes in plants can also be tested with cytochrome *b5* purified from plants as described in this dissertation. Cytochrome *b5* protein generated by overexpression of the cDNA in *Escherichia coli* would not be as valuable as the plant derived protein in studying directive tagging because *E. coli*, having no intracellular membranes, will probably not have tagging mechanisms.

Molecular Genetics Perspectives

Further research could be done on the genomic structure of the cytochrome *b5* gene to determine if the cytochrome *b5* gene is highly conserved among higher plants. If the genes are conserved, cytochrome *b5* might be used as an indicator of divergence as cytochrome *c* is used in human genetics.

The plant cytochrome *b5* cDNA sequence first described in this dissertation allows for many interesting experiments which could not be done previously. Large amounts of cytochrome *b5* protein can now be generated by overexpression of the cDNA constructs in *E.coli*. This protein can be used for 2° and 3° protein structure analysis and for investigations of the porphyrin IX heme binding characteristics. Site directed

mutagenesis of the C-terminal region might provide interesting information on the cytochrome *b5* insertion sequence which allows the protein to anchor in membranes without the aid of the signal recognition peptide-docking protein insertion system commonly used for insertion into the endoplasmic reticulum.

The overexpressed protein might also be used in affinity chromatography purifications of plant proteins such as hydroxylases and desaturases which are known to interact with cytochrome *b5*. However, since the electron transfer interactions are transient, traditional methods for affinity chromatography may need to be modified. The overexpressed protein might also be used in reconstituting the Δ12 desaturase system as well as other cytochrome *b5* requiring systems after the other enzymes involved have been purified.

As described in chapter one, cytochrome *b5* is involved in many electron requiring reactions in animal cells. Experiments addressing the different roles of cytochrome *b5* in different cell types are now limited only by the ability to find a promoter which regulates overexpression of the gene or expression of antisense constructs.

Determination of the copy number of the gene in plants by Southern analysis using the cDNA as a probe is important for genetic engineering of the protein in crop plants. If there is one gene per haploid genome, the manipulation of the gene to achieve a recessive phenotype is much easier than if multiple genes need to be altered. An understanding of the regulation of the gene or genes during development

is important for two reasons. First, so that an altered protein can be expressed in the right tissue at the right time, and, second, because alteration of the pattern of expression of the native protein could be used to regulate phenotype. Regulation of the gene transcript can be studied by Northern analysis using the cDNA as a probe. In the long term, genetic engineering of reactions involving cytochrome *b5* should be possible in existing crop plants. It is hoped that the wise use of data presented in this dissertation may someday provide benefit to mankind through applications such as genetic engineering of crop plants.