ISOLATION AND CHARACTERIZATION OF A LIPID AND PLASTOQUINONE DEFICIENT CYTOCHROME b_6-f COMPLEX

By

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PREFACE

I would like to dedicate this thesis to my husband Paul, who helped me to realize my potential as a scientist. His constant encouragement and support have made this possible. I would also like to dedicate this thesis to our daughter Michelle, our greatest collaborative effort.

I wish to express my gratitutde to all the people here at Oklahoma State University for their assistance and friendship through the years. I am especially indebted to my thesis advisor, Dr. Chang-An Yu, and Dr. Linda Yu for their guidance and friendship. It was a long, rocky road, but we finally made it.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER I

GENERAL INTRODUCTION

Photosynthetic Electron Transport

Photosynthesis is the process by which green plants and certain bacteria convert light energy to chemical energy. This energy is used for the synthesis of ATP and the reduction of pyridine nucleotides, which are required for carbon dioxide assimilation. It is hoped that when the detailed reaction mechanisms of the light harvesting system and the carbon assimilation system are known, the information can be used in the design of herbicides, by increasing chemical potency and species specificity, and in improving crop yield, by artificial stimulation of photosynthesis or genetically improving plants.

Photosynthesis takes place in the chloroplasts of higher plants and in the chromataphores of photosynthetic bacteria. Both systems consists of similar components: chlorophyll, caratenoids, quinones, cytochromes, lipids and iron-sulfur proteins. These components are organized into an electron transport chain in which the movement of electrons can be cyclic or non-cyclic (Figure 1). Purple photosynthetic bacteria have a cyclic system. An electron from a bacteriochlorophyll dimer is excited by 870 nm light to bacteriopheophytin. This portion of the electron transport chain is

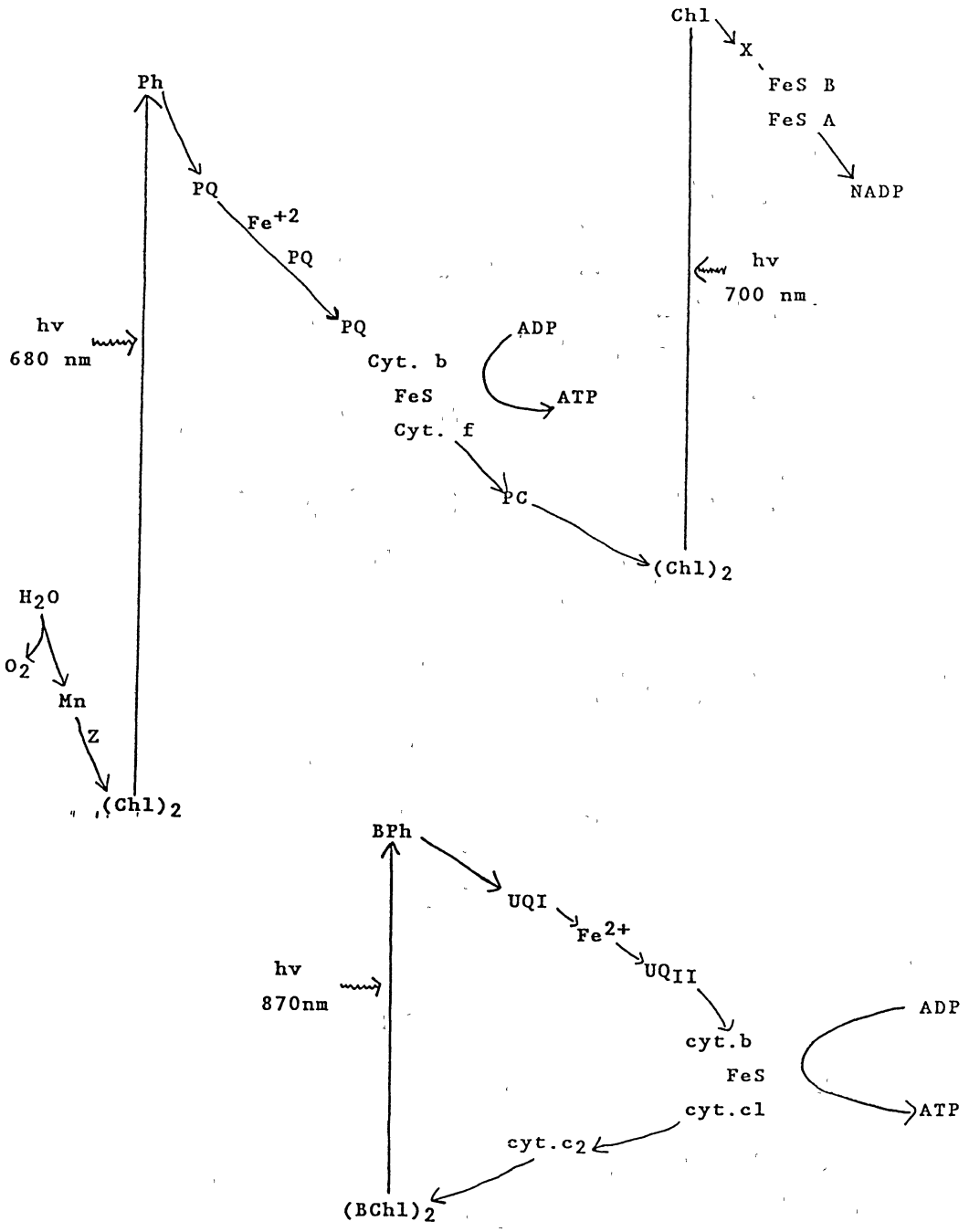

Figure 1: Photosynthetic Electron Transport Chain. Top panel is the electron transfer pathway in higher plants. The bottom panel is the pathway in photosynthetic bacteria like R.sphaeroides. Abbreviations used: Z, uncharacterized electron acceptor; $(Chl)_2$, chlorophyll dimer; Ph, pheophytin; PQ, plastoquinone; cyt.b, cytochrome b; FeS, iron sulfur protein; cyt.f, cytochrome f; PC, plastocyanin; X, uncharacterized electron acceptor; $(BChl)_2$, bacteriochlorophyll dimer; BPh, bacteriopheophytin.

known as the photosystem. The electron is then transferred through a series of electron acceptors, which includes ubiquinone, b and c type cytochromes and an iron-sulfur protein. This converts light energy into chemical energy, which can be used for ATP synthesis. The final electron transfer is back to the bacteriochlorophyll dimer to complete the cycle.

The photosynthetic electron transport chain of higher plants is much more complex. The chain begins with water being split to O_2, with the transfer of electrons through a series of unidentified compounds to photosystem II. The electron donor to photosystem II is thought to be a chlorophyll dimer, which, when excited by 680 nm light, transfers electrons through a series of electron acceptors which includes: plastoquinone, b-type cytochromes, cytochrome f, an iron-sulfur protein and plastocyanin. It is in this region that ATP is formed. The electron is then transferred to a chlorophyll-protein complex which serves as the electron donor to photosystem I. The electron is excited by 700 nm light to ferredoxin, where it is transferred through a flavoprotein to reduce NADP to NADPH. This flavoprotein may also be capable of transferring electrons to plastoquinol, forming a cyclic system around photosystem I.

Quinone Binding Proteins

A common component of the photosynthetic electron transport chains, as well as the mitochondrial electron transport chain, is the quinone compound. For years, ubiquinone was believed to be a mobile molecule which shuttles electrons between electron transfer complexes (1). In recent years, however, evidence that at least a portion of

the quinone is bound to protein has been accumulating (2). One of the first indications of a quinone:protein interaction was the detection of a ubisemiquinone radical. An EPR signal with a g-value of 2.00 and a band width of 10-15 G was found in mitochondrial particles by Raikhman and Blyumenfel'd (3) which was substrate and protein dependent. Inhibition studies on this EPR signal localized the generation site between malonate or amytal and the antimycin A-sensitive site of the NADH or succinate oxidase systems, respectively. In an attempt to determine whether this EPR signal was due to flavin or ubiquinone, the ubiquinone was extracted from the mitochondria, causing a decrease in the EPR signal. Reincorporation of the ubiquinone caused a restoration of the signal. These studies showed that at least part of the EPR signal was due to ubisemiquinone. A later study by Backstrom et. al. (4) on submitochondrial particles demonstrated that ubisemiquinone formation requires a pH range of 6.5-8.5. They also found that the band width can be used to distinguish the ubisemiquinone radical (10G) from the flavin quinone radical (12G). Konstantinov and Runge (5) observed two types of ubisemiquinone radicals in submitochondrial particles by adjusting the oxidation-reduction potential using a fumarate/succinate couple and observing the power saturation behavior. They suggest that the two ubisemiquinone radicals are localized at different sides of the mitochondrial membrane. The detection of a ubisemiquinone radical implies a specific quinone:protein interaction, as the equilibrium constant for the formation of ubisemiquinone is far too low for any detectable amount of ubisemiquinone to be formed unless it is stabilized by interaction

with protein. Further studies on the ubiquinone radical EPR signal in the purified b-c_1 complex (6) showed that the radical formation is concurrent with the reduction of cytochrome b after the reduction of cytochrome c_1 (7). The ubiquinone radical was also found to require phospholipid and to be diminished by the addition of antimycin A, theonyltrifluoroacetone and chymotrypsin (8). Electron transport is required for generation of the ubiquinone radical, so the loss of the ubiquinone radical EPR signal on addition of electron transport inhibitors, such as antimycin A, is reasonable. The decrease in the signal upon addition of a protease, such as chymotrypsin, indicates a dependance of the signal on protein. The ubisemiquinone was further characterized (9) in terms of its midpoint potential (+67 mV at pH 8.0) and pH optimum (pH 9.0).

Further evidence for the existence of ubiquinone binding proteins came from a more traditional biochemical approach. Attempts to isolate an active succinate-cytochrome c reductase complex yielded many preparations (9-13) that varied widely in their purity, activity and quinone content. It was found that a minimum stoichiometry of 1 mole quinone per mole of cytochrome c_1 was needed for maximum activity (14). When the stoichiometry fell below 1:1, maximum activity could only be attained upon addition of exogenous ubiquinone (15). The specific interaction of ubiquinone with protein is further illustrated by the fact that phospholipid can be removed from the succinate-cytochrome c reductase with two ammonium sulfate precipitations, whereas the ubiquinone requires five ammonium sulfate precipitations for removal (14,15). Full restoration of enzymatic activity can only be attained when the ubiquinone is added to the

delipidated enzyme prior to the addition of phospholipids.

Attempts at identifying the quinone-binding proteins in the mitochondrial system began when it was discovered that purified succinate dehydrogenase (16-18) could be reconstituted with soluble cytochrome b-c_1 complex (16) to form an active succinate cytochrome c reductase (6). The succinate dehydrogenase could not be reconstituted with complex III (19,20). It was therefore assumed that there was a component in the soluble cytochrome b-c_1 complex that was not present in the complex III preparations. This protein component, named QPs, was found to bind ubiquinone and converts soluble succinate dehydrogenase into succinate ubiquinone reductase (21). The QPs has been isolated by many groups with various stages of activity and purity (21-25).

From EPR studies of complex I (26) and complex III (7), it is believed that a ubiquinone-binding protein exists in each complex and they are referred to as QPn and QPc, respectively. One method for identifying the quinone-binding protein was the use of photoaffinity ubiquinone derivatives. The first type of derivative used for the identification of QPc was Q_0C_{10}NAPA (27), which has the light-activated azido-group located on the end of the isoprenoid side-chain of ubiquinone (Figure 2). When the ^{14}C-labeled Q_0C_{10}NAPA was incubated with delipidated cytochrome b-c_1 III complex (28) and illuminated, the label was found in the Mr=37000 (cytochrome b) protein and the Mr=17000 protein (29). This label was further characterized with respect to phospholipid and inhibitor effects (30). The binding of Q_0C_{10}NAPA was found to be phospholipid dependent and binds to a different site than antimycin A or 5-n-

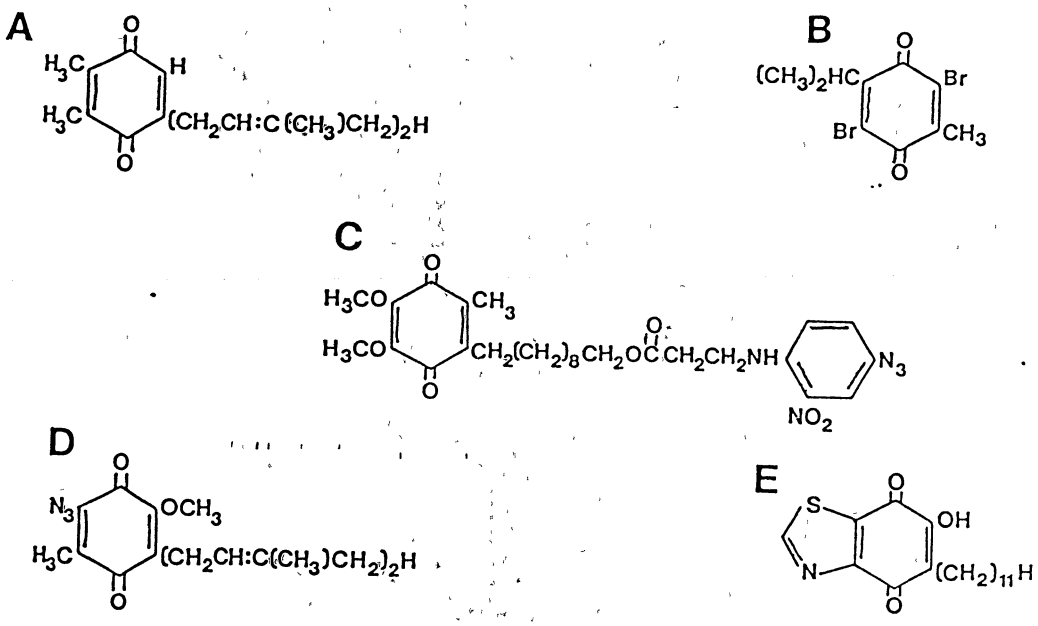

Figure 2: Structures of Quinone Compounds and Inhibitors. A = plastoquinone-2; B = DBMIB; C = $Q_0C_{10}NAPA$; D = Axido-Q; E = UHDBT.

undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT). A similar study on succinate ubiquinone reductase using Q_0C_{10}NAPA identified the Mr=17000 and Mr=15000 proteins as the quinone binding protein (QPs) (31).

The specificity of the Q_0C_{10}NAPA was questioned (32,33) since the azido-group on the end of the side chain was so far from the benzoquinone ring. This sparked efforts to place a light-activated azido-group on the benzoquinone ring. The synthesis of several such derivatives was reported (34). Of the derivatives synthesized, 3-azido-2-methyl-5-methoxy-6-(3,7-dimethyloctyl)-1,4-benzoquinone was found to be the most suitable for photoaffinity labeling studies (35). An extensive study on the binding of this ^{3}H-azido-quinone derivative revealed the Mr=37000 (cytochrome b) and Mr=17000 proteins were heavily labeled. The labeling in the Mr=17000 protein was proportional to enzyme inactivation and was affected by the presence of phospholipids. This would indicate that the Mr=17000 protein is responsible for quinone-binding in the ubiquinol cytochrome c reductase segment of the electron transport chain (35). It was also determined that the inhibitors, antimycin A, 5-n-undecyl-6-hydroxy-4,7-dioxobenzothiazole and n-heptyl-4-hydroxyquinoline-N-oxide, do not act on the quinone-binding site.

A similar study was done on yeast mitochondrial ubiquinol cytochrome c reductase (36). In the yeast mitochondria, the azido-quinone was also found on the cytochrome b and an Mr=14000 protein (37). The labeling of the protein corresponds to inactivation of the enzyme. In contrast to the bovine heart mitochondria (35), both labeled proteins in the yeast were affected by the presence of

phospholipid.

The cytochrome d terminal oxidase complex of Escherichia coli (38) functions by oxidizing ubiquinol and reducing oxygen to water, which generates a transmembrane charge separation (39). Use of the tritiated azido-quinone allowed identification of subunit I of the cytochrome d complex as the ubiquinol-binding site (40). Although some label was found in subunit II, the binding did not correlate with enzyme inactivation.

The involvement of quinones in photosynthetic systems has also been established (41,42). Detection of a semiquinone anion in reaction centers, chromatophores and chloroplasts indicates a specific quinone:protein interaction analogous to the quinone-binding proteins in the mitochondria (43,44). Photoaffinity labeling studies have identified the M subunit of Rhodobacter sphaeroides (formerly referred to as Rhodopseudomonas sphaeroides) reaction centers as the primary quinone-binding site (45). Indirect evidence suggests that the H subunit is the secondary quinone-binding site (42). The isolation of the cytochrome b-c_1 complex from the photosynthetic bacteria Rhodobacter sphaeroides R-26 (46) made it possible to identify the quinone-binding protein that is analogous to the QPc of the mitochondrial system. Using the photoaffinity labeled azido-quinone compound, the cytochrome b and the Mr=12000 protein were identified as the quinone-binding proteins (47). The binding of azido-quinone to the Mr=12000 protein is affected by phospholipid more than the cytochrome b.

The role of plastoquinone in higher plants appears to be similar to that of ubiquinone in the photosynthetic bacteria. The primary

acceptor in photosystem II has been identified as a plastosemiquinone anion (48,49) and is believed to be bound to protein (50). Reconstitution experiments identified plastoquinone as the secondary electron acceptor and photoaffinity labeling experiments identified the Mr=32000 protein as responsible for quinone-binding (51). The isolation of a cytochrome b_6-f complex (52) opened the door to investigation into the existence of a plastoquinone-binding protein analogous to the QPc of the mitochondrial system. The use of an arylazido-plastoquinone derivative identified the cytochrome b_6 and the iron-sulfur protein as the plastoquinone-binding proteins (53). A similar labeling pattern was obtained using an azido analog of the inhibitor 2-iodo-2',4,4'-trinitro-3-methyl-6-isopropyl-diphenylether (DNP-INT) which is thought to inhibit plastoquinone oxidation (54). These experiments lack any correlation to enzyme activation and are performed in the presence of exogenous lipid (1% lipid present). The presence of lipid in these enzyme complexes has been shown to interfere with quinone binding in other systems (35,37,40). Further investigation into the identity of the plastoquinone-binding protein is required using a lipid and quinone depleted cytochrome b_6-f complex.

The Cytochrome b_6-f Complex

The cytochrome b_6-f complex from photosynthetic plants shuttles electrons from plastoquinol to plastocyanin between the two photosystems. The first indication that cytochrome b_6 and cytochrome f were part of an enzyme complex came when they were first isolated together on a sucrose density gradient from digitonin-solubilized

chloroplasts (55). Nelson and Neumann (56) reported the isolation of a cytochrome b_6-f particle from photosystem I particles using salt fractionation, Bio-Gel and DEAE column chromatography. This cytochrome b_6-f particle was found to contain non-heme iron, phospholipid and 2 b-type cytochromes per cytochrome f. It was later shown to contain cytochrome b_{559} (57,58). Another isolation procedure details a cytochrome b_6-f complex containing cytochromes b_6 and f, non-heme iron and plastocyanin (59). These early preparations were not tested for enzyme activity or polypeptide composition.

The discovery that disrupted chloroplast membranes catalytically stimulated the reduction of plastocyanin by plastoquinol (60) was the first direct evidence of a plastoquinol-plastocyanin oxidoreductase activity in chloroplasts that is analogous to the mitochondrial ubiquinol cytochrome c reductase activity. It was determined that the cytochrome b_6-f complex was responsible for this activity. In testing previous cytochrome b_6-f complexes (55,56,59), it was found that they were active, but impure. Attempts at that time to further purify the cytochrome b_6-f complex left the enzyme inactive.

It was not until 1981 that a procedure for the isolation of a pure, active cytochrome b_6-f complex was reported (52). This complex was found to contain 5 polypeptides with Mr=34000,33000,23500,20000 and 17500. The complex was isolated from spinach chloroplasts by octylglucoside/sodium cholate solubilization, followed by ammonium sulfate fractionation and sucrose gradient ultracentrifugation (52). When the enzyme is isolated in the absence of triton X-100, it had an activity of 40 nmoles of cytochrome c_{552} reduced per nmole of cytochrome f per hour (61). The enzyme was found to be sensitive to

electron transfer inhibitors such as DNP-INT, DBMIB and UHDBT, but showed little inhibition by antimycin A, HQNO or myxothiazol, which are potent inhibitors of mitochondrial electron transfer (63).

The three larger subunits of the cytochrome b_6-f complex (Mr=34000, 33000 and 23500) were found to contain heme. After further investigation, the Mr=34000 and 33000 were assigned to a heterogeneous cytochrome f, while the Mr=23500 subunit is believed to be cytochrome b_6 (61). The Rieske-type iron sulfur protein has Mr=20000 (73). The identity of the Mr=17500 subunit is, as yet, unknown. This subunit has been shown to contain a great deal of sequence homology with the carboxyl terminal of the mitochondrial cytochrome b (62), and it has been suggested to be responsible for plastoquinone binding in the cytochrome b_6-f complex (52,62). The stoichiometry of the subunits was originally reported as 1:1:1:1 (52) for cytochrome f, cytochrome b, iron-sulfur protein and Mr=17500 protein, respectively, but was late corrected to 1:2:1:2 (61).

Since the isolation of the cytochrome b_6-f complex (52), much time has been spent trying to improve the isolation procedure (83,84,85). One such procedure uses protease inhibitors and affinity chromatography to isolate a cytochrome b_6-f complex with an extra polypeptide (Mr=37000) on electrophoresis (83). This complex was obtained in higher yields and with less chlorophyll contamination than the previously reported method (52,61), but it was later determined that the extra polypeptide was ferredoxin:NADP oxidoreductase (86). Another procedure started with the cytochrome b_6-f complex prepared by the method of Hurt and Hauska (52,61) and applied it to a second sucrose gradient ultracentrifugation in an

attempt to purify a lipid and quinone depleted cytochrome b_6-f complex (84). The major problem with this procedure is that only small sample volumes can be processed and the long centrifugation times (up to 72 hours) may cause sample denaturation.

The cytochrome b_6-f complex from plants shows a great deal of similarities to the cytochrome b-c_1 complex from mitochondria and photosynthetic bacteria (63,68). The common subunits of these oxidoreductase complexes are cytochrome b, cytochrome f or c_1, the Rieske-type iron sulfur protein and a small molecular weight polypeptide. The molecular weight of the b-type cytochromes range from Mr=23500 in spinach chloroplasts (52,61) and Anabaena variabilis (64), to Mr=30000 in the mitochondrial from yeast (36) and N.crassa (64), to Mr=40000 (66) or 48000 (15,67) in R.sphaeroides. Bovine heart mitochondrial cytochrome b has been reported to have a molecular weight ranging from Mr=30000 to 37000 (6,16,69). Studies on the gene sequence of the b cytochromes indicate that the apparent molecular weight is 42000 in yeast (70) and bovine heart mitochondria (71), and 23500 in chloroplasts (62). The mitochondrial b-cytochromes were found to have a great deal of sequence homology while the chloroplast b-cytochrome shows homology with the amino terminal of the mitochondrial cytochrome b (62).

The cytochrome c_1 or f from various sources appears to be very similar with apparent molecular weights of 29000 to 34000 (63). Cytochrome f from spinach was reported to be heterogeneous (61), possibly due to proteolysis during isolation. The iron-sulfur protein also shows similar molecular weights from the various sources (Mr=20000 to 25000). Each of these enzyme complexes also contains a

low molecular weight subunit that does not contain heme (63). Evidence is mounting that this low molecular weight protein is responsible for the binding of quinones (35,37,40). The only major difference between the mitochondrial and photosynthetic enzymes is the presence of two core proteins in the mitochondrial enzymes (69,72). The absence of these core proteins in the functionally active photosynthetic oxidoreductases indicates that these proteins are not necessary for enzymatic activity. They may, however, be required for structural integrity.

Since the isolation of an active cytochrome b_6-f complex from spinach was reported (52), extensive studies on the enzyme have been reported. The Rieske-type iron sulfur protein has been isolated from the complex (73). The isolated protein, with an apparent molecular weight of 20000, retained its EPR characteristics. The study also showed that, although DBMIB drastically alters the EPR spectra of the iron sulfur protein in the complex, the inhibitor has no effect on the isolated protein (73). This would indicate that the DBMIB can not act on the iron-sulfur protein alone, but rather with a closely associated subunit.

The isolated cytochrome b_6-f complex was found to contain a bound plastoquinone (52,61). The complex, isolated in the presence of triton X-100, was found to contain only 0.4 moles of plastoquinone per mole of cytochrome f, and had a maximal turnover of cytochrome f of 14 sec^{-1} (52). When the enzyme is isolated in the absence of triton X-100, the enzyme contains only 1 mole of plastoquinone per mole of cytochrome f and has a maximal turnover of cytochrome f of 60 sec^{-1} (61). The bound plastoquinone could be removed from the

cytochrome b_6-f complex by acetone extraction (74). Removal of the plastoquinone caused the oxidant induced reduction of the cytochrome b_6 to be lost, but reconstitution with either plastoquinol-1 or plastoquinol-9 restored the oxidant induced reduction of cytochrome b_6 (74). This suggests that bound plastoquinone is involved in the oxidant induced reduction of cytochrome b_6.

The isolated cytochrome b_6-f complex can be incorporated into lipid vesicles where it functions in the reduction of external plastocyanin by plastoquinol (75). This reduction of plastocyanin can be greatly stimulated by the addition of uncouplers (75). One proton per electron is ejected from the vesicle, in addition to the scalar proton liberated by the plastoquinol. The ejection of a proton is stimulated by the addition of valinomycin and K^+, and abolished under uncoupling conditions. The formation of a membrane potential was observed during the reaction, further indicating that the isolated cytochrome b_6-f complex can function as an electrogenic proton translocator.

The effect of inhibitors can be a useful tool in determining the mechanism of electron transfer. DNP-INT has been shown to block the reduction of cytochrome b by a semiquinone (76,77) in the cytochrome b_6-f complex. DNP-INT has also been reported to be required for the reduction of cytochrome b by ferredoxin in a cyclic electron transport chain (78), via a plastosemiquinone intermediate. Since DBMIB does not stimulate this cyclic electron transfer, it is believed to have a different binding site. The binding site for DBMIB has been suggested to be on the iron sulfur protein based on EPR studies (73).

UHDBT has also been found to be a potent inhibitor of the cytochrome b_6-f complex (52). It is believed to interact with the iron sulfur protein, based on EPR studies (79-81). It acts by blocking the formation of semiquinone from quinol and inhibits oxidant-induced reduction of cytochrome b (77,82).

Statement of Problem

A common component of the electron transport chains from the mitochondrial and photosynthetic systems is the quinone compound. There is extensive evidence that indicates that at least a portion of the quinone present in the photosynthetic and mitochondrial membranes is bound to protein. The existence of specific plastoquinone-binding proteins in the cytochrome b_6-f complex from spinach chloroplasts is expected, based on the similarities to the mitochondrial cytochrome b-c_1 complex, yet the evidence is still lacking. One study used an arylazido-plastoquinone derivative to show that cytochrome b_6 and the iron-sulfur protein were responsible for plastoquinone binding (53). These findings are somewhat inconclusive in that the enzyme used (52,61) contains bound quinone and excess phospholipid, which can block access of the azido-plastoquinone derivative to the plastoquinone binding site. It has also been reported that the removal of bound plastoquinone does not affect the photoaffinity labeling pattern (63), which indicates that the label is not in the plastoquinone binding site.

In an attempt to provide evidence for the existence of a quinone-binding protein, it is necessary to isolate a lipid and quinone deficient cytochrome b_6-f complex in quantities large enough

to allow for extensive studies. This complex must be capable of reconstituting with plastoquinone and lipid to form a functionally active oxidoreductase. Once this complex has been isolated and characterized, photoaffinity labeling studies can be performed using an azido-plastoquinone derivative that has the light-activated azide-group on the benzoquinone ring. This should provide evidence for the specific subunit(s) involved in plastoquinone binding.

Further evidence for the existence of a plastoquinone binding protein in the cytochrome b_6-f complex can be obtained from EPR studies on the lipid deficient and lipid reconstituted forms of the complex. EPR studies can also indicate changes in the heme environment of the components in the cytochrome b_6-f complex upon changing from the inactive, lipid deficient form to the active, reconstituted form. These EPR spectra can then be compared to those of the cytochrome b-c_1 complex of the photosynthetic bacteria R.sphaeroides.

CHAPTER II

PREPARATION AND RECONSTITUTION OF A PHOSPHOLIPID DEFICIENT CYTOCHROME b_6-f COMPLEX FROM SPINACH CHLOROPLASTS

Summary

A simple, rapid procedure suitable for large scale preparation of a lipid deficient cytochrome b_6-f complex from spinach chloroplasts has been developed. The procedure involves solubilization with a mixture of sodium cholate and octylglucoside, ammonium sulfate fractionation and calcium phosphate column chromatography. The purified complex contains, in nanomoles per milligram protein, 20.6 cytochrome b, 10.8 cytochrome f and 54 phospholipids. The purified complex has little plastoquinol-cytochrome c reductase activity in the absence of added lipid. Full reductase activity was reconstituted by the addition of plastoquinone prior to the addition of lipid.

Introduction

The cytochrome b_6-f complex mediates electron transfer from plastoquinol to plastocyanin in the chloroplast membranes of photosynthetic plants. Several methods have been developed for the isolation of this enzyme complex, at different stages of purity and

various degrees of activity. The requirement of lipids in the preparation of Hurt and Hauska (52,61) was not clear as the complex was purified in the presence of exogenous lipids. The cytochrome b_6-f complex of Clark and Hind (81) was found to contain ferredoxin-NADP reductase (86). Recently, Chain (84) reported the isolation of a quinone-depleted cytochrome b_6-f complex, based essentially on the method of Hurt and Hauska (52,61). This preparation was found to be reconstitutively active upon the addition of exogenous plastoquinone and lipid. While both lipid and plastoquinone are required for reconstitution, no preference for addition sequence of plastoquinone and lipid was observed. The preparation suffers with high residual lipids and plastoquinone, as judged from the high residual activity. The procedure involves long, time-consuming sucrose gradient ultracentrifugation and is unsuitable for large-scale preparations.

In this communication we report a simpler procedure for lipid and plastoquinone depleted cytochrome b_6-f complex. The isolated complex has little plastoquinol-cytochrome c reductase activity in the absence of added plastoquinone and lipid, but the activity can be reconstituted upon addition of exogenous plastoquinone and lipid. The procedure is suitable for large scale preparation of the cytochrome b_6-f complex.

Materials and Methods

Horse heart cytochrome c, type III, sodium cholate, and octylglucoside were purchased from Sigma. Calcium phosphate was prepared according to the method of Jenner (87). Plastoquinol-2 (35) and lipids from spinach chloroplasts (88) were prepared by the

reported methods. Lipids were dried under nitrogen and dispersed in 1% sodium cholate by sonication prior to their use.

Cytochromes b_6 and f were determined as reported (52). Enzymatic activity was determined spectrophotometrically at 25°C in a Cary spectrophotometer, model 219. The assay mixture contained in 1 ml: 20 mM MES buffer, pH 6.2, 10 uM cytochrome c, 20 uM plastoquinol-2 and cytochrome b_6-f complex, as indicated in the figure legends. The change in absorbance at 550 nm was followed with time. Protein was determined by the method of Lowry (89). Sodium dodecyl sulfate polyacrylamide gel electrophoresis was by the method of Weber and Osborn (90).

Results And Discussion

Purification of the cytochrome b_6-f complex

Spinach chloroplasts, which were prepared as in (52) with the omission of the second NaBr wash, were solubilized using the conditions reported by Hurt and Hauska (52). The 300,000 x g supernatant was brought to 40% ammonium sulfate saturation and the pellet was removed by centrifugation at 40,000 x g for 10 minutes. The resulting supernatant was brought to 65% ammonium sulfate saturation and the resulting pellet was collected and resuspended in30 mM Tris-succinate, pH 6.5, containing 1% sodium cholate. The sample was diluted to 40 uM cytochrome f after a short dialysis (one hour) to remove excess salt. The diluted sample was then applied to a calcium phosphate:cellulose (3:1) column (3 x 10 cm), equilibrated with 50 mM potassium phosphate buffer, pH 8.0, containing 1 % sodium cholate. The column is washed with three column volumes of the

equilibrating buffer and eluted with 0.2 M potassium phosphate buffer, pH 8.0 containing 1 % sodium cholate. The eluted sample was subjected to ammonium sulfate fractionation. The purified plastoquinone and lipid depleted cytochrome b_6-f complex was recovered in the pellet formed between 25% and 35% ammonium sulfate saturation. The pellets were collected and resuspended in 30 mM Tris-succinate buffer, pH 6.5, containing 1% sodium cholate and 10% glycerol and stored at -70°C until use.

A summary of the purification data is given in Table I. This procedure yields 28% of the cytochrome f in the solubilized chloroplasts. This yield can be improved if the final ammonium sulfate saturation is increased to 45%, but this can decrease the purity of the isolated complex.

Properties of the Cytochrome b_6-f complex

As indicated in Table I, the purified cytochrome B_6-f complex contains 20.6 nanomoles of cytochrome b, 10.8 nanomoles of cytochrome f and 54 nanomoles of phospholipids (based on total phosphorus) per milligram of protein. The phospholipid represents only 4.2% of the total complex, which is quite different compared to other isolated lipid-protein complexes. The active lipid-protein complexes generally contain about 20% phospholipid. The low absorption in the 600-700 nm region and the 450-500 nm region of the spectra indicates very little chlorophyll or carotenoid contamination in the isolated complex (Figure 3). The cytochrome f is partially reduced in the purified complex as seen by the peak at 554 nm in the spectra of the isolated complex. The cytochrome peaks at 563 nm for cytochrome b_6

TABLE I

SUMMARY OF PURIFICATION DATA

Treatment	Volume	Protein	Activity	PL*	Cytochrome f	
					Conc.	Recovery
	ml	mg/ml	units**	%	uM	%
300,000 x g Supernatant	338	2.2	9.2	41	3.0	100
$AmSO_4$, 45-65% Sat'n	7.2	35.0	5.2	28	126	90
$CaPO_4$ Eluant	86.4	0.7	1.7	19	6.3	54
$AmSO_4$, 25-35% Sat'n	6.0	4.3	0.6	4	46.9	28

*Based on total phosphorus
**Units of umoles c reduced/nmole f/hour

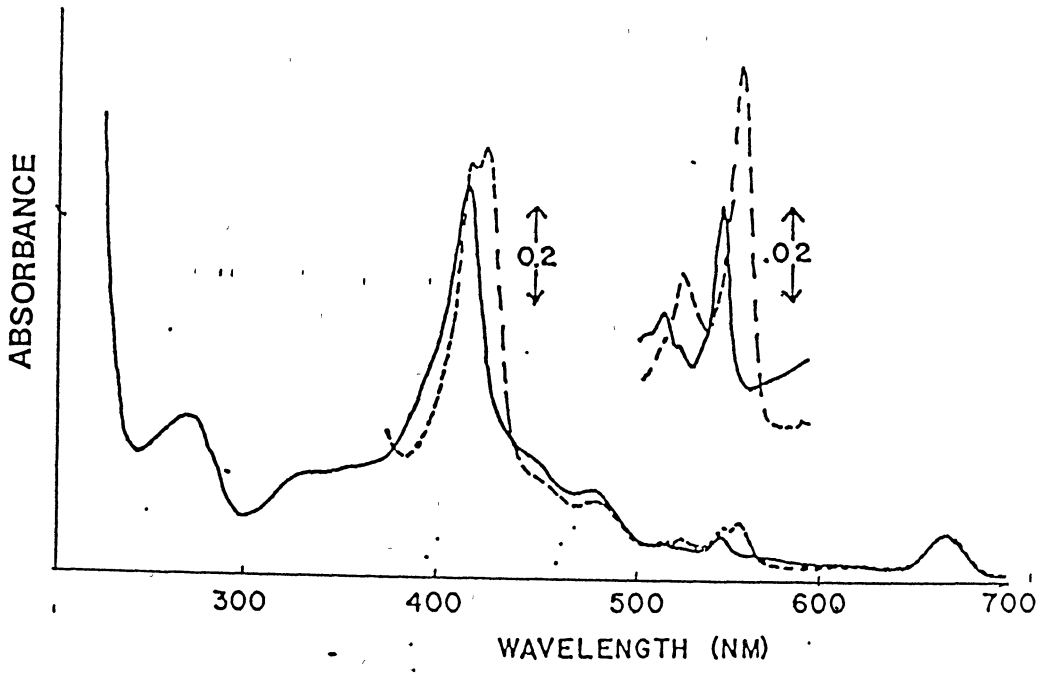

Figure 3: Absorption Spectra of the Cytochrome b_6-f Complex. Diluted cytochrome b_6-f complex, 0.2 mg/ml in 30 mM Tris-succinate, pH 6.5, was used. Solid lines (—) represent the purified complex as prepared and the dashed lines (- -) represent $Na_2S_2O_4$ reduced enzyme complex. The inset represents a difference spectra of the same sample. The solid line is ascorbate reduced minus $K_3Fe(CN)_6$ oxidized. The dashed line is dithionite reduced minus ascorbate reduced.

and 554 for cytochrome f correspond to those previously published (52). The purified complex shows five protein bands on sodium dodecyl sulfate polyacrylamide gel electrophoresis, with molecular weights of 34000, 33000, 23000, 20000 and 17500. These molecular weights for the protein subunits are very similar to those reported by Hurt and Hauska (52). The lack of a 38000 molecular weight subunit indicates that this preparation is not contaminated with ferredoxin-NADP reductase.

The purified cytochrome b_6-f complex shows little plastoquinol-cytochrome c reductase activity in the absence of added plastoquinone and lipid. The addition of exogenous lipid and plastoquinone restores the enzymatic activity. The activity of the b_6-f complex is highly dependent on the concentration of exogenous plastoquinone. As shown in Figure 4, when lipid concentration is held constant (2 mg/mg protein), a maximal activity is obtained at a level of 2 moles of plastoquinone per mole of cytochrome f. This number is higher than the concentration of 1 mole of plastoquinone per mole of cytochrome f obtained from the quinone-depleted complex reported by Chain (84). Reconstitutive activity is also dependent on lipid concentration, as shown in Figure 5. Maximum reconstitutive activity is obtained at 2 milligrams lipid per milligram protein. To achieve maximum reconstitutive activity, it is necessary to incubate the enzyme with the plastoquinone and lipid for 90 minutes prior to assay, as can be seen in Figure 6. It is important to note that under the assay conditions described, lipids alone can catalyze a substantial amount of electron transfer from plastoquinol to cytochrome c. This phenomenon may be due to the antimycin A insensitive ubiquinol-

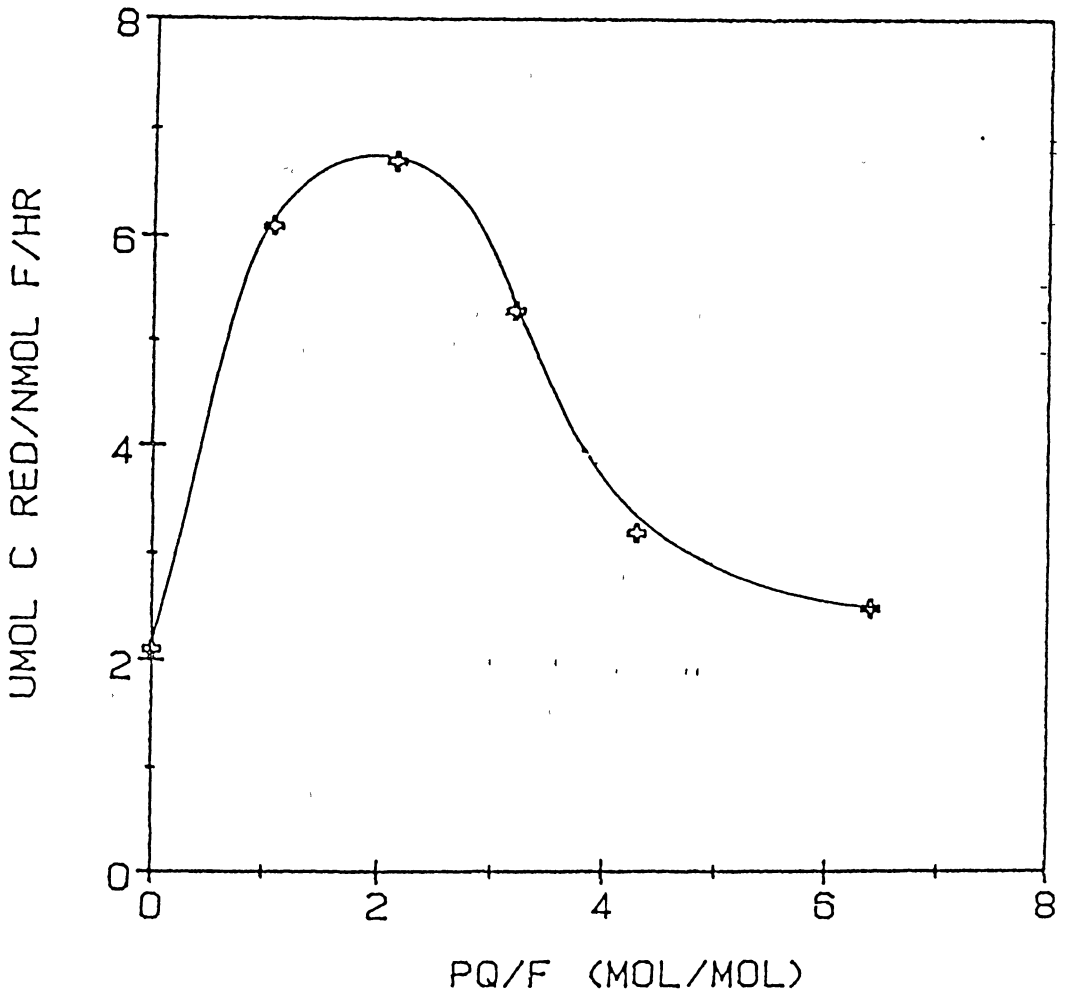

Figure 4: Effects of Varying Plastoquinone Concentration on the Reconstitutive Activity at Constant Lipid Concentration. The cytochrome b_6-f complex was diluted to 8uM cytochrome f. The indicated amount of plastoquinone was added and the sample incubated for 30 minutes. Lipid was then added to a final concentration of 2 mg/ml and the sample incubated for 90 minutes prior to assay.

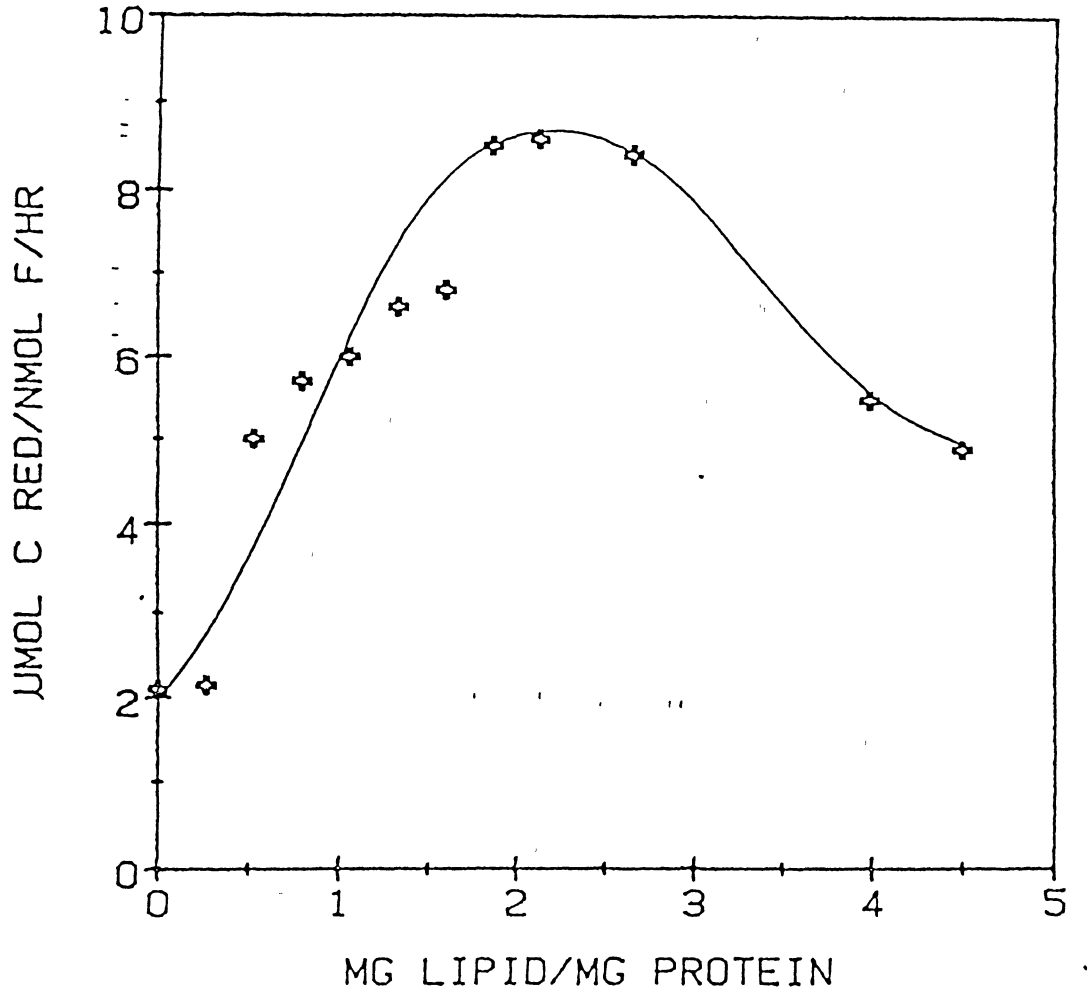

Figure 5: Effect of Lipid Concentration on Reconstitutive Activity at Constant Plastoquinone Concentration. Assay conditions were as in Figure 4, except the plastoquinone was constant at a final concentration of 45 uM, while the lipid concentration was varied as indicated.

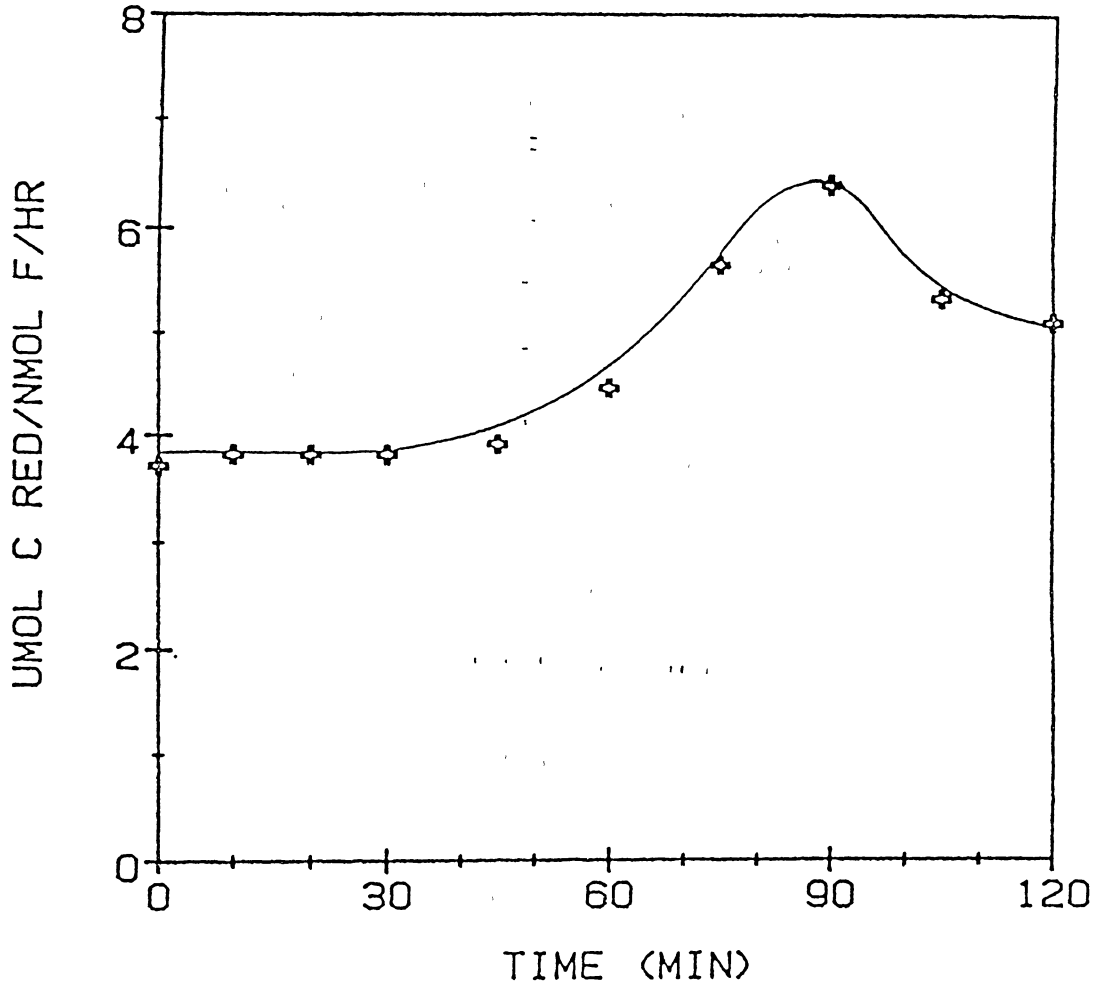

Figure 6: Effect of Incubation Time on Reconstitutive Activity. The cytochrome b_6-f complex was diluted to a final concentration of 45 uM. After a 30 minute incubation, lipid was added to a final concentration of 2 mg/ml. The sample was then assayed for the indicated time.

cytochrome c reductase activity previously described (91). For this reason it is important that a lipid control assay be conducted and the result subtracted from the activity obtained from the reconstituted system.

Table II shows the effect of added exogenous plastoquinone on the delipidated complex. The addition of plastoquinone alone has little effect on the activity. The addition of plastoquinone followed by the addition of lipid restores 93% of the activity present in the original solubilized complex. On the other hand, addition of lipid prior to the addition of plastoquinone shows only partial (58%) restoration of the activity present in the solubilized complex. This is in contrast to the results by Chain (84), whose quinone-depleted complex showed no preference for the order of lipid and plastoquinone reconstitution. It seems reasonable that the addition of plastoquinone prior to the addition of lipid restores more activity, as adding lipid first would most likely block some plastoquinone binding sites. A similar phenomenon has also been observed in the reconstitution of ubiquinone and phospholipid depleted mitochondrial succinate cytochrome c reductase (14).

Electron paramagnetic resonance spectra of the depleted and reconstituted cytochrome b_6-f complex in oxidized and ascorbate reduced forms were compared to the spectral characteristics of the cytochromes b_{563} and f, and the Rieske-type Fe-S clusters reported by Salerno et. al. (92). In the lipid deficient complex, EPR spectra of the cytochrome b_{563}, cytochrome f and the Rieske-type Fe-S show a slight shift to higher field compared to those of the lipid sufficient or the lipid reconstituted preparations. The shift in the

TABLE II

THE RECONSTITUTION OF PLASTOQUINOL-CYTOCHROME c
REDUCTASE BY ADDITION OF PLASTOQUINONE
AND LIPID

Treatment	umol c red/nmol f/hr
Cytochrome b_6-f	1.00
Cytochrome b_6-f + PQ_2	1.37
Cytochrome b_6-f + lipid	3.29
(Cytochrome b_6-f + PQ_2) + lipid	8.54
(Cytochrome b_6-f + lipid) + PQ_2	5.37

The cytochrome b_6-f complex was diluted to 8 uM cytochrome f. Plastoquinone was added to a final concentration of 45 uM, where indicated. Lipid was added to a final concentration of 2 mg/ml, where indicated. The complex was incubated for 60 minutes before assay. Controls containing buffer and the same amounts of PQ_2 and/or lipid were assayed and the activity subtracted.

cytochrome f peak upon reconstitution with lipids is of interest. A similar study on delipidated mitochondrial cytochrome b-c_1 complex showed no shift in the cytochrome c_1 peak upon reconstitution with phospholipid. This phenomenon is under further investigation.

CHAPTER III

IDENTIFICATION OF THE Mr=17500 PROTEIN AS THE PLASTOQUINONE-BINDING PROTEIN IN THE CYTOCHROME b_6-f COMPLEX FROM SPINACH CHLOROPLASTS

Summary

An azido-ubiquinone derivative, 3-azido-2-methyl-5-methoxy-6-(3,7-dimethyloctyl)-1,4-benzoquinone, was used to study the plastoquinone:protein interaction and to identify the plastoquinone-binding protein in the cytochrome b_6-f complex from spinach chloroplasts. When the lipid and plastoquinone deficient cytochrome b_6-f complex is photolyzed in the presence of the azido-ubiquinone derivative, a 40% inactivation of the lipid-reconstituted activity is observed. Optimal conditions for this inactivation are 30 moles azido-ubiquinone per mole of cytochrome f, and a photolysis time of 7 minutes. The photoinactivation of the enzyme activity correlates closely with the amount of azido-ubiquinone incorporation, as determined from the use of the tritiated azido-ubiquinone derivative. The 40% inactivation is in good agreement with that expected based on the amount of plastoquinone deficiency of the isolated enzyme complex. The photolyzed sample is extracted to remove unreacted azido-ubiquinone and subjected to SDS PAGE for analysis of the distribution of radioactivity among the subunits of the complex. The

radioactivity was found mainly in the Mr=17500 dalton protein, suggesting that this protein is responsible for the binding of plastoquinone. The binding of the azido-ubiquinone derivative is masked by the presence of phospholipids. 2,5-dibromo-3-methyl-6-isopropylbenzoquinone, an inhibitor of electron transfer in the cytochrome b_6-f complex, showed a 35% decrease in the incorporation of azido-ubiquinone into the complex, while 5-n-undecyl-6-hydroxy-4,7-dioxobenzothiazole, another inhibitor, showed a 65% decrease in the azido-ubiquinone incorporation.

Introduction

The existence of specific ubiquinone-binding proteins in the mitochondrial and photosynthetic electron transfer complexes has been established through biochemical and biophysical studies (2). Photoaffinity labeled quinone derivatives have been useful in identifying the quinone-binding proteins in the mitochondrial (35), bacterial (47) and yeast (37) cytochrome b-c_1 complexes, as well as the cytochrome d terminal oxidase complex of E.coli (40). These studies employed an ubiquinone derivative with a light-activated azido-group located on the benzoquinone ring. Studies on the mitochondrial cytochrome b-c_1 complex have shown that this type of azido-ubiquinone derivative gives more specific labeling of the quinone binding site(s) than arylazido-quinone derivatives whose azido group is on the end of the isoprenoid side chain (35). In each of the cytochrome b-c_1 complexes, the quinone-binding proteins were found to be the polypeptide corresponding to cytochrome b and a low molecular weight (Mr=14000 to 17500) polypeptide.

The photosynthetic electron transfer system of higher plants contains an enzyme, the cytochrome b_6-f complex, that is similar in structure and function to the mitochondrial cytochrome b-c_1 complex. The cytochrome b_6-f complex contains four major polypeptides (52,61): cytochrome f (Mr=33000, Mr=34000) which appears as a double band on electrophoresis, cytochrome b_6 (Mr=23500), an iron sulfur protein (Mr=20000) and a low molecular weight protein (Mr=17500) with no assigned function. Some small polypeptides (Mr=5000 to 8000) have also been reported to be associated with the enzyme (61). The cytochrome b_6-f complex contains no core proteins as found in the mitochondrial system (69,72). The enzyme is responsible for the shuttle of electrons from plastoquinol to plastocyanin (60).

Photoaffinity labeling of a cytochrome b_6-f complex using an arylazido-quinone derivative reports the iron-sulfur protein (Mr=20000) and the cytochrome b_6 (Mr=23000) as the plastoquinone binding proteins (53). The binding of the photoaffinity label to the cytochrome b_6 is expected based on results from similar studies on other systems (2,35,37,40,47). The binding of the plastoquinone to the iron-sulfur protein is unprecedented in photoaffinity labeling studies on similar systems. The cytochrome b_6-f complex used for this study is isolated in the presence of excess exogenous phospholipids (52) and is reported to contain 1 mole of plastoquinone per mole of cytochrome f (61). The presence of lipids and bound quinone can prevent the azido-quinone access to the quinone binding site (2). There was also no attempt to correlate the binding of the photoaffinity label to the inactivation of the enzyme.

The procedure for the isolation of a plastoquinone and lipid

deficient cytochrome b_6-f complex from spinach chloroplasts has been reported (85). The delipidated cytochrome b_6-f complex is suitable for studies using photoaffinity labeled quinone derivatives as it allows the quinone-label access to its binding site, while still retaining the ability to reconstitute with lipids to regain full reconstitutive activity. The results of the photoaffinity labeling studies of the lipid and plastoquinone deficient cytochrome b_6-f complex are reported here.

Materials and Methods

The plastoquinone and lipid deficient cytochrome b_6-f complex was prepared according to the previously reported method (85). Spinach lipids were prepared by extraction with organic solvents and separated into lipid classes using a silica gel column as described (88). The synthesis of 3-azido-2-methyl-5-methoxy-6-(3,7-dimethyl-[^{3}H]octyl)-1,4-benzoquinone (azido-Q), and plastoquinone (PQ_2) was carried out according to the published method (35). Protein was determined by the Lowry method (89). UHDBT was synthesized as reported (94). Estimation of plastoquinone concentration was determined by the reported method (95). Phosphate (100) and sugar (88) were determined by the reported method. Sodium cholate, octylglucoside and horse heart cytochrome c, type III, were purchased from Sigma. Other chemicals were of the highest purity commercially available.

Illumination

The cytochrome b_6-f complex was diluted to a cytochrome f

concentration of 10 uM with 30 mM Tris-Cl buffer, pH 6.8, containing 0.5% sodium cholate. The azido-Q was added as described in the legends. The sample was incubated at 2°C for 20 minutes in the dark before it was transferred to a 0.1 cm light path quartz-windowed dewar flask at a constant temperature of 2°C. The sample was then illuminated with a long wavelength UV lamp for the time indicated in the figure legends. For activity assays, the photolyzed samples were diluted to 2 uM cytochrome f in 30 mM Tris-Cl buffer, pH 6.8, containing 0.5% sodium cholate and 1.2 mg/ml spinach glycolipids. The samples were incubated 30 minutes at 2°C and the reduction of cytochrome c at 550 nm was monitored ar reported previously (85).

Radioactive Azido-Q Incorporation

To determine the radioactive incorporation of azido-Q into the protein, 2 ul aliquots were withdrawn from the reaction cuvette at the given times during photolysis and spotted in the dark on Whatman paper, No. 3. When all samples were spotted and dried, the paper was developed in a solvent system of chloroform:methanol (2:1) (v/v). This leaves the denatured proteins at their original spots, while unreacted azido-Q and residual lipids move up the paper with the solvent. After the paper has dried, the protein spots were cut out and placed into scintillation vials with 5 ml of Insta-gel. The radioactivity was determined in a Beckman liquid scintillation counter, model SL-3150T.

Radioactive Distribution

The photolyzed samples were dialyzed against water and extracted

with organic solvents, as described previously (35). The lyophilized protein was dissolved in 30 mM Tris-Cl buffer, pH 6.8, to a protein concentration of 1 mg/ml. SDS and 2-mercaptoethanol were added to a final concentration of 1 % and the samples were incubated at 37°C for 2 hours. Aliquots containing 50 ug of protein were loaded onto SDS polyacrylamide gels, prepared according to the method of Laemlli (93), with the substitution of N,N'-diallyltartardiamide as the crosslinker. After electrophoresis, the gels were stained with Commassie Blue and destained. The gels were sliced into 3 mm slices and each slice was dissolved in 0.2 ml of 2% periodic acid at room temperature for 30 minutes. Insta-gel (5 ml) was added to each tube and the radioactivity determined.

Results and Discussion

Properties of the Lipid and Plastoquinone Deficient Cytochrome b_6-f Complex

It has been shown in bovine heart (35) and yeast (37) mitochondrial cytochrome b-c_1 complexes that endogenous ubiquinone and phospholipid must be removed from the complex for specific interaction of azido-Q derivatives. The cytochrome b_6-f complex isolated using calcium phosphate column chromatography has been shown (85) to be deficient in lipid and plastoquinone, as is evident from the low enzymatic activity that can only be restored by the addition of exogenous plastoquinone and lipid. The degree of lipid and plastoquinone deficiency is dependent on the amount of sample applied to the column, the flow rate of the column and the amount of buffer used to wash the column. The sample used for these experiments

contains 10.1 nanomoles cytochrome f, 19.8 nanomoles cytochrome b_6, 12.1 nanomoles of phospholipid (based on total phosphorus), 1.24 nanomoles of glycolipid (based on total sugar), and 6.13 nanomoles of plastoquinone per milligram of protein. Assuming a one-to-one stoichiometry between plastoquinone and cytochrome f, as was previously reported (84), then the complex is about 40% deficient in plastoquinone.

Effect of Azido-Q Concentration

Figure 7 shows the effect of azido-Q concentration on the inactivation of the cytochrome b_6-f complex after photolysis. Maximum inactivation of approximately 43% is obtained when the azido-Q concentration is 30 moles of azido-Q per mole of cytochrome f. The inactivation of 43% is in close agreement with the 40% inactivation expected based on the degree of plastoquinone deficiency. The concentration of azido-Q needed for maximal inhibition is higher than that found in the mitochondrial system (35) and may be due to a lower affinity of the azido-Q for the cytochrome b_6-f complex. Figure 7 also shows the amount of azido-Q incorporation, based on the amount of radioactivity incorporated into the protein, as a function of azido-Q concentration. The azido-Q incorporation increases sharply up to a concentration of 40 nanomoles azido-Q per nanomole of cytochrome f. At increasingly higher concentrations of azido-Q, the incorporation continues, but at a much lower rate. The azido-Q-incorporation at the lower concentrations of azido-Q correlates well to the inactivation of the cytochrome b_6-f complex, while the

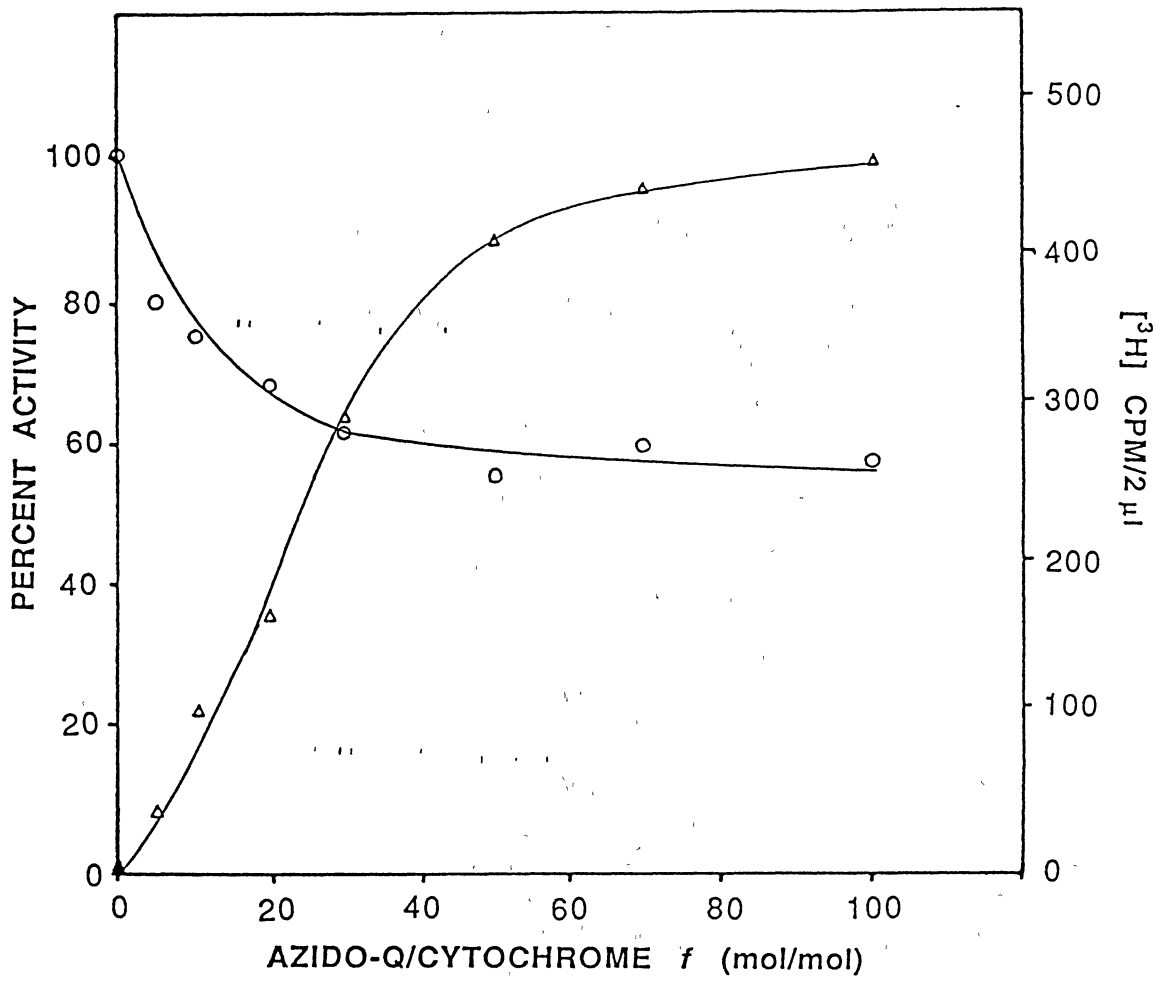

Figure 7: Effect of Azido-Q Concentration on Azido-Q Uptake and Cytochrome b_6-f Inactivation. The cytochrome b_6-f complex (10 uM cytochrome f) was incubated with the indicated amount of azido-Q for 20 minutes in the dark. The samples were then photolyzed for 7 minutes at 2°C. Activity (o) and radioactivity (Δ) were determined as described in Materials and Methods. 100% activity corresponds to 13 umoles cytochrome c reduced/nmole f/hour.

incorporation at high concentrations, where no further activity loss is observed, is due to nonspecific labeling. This nonspecific labeling at high concentrations is further substantiated by the labeling pattern on SDS polyacrylamide gels, which show high levels of radioactivity in all protein bands at azido-Q concentrations above 30 nanomoles azido-Q per nanomole cytochrome f. It is important to demonstrate a correlation between activity loss and azido-Q binding to determine whether the azido_q is bound to the plastoquinone binding site. Previous investigations (53) on the plastoquinone binding protein showed that a Q-derivative that was similar in structure to the arylazido-plastoquinone could function as an electron donor. They therefore assumed that the arylazido-plastoquinone was acting on the plastoquinone binding site. This may not be the case, since the enzyme was not plastoquinone deficient. It is possible that the arylazido-plastoquinone served as an electron donor for the bound plastoquinone, causing the labeling of the arylazido-plastoquinone at a site other than the actual plastoquinone binding site. By correlating the loss of activity to the incorporation of azido-Q, this possibility has been eliminated. This is further substantiated by the 43% inactivation of the enzyme that was shown to be 40% deficient in bound plastoquinol.

Effect of Illumination Time

Figure 8 shows the effect of illumination time on the plastoquinol-cytochrome c reductase activity and the azido-Q incorporation into the cytochrome b_6-f complex. Maximum inactivation (40%) is reached in about 7 minutes. A control sample containing a

similar volume of ethanol shows less than 5% photoinactivation over the time period studied. The incorporation of azido-Q into the cytochrome b_6-f complex is also dependent of illumination time. The azido-Q incorporation increases sharply as the enzyme becomes inactive, indicating that the inactivation is a result of azido-Q binding to the plastoquinone binding site. The light increase in azido-Q incorporation after the maximal inactivation has been reached is believed to be due to nonspecific labeling. Once again, analysis of the incorporation of radioactive azido-Q in the polypeptides of the cytochrome b_6-f complex showed high levels of incorporation in all the polypeptides at longer photolysis times, indicating nonspecific labeling.

Radioactivity Distribution of Azido-Q among the Subunits of the Cytochrome b_6-f Complex

Using the optimum conditions of 30 nanomoles azido-Q per nanomole cytochrome f and a photolysis time of 7 minutes, the cytochrome b_6-f was photolyzed and the protein was extracted with organic solvents in the dark to remove unreacted azido-Q and azido-Q bound to phospholipid, as discussed in the Materials and Methods section. Since the incorporation of azido-Q into the cytochrome b_6-f complex parallels the inactivation of the enzyme and the percent inactivation correlates with the percent plastoquinol deficiency, the distribution of the azido-Q among the subunits of the cytochrome b_6-f complex can be used to identify the plastoquinone binding protein. Figure 9A shows the radioactivity distribution among the subunits of the cytochrome b_6-f complex. The major peak of radioactivity corresponds with the Mr=17500 subunit. Lower levels of radioactivity

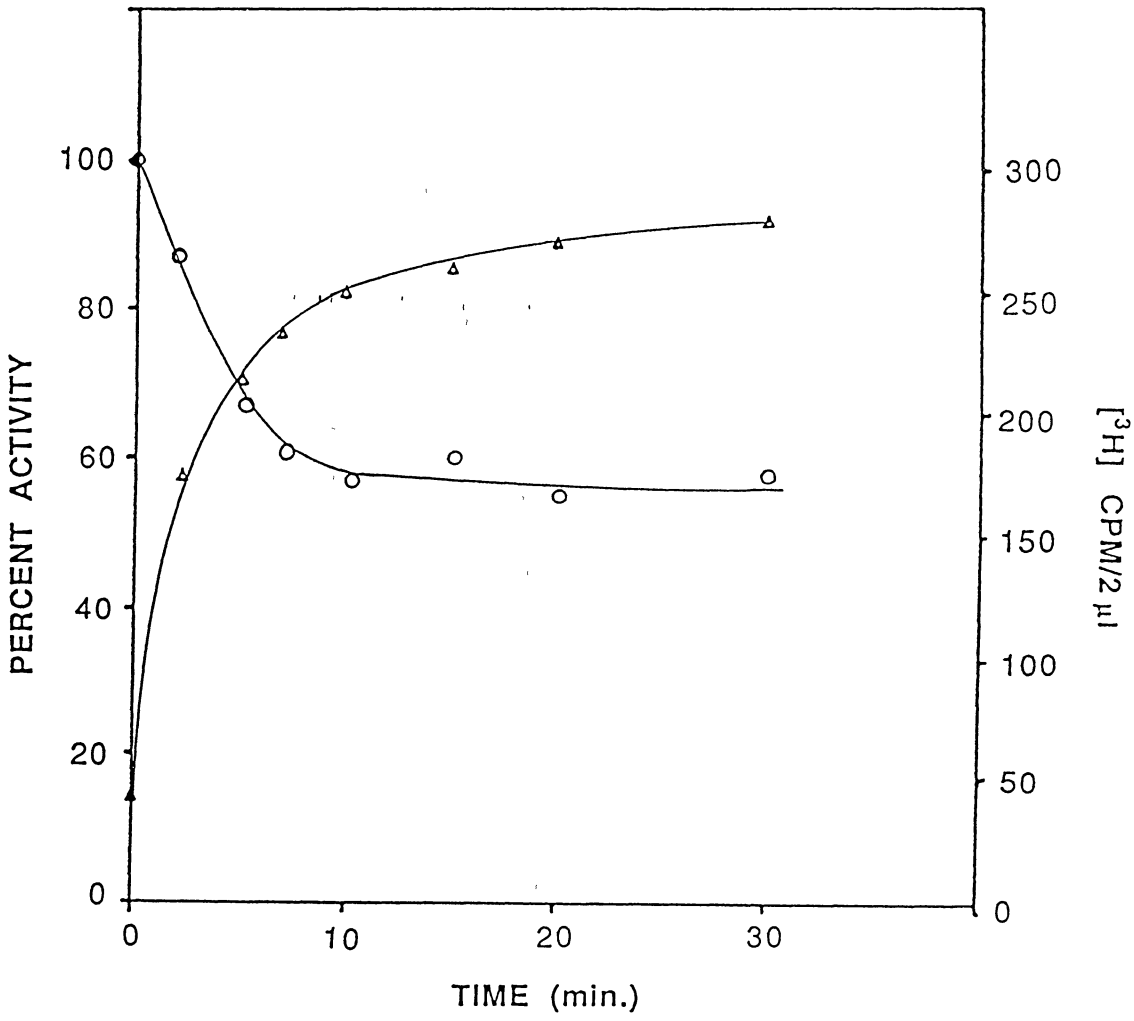

Figure 8: The Effect of Photolysis Time on Azido-Q Uptake and Cytochrome b_6-f Inactivation. The cytochrome b_6-f complex (10 uM cytochrome f) was incubated with azido-Q (300 uM) for 20 minutes in the dark. The samples were then photolyzed for the indicated times and the samples were assayed for activity (o) and radioactivity (Δ) as described in Materials and Methods. A control sample containing an equivalent amount of ethanol was photolyzed and showed no loss of enzymatic activity over the time period studied.

were found in the iron sulfur protein (Mr=20000) and the cytochrome b_6 (Mr=23500). Moderate levels of radioactivity were found in a poorly stained band with low molecular weight (Mr=10000 to 12000). This band may be due to band smearing on the electrophoresis or possibly to a lipid-azido-Q complex that was not removed by the extraction procedure. Since the extraction procedure used was one developed for the mitochondrial system, it may not be effective in removing lipids that are unique to the photosynthetic system. The identity of this low molecular weight band has yet to be determined.

The results of this study are in contrast to previous studies into the identification of the plastoquinone binding protein (53). Previously, the cytochrome b_6 and the iron sulfur protein were reported to be responsible for plastoquinone binding. These studies employed an arylazido-plastoquinone derivative where the light activated azido-group is located on the isoprenoid side chain. Since the benzoquinone ring is the functional moiety in the electron transfer reaction, the arylazido label may be labeling near, but not at, the active site, even though the isoprenoid side-chain on this particular photoaffinity label is only two units long as compared with the ten unit isoprenoid side chain used for mitochondrial labeling experiments (30).

Another factor in the incorporation of azido-Q derivatives is the presence of lipid and plastoquinone. Previous labeling experiments used a cytochrome b_6-f complex that was isolated in the presence of excess exogenous lipid. Studies on the cytochrome b-c_1 complex from mitochondria (35) and yeast (37) showed that the presence of lipid can prevent azido-Q binding. If this were true,

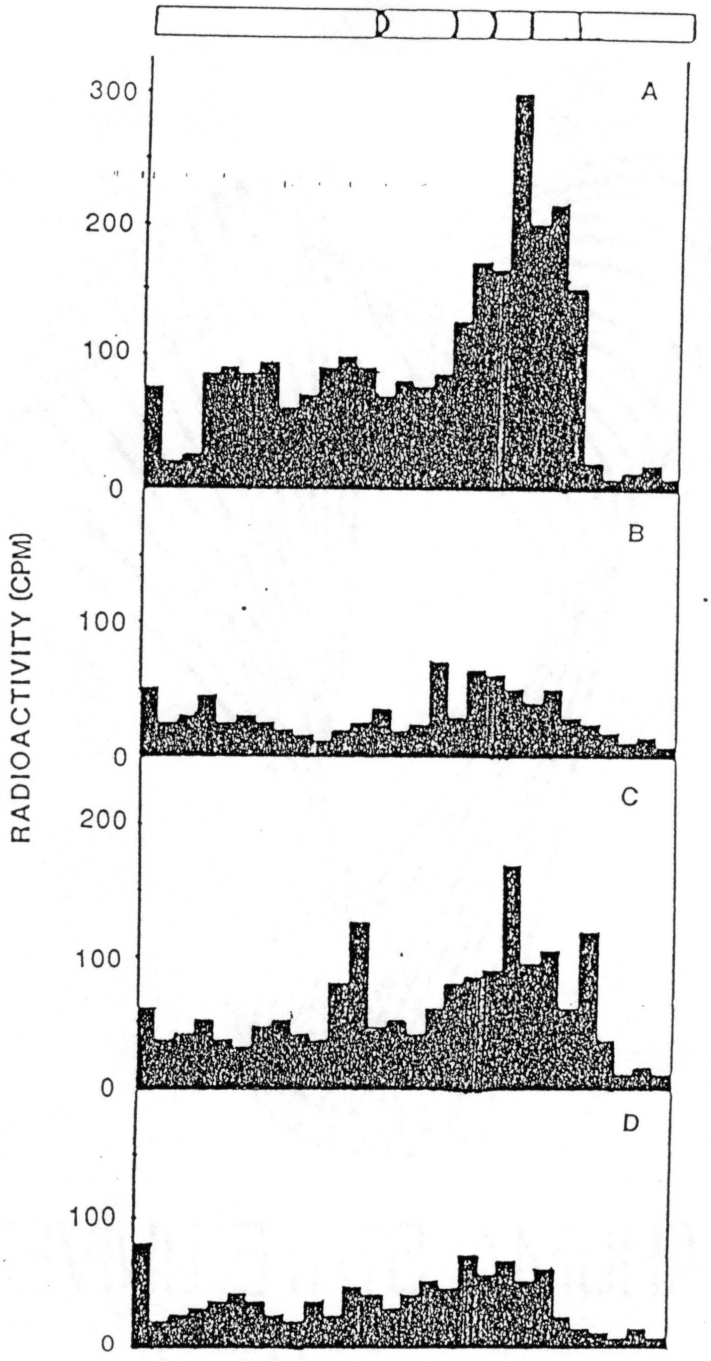

Figure 9: Radioactivity Distribution Among the Subunits of the Cytochrome b_6-f Complex under Various Conditions. Photolyzed samples were extracted with organic solvents and subjected to SDS PAGE. The gels were sliced and radioactivity determined as described in Materials and Methods. Graph A is the cytochrome b_6-f complex + azido-Q. Graph B is the cytochrome b_6-f complex + lipid + azido-Q. Graph C is the cytochrome b_6-f complex + DBMIB + azido-Q. Graph D is the cytochrome b_6-f complex + UHDBT + azido-Q.

then the incorporation of azido-Q would be far less specific and more likely to bind readily accessible proteins that may be protruding from the lipid environment, perhaps near the plastoquinone binding site.

Effects of Lipid on the Binding of Azido-Q to the Cytochrome b_6-f Complex

To test for the effect of lipid on the azido-Q binding, one sample was incubated with azido-Q, then photolyzed, while another sample was reconstituted with lipids prior to the addition of azido-Q and then photolyzed. The results show that the sample that was photolyzed in the absence of lipids lost 42% of its reconstitutive activity upon photolysis. The sample that was reconstituted prior to the addition of azido-Q showed less than 5% loss of activity upon photolysis. As shown in Figure 9B, the labeling pattern of the lipid sufficient preparation shows almost no incorporation of azido-Q. The small amount of label that is incorporated is found in the cytochrome b_6 and the iron sulfur protein. This labeling pattern is very similar to that reported using the arylazido-plastoquinone (53). Since this incorporation does not correlate to a loss on enzymatic activity, it is most likely due to nonspecific labeling. As a further control, the cytochrome b_6-f complex was incubated with azido-Q prior to reconstitution with lipids. The complex was then photolyzed and the results were the same as the sample photolyzed in the absence of lipid, indicating that the presence of lipid during photolysis does not affect the labeling.

The lipid-dependent binding of the azido-Q to the Mr=17500 protein of the cytochrome b_6-f complex is very similar to that of the

Mr=17000 protein of the mitochondrial cytochrome b-c_1 complex (35). The mitochondrial cytochrome b-c_1 complex also shows significant azido-Q binding in the cytochrome b (Mr=37000), which shows no correlation to enzyme inactivation and is not phospholipid dependant. There is not a significant amount of azido-Q incorporation in the cytochrome b_6 (Mr=23000) in the cytochrome b_6-f complex. A comparison of the gene sequences (62) of the cytochrome b_6 and the Mr=17500 protein with the cytochrome b form mitochondria showed that the cytochrome b_6 has a great deal of sequence homology with the amino terminal end of the mitochondrial cytochrome b, while the Mr=17500 protein from the cytochrome b_6-f complex shows homology with the carboxyl terminal of the mitochondrial cytochrome b. The sequence homology of the chloroplast Mr=17500 protein with the mitochondrial cytochrome b may explain why the mitochondrial cytochrome shows azido-Q incorporation that does not correlate with enzymatic activity (35). The homology may be indicative of a structural similarity between the quinone-binding protein (Mr=17500) and the mitochondrial cytochrome b that either looks enough like a quinone binding site to cause non-specific labeling, or is a second type of quinone binding site that is not present in the chloroplast system. Another explanation may be that only one of the two types of plastoquinone binding sites (96) in the cytochrome b_6-f complex has been exposed during the isolation of the plastoquinone deficient complex, so only one class of plastoquinone binding site is exposed for labeling.

Effect of Inhibitors of Electron Transfer on the Binding of Azido-Q to the Cytochrome b_6-f Complex

The inhibitory effect of UHDBT and DBMIB on the electron transfer activity of the cytochrome b_6-f complex has been well established (63,80,97). These quinone analogs are known to interfere with plastoquinol oxidation. It is therefore of interest to determine what effect, if any, these inhibitors have on the binding of azido-Q to the cytochrome b_6-f complex. Figure 9C shows the radioactivity distribution among the subunits of the cytochrome b_6-f complex when the enzyme is preincubated with DBMIB prior to the addition of the azido-Q. The radioactivity in the Mr=17500 protein is decreased by approximately 40% with a corresponding increase in the cytochrome f and an Mr=8000 band. One explanation for this is that the DBMIB does not appear to bind in the plastoquinone binding sit, but it does mask the site, causing an increase in the non-specific labeling. It has been suggested (96) that DBMIB binds to one of the two proposed plastoquinone binding sites in the cytochrome b_6-f complex. Since there is still a substantial amount of plastoquinone bound to the cytochrome b_6-f complex, the DBMIB site may only be partially depleted. This is an interesting possibility, as the enzyme is 40% plastoquinone deficient and the radioactivity in the Mr=17500 protein is decreased by 40%.

The addition of UHDBT to the cytochrome b_6-f complex prior to the addition of azido-Q and photolysis caused a 65% decrease in the total radioactivity incorporated into the protein. The effects of UHDBT on azido-Q incorporation varies greatly in the different cytochrome b-c_1 complexes. UHDBT showed no effect on the labeling of

the mitochondrial cytochrome b-c_1 complex (35). The photosynthetic bacteria R.sphaeroides cytochrome b-c_1 complex showed an increase in the amount of label found in the cytochrome b upon preincubation with UHDBT (47). In this study, the UHDBT almost completely blocks azido-Q binding to the cytochrome b_6-f complex. UHDBT has been reported to react with the iron-sulfur protein (80,98). Binding to the iron-sulfur would make an explanation for the experimental results somewhat difficult. Recently, UHDBT has been suggested to be phospholipid associated (99). Since the enzyme complexes from the different sources contain different types and amounts of lipids, the association of the UHDBT to lipid could cause different effects to the azido-Q labeling patterns. In the cytochrome b_6-f complex, the UHDBT binds in such a way as to prevent the binding of the azido-Q.

CHAPTER IV

EPR CHARACTERISTICS OF THE LIPID DEFICIENT CYTOCHROME b_6-f COMPLEX

Summary

The cytochrome b_6-f complex has been isolated in a lipid deficient form. The lipid deficient enzyme exhibits a slight shift in the cytochrome f signal (g~3.5) to higher field, compared to the lipid reconstituted form. The iron-sulfur protein showed a pronounced shift in the g~2.022 and the g~1.77 signals when comparing the lipid deficient and the reconstituted forms. In the lipid deficient form, the cytochrome b exhibited a single EPR signal with g~3.53. Upon lipid reconstitution, two distinct cytochrome b signals can be observed, indicating that two spectrally distinct b-cytochromes exist in the active, lipid sufficient cytochrome b_6-f complex. A plastosemiquinone signal is observed with g~2.005 and linewidth of 8 gauss. The signal has a pH optimum of 8.5-9.0. Electron transfer inhibitors, such as UHDBT, myxothiazol and antimycin A, greatly diminish the plastosemiquinone signal, while DBMIB has little effect.

Introduction

The cytochrome b_6-f complex shuttles electrons from plastoquinol

to plastocyanin in the chloroplasts of higher plants. The isolated complex (52) consists of five polypeptide subunits with Mr=34000, 33000, 23500, 20000 and 17500. Cytochrome f was found to be heterogeneous and was assigned to the Mr=34000 and 33000 subunits (61). The Mr=23500 subunit is thought to be cytochrome b_6 (61) and shows a great deal of sequence homology to the amino terminal of the mitochondrial cytochrome b (62). The Mr=20000 subunit is a Rieske-type iron sulfur protein (73). The Mr=17500 subunit shows a great deal of sequence homology with the carboxyl terminal of the mitochondrial cytochrome b (62) and may be involved in plastoquinone binding (52,61,62).

As is the case with many membrane-bound enzymes (20,28,101-103), the cytochrome b_6-f complex requires lipid for enzymatic activity (84,85). Evidence for this lies in the fact that the cytochrome b_6-f complex can be isolated in a lipid deficient form (85) with approximately 10% of the enzymatic activity of the lipid-containing form (61). The enzymatic activity can be fully restored upon reconstitution with plastoquinone and lipid (85). It is of interest to determine how this lipid reconstitution effects each of the components in the cytochrome b_6-f complex.

Materials and Methods

The cytochrome b_6-f complex was isolated by the method of Doyle and Yu (85). Activity was assayed as in (60), using horse heart cytochrome c, type III, as the electron acceptor and plastoquinol-2 as the electron donor (85). Lipids were prepared as in (88) and further purified using a silica gel column. Octylglucoside,

antimycin A and myxothiazol were from U.S. Biochemicals. UHDBT was synthesized in our laboratory using the method of Friedman et. al. (94).

To reconstitute the cytochrome b_6-f complex for EPR studies, the sample was diluted to 10 uM cytochrome f, incubated for 10 minutes with plastoquinone (10 uM), then reconstituted with glycolipid-enriched spinach chloroplast lipids (2 mg/ml) and incubated another 20 minutes. The samples was then concentrated to 100 uM cytochrome f using an Centricon-10 (Amicon) microconcentrator. For inhibition studies, inhibitors were added prior to the addition of plastoquinone and lipid and incubated 10 minutes. EPR spectra were recorded using a Bruker ER-200D spectrometer with an Air Products flowing helium cryostat.

Results and Discussion

Effects of Reconstitution on the EPR of the Cytochrome b_6-f Complex

The cytochrome b_6-f complex prepared by the method of Doyle and Yu (85) was shown to be deficient in plastoquinone and lipid. This preparation was inactive in its isolated state. The enzyme can be fully reactivated upon addition of plastoquinone and lipid to a fully active state. Figure 10A shows the EPR spectra in the cytochrome f region of the fully oxidized lipid-deficient cytochrome b_6-f complex. Figure 10B shows the same spectra using the lipid and plastoquinone reconstituted complex. The inactive lipid-deficient complex exhibits a signal at g~3.494, whereas the active reconstituted sample has a signal at g~3.512. This slight shift to lower field upon

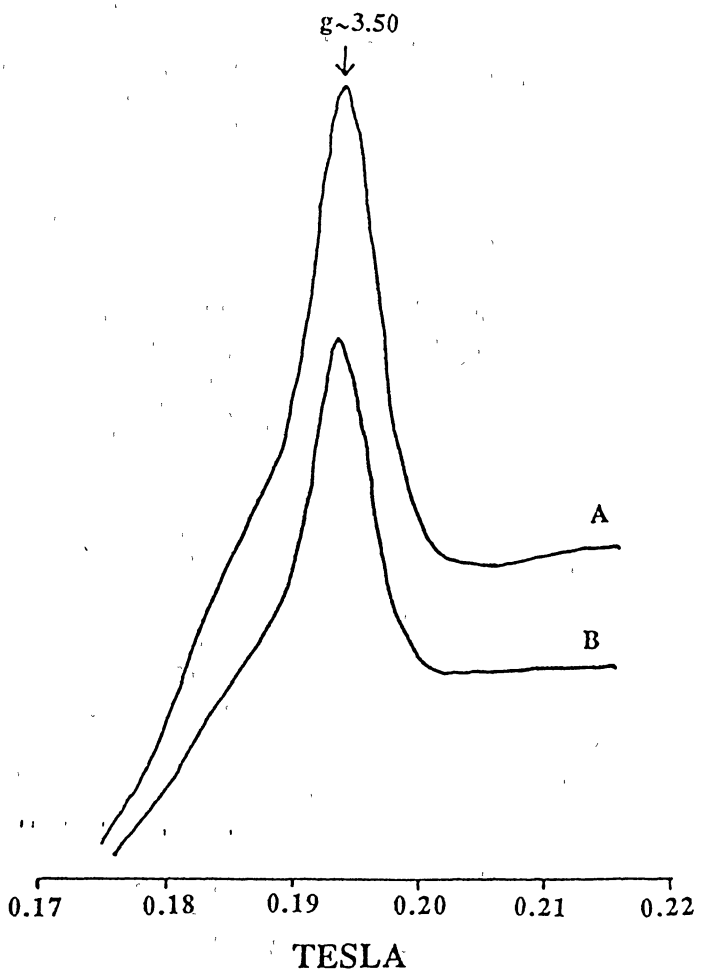

Figure 10: EPR Spectra of the Cytochrome b6-f Complex in the Oxidized Form. Instrument settings were as follows: field modulation frequency, 100 KHz, microwave frequency 9.49 GHz, modulation amplitude 2 x 10-3 TESLA and microwave power 2 mW. The sample temperature is 5°K. Spectrum A shows the cytochrome f (130 uM) signal as prepared. Spectrum B shows the cytochrome f (100 uM) signal after reconstitution with PQ_2 and lipids as in Materials and Methods.

reconstitution, along with some peak sharpening indicates that there is a small change in the heme environment of the cytochrome f upon reconstitution.

The cytochrome b_6-f complex contains two b-type cytochromes per cytochrome f (52,61). Figure 11A and 11B show the ascorbate reduced cytochrome b_6-f complex in the delipidated and reconstituted forms, respectively. The delipidated complex shows a single asymmetric signal at g~3.5. Upon reconstitution with plastoquinone and lipid, two distinct b-cytochromes exist in the cytochrome b_6-f complex. The existence of two spectrally distinct b-cytochromes in the cytochrome b_6-f complex was previously postulated by Salerno et. al. (92). Although their preparation exhibited only one cytochrome b signal (g~3.7), the shape of the signal was very similar to the cytochrome b signal in a delipidated mitochondrial succinate-cytochrome c reductase complex. Since the mitochondrial cytochrome b showed two signals on lipid reconstitution, they postulated that the same might happen in the cytochrome b_6-f complex. The results shown here indicate that this is indeed the case, that the spectrally distinct b-cytochromes are apparent only in the active form of the enzyme.

Using redox titrations, Bergstrom et. al. (107) reported that two spectrally distinct b-cytochromes do, indeed, exist in the cytochrome b_6-f complex. They used low temperature difference spectroscopy and EPR to monitor the changes in the cytochrome b_6 at different stages of reduction. The EPR spectra showed one broad signal (g~3.5) that appears to shift to lower field as the cytochrome becomes reduced. The liquid nitrogen temperature difference spectra of partial reduced cytochrome b versus ascorbate reduced cytochrome f

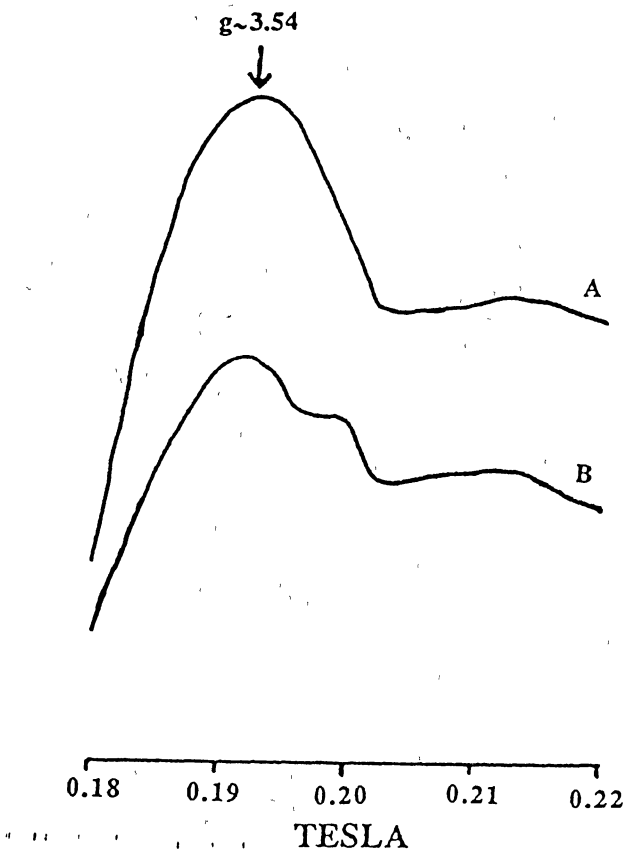

Figure 11: EPR Spectra of Cytochrome b6-f Complex in the Ascorbate Reduced Form. Instrument Settings are the same as in Figure 10. Spectrum A shows the cytochrome b (260 uM) signal upon reduction of the sample with ascorbate. Spectrum B shows the cytochrome b (200 uM) signal after reconstitution with PQ_2 and lipids and reduction with ascorbate.

showed two peaks corresponding to cytochrome b_6. This is of interest since Hurt and Hauska (52) reported only a single peak for cytochrome b_6 at low temperatures.

The results reported here indicate that the cytochrome b_6 does indeed exist as two spectrally distinct cytochromes, but only when the complex is in a lipid sufficient, active state. The delipidated state, which is enzymatically inactive, exhibits only a single signal. This would indicate that the lipids are required to maintain the b-cytochromes in their distinctive, active conformation.

Figure 12A and 12B show the Rieske-type iron sulfur signals in the delipidated and the lipid reconstitutive forms. The lipid deficient form of the cytochrome b_6-f complex exhibits the three characteristic EPR signals for the iron sulfur protein, with g-values (g_x~1.80, g_y~1.91 and g_z~2.02) similar to those reported by Salerno et. al. (92). Upon reconstitution with plastoquinone and lipid, the g_z signal is shifted to lower field and the g_x signal became sharper. This indicates that the iron sulfur protein also requires lipid to achieve an active conformation.

Figure 12A and 12B both show a large free radical signal at g~2.005, which may be due to a plastosemiquinone radical. In an attempt to characterize this g~2.005 signal, inhibitor effects were studied. Table III shows the effect of different electron transfer inhibitors on the plastoquinol-cytochrome c reductase activity. At the concentrations used, DBMIB and UHDBT both showed approximately 80% inhibition or the reconstitutive activity. These results agree with previous inhibition studies (52). Hurt and Hauska (52) reported that antimycin A yields only 30% inhibition even at a concentration

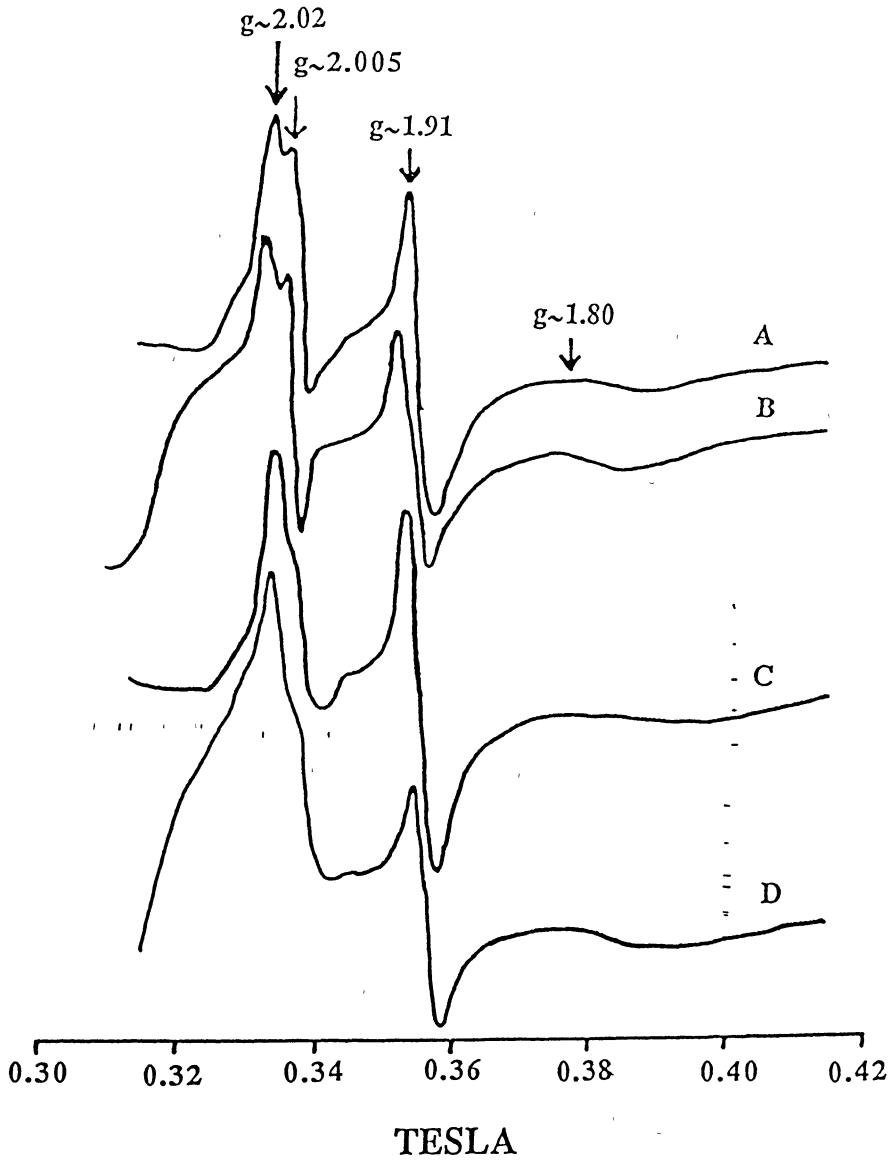

Figure 12: EPR Spectra of Fe-S Protein of the Cytochrome b_6-f Complex in the Ascorbate Reduced Form. Instrument settings are the same as in Figure 10. The sample temperature is 15oK. Spectrum A is the complex (100 uM cytochrome f) as prepared. Spectrum B is the complex (68 uM cytochrome f) after reconstitution with PQ_2 and lipids. Spectrum C is the reconstituted complex (68 uM cytochrome f) after the addition of myxothiazol (0.7 mM). Spectrum D is the reconstituted complex (100 uM) cytochrome f) after the addition of UHDBT (0.3 mM).

TABLE III

EFFECT OF ELECTRON TRANSFER INHIBITORS ON THE CYTOCHROME b_6-f ACTIVITY

Inhibitor	Concentration mol I/mol f	Activity umol c red/nmol c/hr	Inhibition %
-	-	13.2	0
DBMIB	5	2.2	83
UHDBT	5	2.9	78
Antimycin A	50	7.7	42
Myxothiazol	50	7.3	45

of 2000 moles inhibitor per mole of cytochrome f. The lipid reconstituted enzyme shows 42% inhibition at a concentration of only 50 moles of antimycin A per mole of cytochrome f. Increasing the inhibitor concentration, however, did not yield any further inhibition. Similarly, myxothiazol was reported to be ineffective in the cytochrome b_6-f complex (63), although the results reported here indicate that partial inhibition (45%) is possible at a myxothiazol to cytochrome f ratio of 50:1. As was the case with the antimycin A, increasing the myxothiazol concentration did not yield more inhibition.

Figure 13 shows the effects of these inhibitors on the g~2.005 signal. Figure 13A shows the lipid reconstituted plastosemiquinone signal. The addition of DBMIB to the enzyme resulted in no change to the g~2.005 signal. Addition of UHDBT, antimycin A or myxothiazol diminished the g~2.005 by 65-70% in each case. The decrease in the free radical signal upon addition of electron transfer inhibitors is expected because electron transfer through the plastoquinone region of the electron transfer chain is required to generate the plastosemiquinone, and because these inhibitors are thought to act on an electron transfer step involving plastoquinone. Of particular interest is the fact that UHDBT diminishes the plastosemiquinone, but DBMIB does not. These two inhibitors are thought to act on the transfer of electrons from plastoquinol to the Rieske-type iron sulfur protein (63). These results would indicate that the two inhibitors are acting on different sites. Another explanation may be that the shifted iron-sulfur signal upon addition of DBMIB to a g-

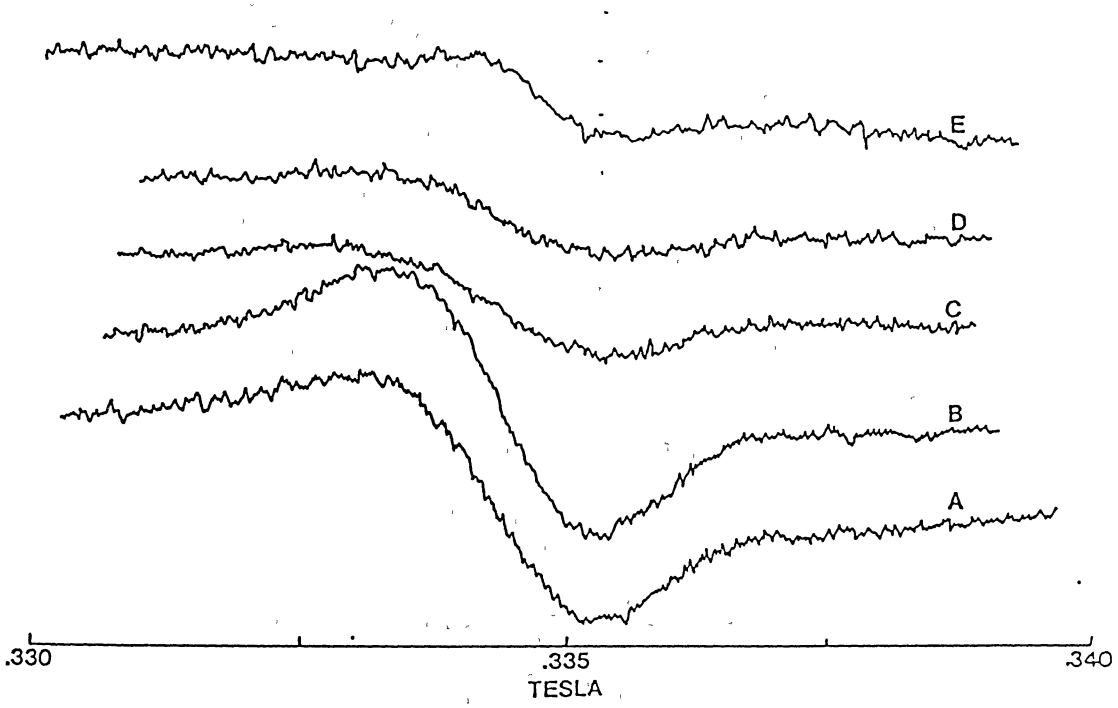

Figure 13: EPR Spectra of the Plastosemiquinone in the Cytochrome b_6-f Complex. Spectrum A is the reconstituted complex. Spectrum B is sample A plus DBMIB. Spectrum C is sample A plus UHDBT. Spectrum D is sample A plus myxothiazol. Spectrum E is sample A plus anitmycin A. Inhibitor concentrations are as in Table III. Instrument setiings are: microwave frequency 9.34 GHz, modulation amplitude 2 x 10^{-3} TESLA and microwave power 20 mW.

value of approximately 2.01 (73) may be overlapping with the free radical signal. In fact, the DBMIB is reported (73) to form stable free radicals, and this may be contributing to the g~2.005 signal.

Figure 14 shows the pH dependence of the plastoquinol-plastocyanin oxidoreductase activity and the plastosemiquinone radical. The enzymatic activity shows a pH optimum of 7.5-8.0, while the plastosemiquinone radical has a pH optimum of 8.5-9.0. This higher pH optimum of the plastosemiquinone radical is similar to the results previously reported for the mitochondrial semiquinone (9) and indicates that the plastoquinone is associated with protein and in the anion form. Free semiquinone species are not stable enough to be seen at pH values of less than 12 (9). This figure also illustrates why plastosemiquinone radicals are often not detected. The conditions for enzymatic activity are at a pH where the plastosemiquinone radical is a transient species, but at higher pH, where it is more stable, it can be more easily detected.

Figure 15 shows the power saturation behavior of the g~2.005 signal. The signal begins to show saturation at 200 uW. This behavior is also very similar to the results obtained from the mitochondrial ubisemiquinone (9). This indicates that the plastosemiquinone is protein associated, but not with another paramagnetic center such as the iron sulfur protein.

In summary, the EPR characteristics of the lipid-deficient cytochrome b_6-f complex are quite similar to those previously reported (92). The reconstituted complex shows shifts in the signals representing the cytochrome f, cytochrome b and the iron sulfur

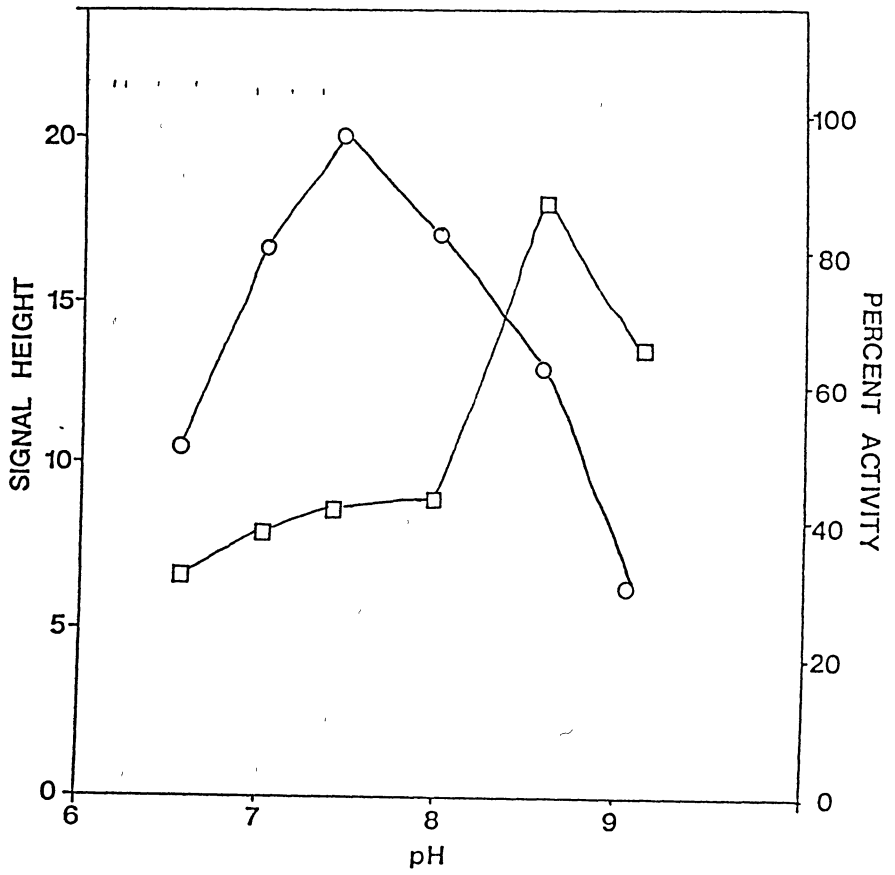

Figure 14: pH Dependence of the Cytochrome b_6-f Complex Activity and the Generation of the Plastosemiquinone Signal. The open circles represent the plastoquinol-cytochrome c reductase activity (100% activity is 13.2 umol c red/nmol f/hr). The open squares represent the height of the plastosemiquinone signal.

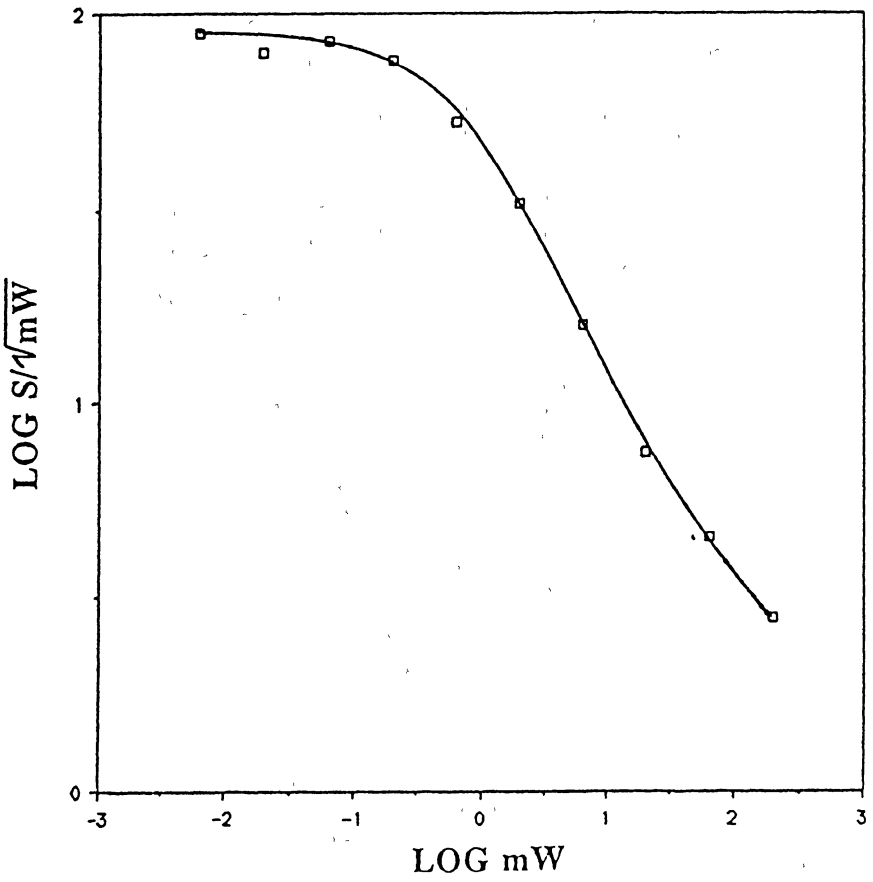

Figure 15: Power Saturation of the Plastosemiquinone Signal. The cytochrome b_6-f complex (100 uM cytochrome f) was reconstituted with PQ_2 and lipids. Mitochondrial succinate-cytochrome c reductase (5 uM cytochrome b) and a fumarate/succinate mixture (100 uM/10 uM) were added to stabilize the plastoquinone radical. The sample temperature was 100°K. The signal height (S) was followed at varying power (mW).

protein. The most significant change occurs in the cytochrome b spectra. The lipid deficient cytochrome b_6-f complex exhibits only a single asymmetric signal, whereas the active, lipid reconstituted enzyme shows two distinct, but overlapping, cytochrome b signals. These results indicate that changes in the heme environment of one or both b-cytochromes has occurred upon reconstitution and this change can be correlated to enzymatic activity. The EPR spectra in the iron sulfur region shows a signal at g~2.005 which corresponds to plastosemiquinone. The plastosemiquinone signal has a line width of 8 gauss, a pH optimum of 8.5-9.0 and begins to power saturate at 220 uW. The addition of electron transfer inhibitors, such as UHDBT, antimycin A and myxothiazol diminished the plastosemiquinone signal, while DBMIB did not. The DBMIB may be forming a free radical which interferes with the detection of the plastosemiquinone signal. These results further suggest that plastoquinone is bound to protein.

CHAPTER V

DISCUSSION

Isolation Procedure

Although many procedures have been published for the isolation of the cytochrome b_6-f complex, these procedures were time-consuming and produced only limited amounts of sample. In an attempt to scale up the preparation, a method was developed for depleting the enzyme of bound plastoquinone and lipid to the extent of abolishing the enzymatic activity. Fortunately, the activity could be restored by adding exogenous lipid and plastoquinone. It is this property of the enzyme complex that allows investigation into the lipid and plastoquinone binding properties, which is the goal of this work.

The procedure described in Chapter II for the isolation of a lipid deficient cytochrome b_6-f complex from spinach chloroplasts is a relatively simple one. The choice of the detergents octylglucoside and sodium cholate to solubilize the enzyme complex from the thylakoid membrane is a fortuitous one. Attempts to solubilize the cytochrome b_6-f complex with other detergents, such as deoxycholate, CHAPS, LDAO or zwittergent, alone and in combination, provided very low yields. The only detergent that was capable of solubilizing the complex was triton X-100, but the concentration required for solubilization also solubilized many contaminants and diminished the

oxidoreductase activity compared to the octylglucoside/sodium cholate solubilized enzyme. This may be due to the inhibitory effect that triton X-100 has on the activity (52) or it may be that the concentration of triton X-100 used may have partially removed the Rieske-type iron sulfur protein (61),

Calcium phosphate column chromatography provided a simple method by which the cytochrome b_6-f could be depleted of lipid and bound quinone. The extent to which the column is washed after the sample is applied seems to correlate with the extent of lipid depletion. If the column is not washed before elution, there is more residual lipid remaining, as evidenced by higher enzymatic activity. If the column is washed too extensively, then the ability to reconstitute enzymatic activity by replenishing plastoquinone and lipid is lost. This indicates that there may be some minimal requirement for lipid and/or plastoquinone to retain a reconstitutively active conformation. By washing the column with three column volumes, the optimum lipid-deficient enzyme was isolated that retained its reconstitutive activity.

The lipid deficient cytochrome b_6-f complex contains only 4.2 % phospholipid and approximately 1% glycolipid. The determination of glycolipid is based on an assay of total sugar (109). Since octylglucoside contains a glucose moiety, any residual octylglucoside will contribute to the glycolipid determination and may explain the large variability in glycolipid determinations. Preliminary attempts to determine the nature of the remaining bound lipid by thin layer chromatography (88) provided little information. Perhaps a method of lipid separation using HPLC would produce more satisfactory results.

The nature of these residual lipids could provide valuable information into the conformational requirements of this enzyme complex.

The lipid used for reconstitution experiments was prepared from fresh market spinach. Attempts to use commercially prepared soybean lipid mixtures, such as azolectin or lecithin, were unsuccessful as these preparations showed a significant amount of plastoquinol-cytochrome c reductase activity in the absence of enzyme. This may be due to the sensitive assay conditions required for activity in this system, as compared to the mitochondrial where this activity would be negligible. It may also be due to an antimycin A-insensitive ubiquinol cytochrome c reductase activity which was found to co-purify with lipid from R.sphaeroides (91).

The preparation of crude lipid extract from spinach leaves entails blending the destemmed leaves in chloroform:methanol (1:2), filtration, and washing of the retentate with chloroform. The filtrate is then separated into two phases by the addition of water. Centrifugation is used to aid in the phase separation and the chloroform layer, which contains the lipid, is collected and concentrated on a rotarevaporator. Further purification into lipid classes was achieved on a silica gel column. Most of the quinones, chlorophylls and carotenoids elute in the chloroform wash, while glycolipids elute in the acetone wash and phospholipid elutes with the methanol wash. Lipid solutions were prepared by evaporating off the organic solvent and sonicating the residue into water under an argon atmosphere, immediately prior to use.

Preliminary reconstitution experiments with the isolated

cytochrome b_6-f complex showed maximum reconstitutive activity required incubations of 60-90 minutes. Further study showed that the purified complex is highly unstable and loses its reconstitutive ability within a few hours. For this reason, the enzyme is aliquoted and frozen at -80°C immediately upon isolation. When the sample is thawed for experimentation, it is immediately reconstituted with plastoquinone and lipid. Under these conditions, maximum reconstitutive activity is obtained with a 15-30 minute incubation with the plastoquinone and lipid. When the enzyme is allowed to sit on ice prior to reconstitution, the longer incubation times (60-90 minutes) are required for maximum activity. It is for this reason that the subsequent experiments in Chapter III and IV have 15-30 minute incubation times.

It is of interest to note the differences between the results reported here and those presented by Richard Chain (84). The lipid and quinone depleted cytochrome b_6-f complex of Chain was isolated using the procedure of Hurt and Hauska (61) with the addition of a second sucrose density gradient ultracentrifugation. This second centrifugation yielded three bands that varied in activity, plastoquinone content and constituent stoichiometry. The top two bands on the gradient could not be reconstituted with plastoquinone and lipid to replenish lost activity. It is of interest to note that these bands also show a decrease in the amount of iron sulfur protein, which may be contributing to the activity loss. The lower band, which contains only 0.12 moles of plastoquinone per mole of cytochrome f was capable of reconstitutive activity. The addition of plastoquinone and lipid showed a four fold increase in the activity.

A comparison of the reconstitutive activities of the Chain preparation and the preparation reported here is shown in Table IV. Since the methods for assaying activity are vastly different, only relative differences can be compared. Some of the major differences between the two preparations is the amount of plastoquinone present in the deficient complex. Chain reports a PQ/cytochrome f ratio of 0.12 while the preparation used in the present study contains 0.6 PQ/cytochrome f. These numbers become even more interesting when comparing the amount of plastoquinone required for maximum reconstitutive activity. The preparation of Chain required the addition of 1 PQ/cytochrome f, while the procedure here requires 2 PQ/cytochrome f. The reason for this difference is not readily apparent. Direct comparisons may prove dangerous as different types of plastoquinone compounds were used for reconstitution and different quinols were used as electron donors. The differences may be only a difference in affinity of the plastoquinone for the complex or of the quinols for the plastoquinones. One must also question how much of the added quinone is actually bound to the enzyme complex and what affect the free quinone has on the assay, as increased plastoquinone concentration causes a decrease in the maximum reconstitutive activity in both preparations.

Perhaps the most significant information that can be obtained from a comparison of the two preparations is the requirement for preincubation of the preparation reported here with the plastoquinone prior to the addition of lipid. The Chain preparation did not require preincubation, indicating that there may be two distinct

TABLE IV

COMPARISON OF THE RECONSTITUTIVE ACTIVITIES
OF CHAIN VS. DOYLE AND YU

Treatment	Relative Activity[A] Chain	Relative Activity[B] Doyle and Yu
Cyt. b_6-f	1.00	1.00
Cyt. b_6-f + PQ	1.86	1.37
Cyt. b_6-f + lipid	2.16	3.29
Cyt. b_6-f + PQ +lipid	3.88	8.54
Cyt. b_6-f + lipid + PQ	3.88	5.37

A: Relative activity of 1.00 corresponds to an activity of 5.0 umol plastocyanin reduced/nmol f/hr using duroquinol as the electron donor.

B: Relative activity of 1.00 corresponds to an activity of 1.0 umol cytochrome c reduced/nmol f/hr using plastonquinol-2 as the electron donor.

plastoquinone binding sites in the cytochrome b_6-f complex. One of these sites may be easily accessible to lipid, while the other is not. The site that is not accessible in the presence of lipid is probably more difficult to remove from the enzyme than the easily accessible site. If the procedure of Chain had only removed the easily accessible plastoquinone, then that would explain that the 1 plastoquinone per cytochrome f required for reconstitution and the lower reconstitutive activity. The only problem is the determination of 0.12 moles of plastoquinone per mole of cytochrome f in the enzyme complex. Another explanation could be that one of the plastoquinone binding sites, the inaccessible site, has been altered during isolation, so that it no longer binds plastoquinone. This explanation fits all of the observed data.

The hypothesis of two plastoquinone binding sites on the cytochrome b_6-f complex fits the data presented here. When the lipid is added prior to the addition of plastoquinone, only 60% of maximal reconstitutive activity is obtained. This activity represents the binding site that is accessible in the presence of lipid. When preincubated with plastoquinone prior to the addition of lipid, a higher activity is obtained, indicating the occupation of all the plastoquinone binding sites.

Plastoquinone Binding

The development of a procedure to isolate the cytochrome b_6-f complex in a lipid and plastoquinone deficient form made it possible to study the plastoquinone binding properties of the enzyme complex. Initial experiments done using the preparation of Hurt and Hauska

(52,61), and the azido-Q derivative showed no inhibition of enzymatic activity, although some incorporation of the azido-Q derivative was evident. This incorporation appeared to be mostly in the cytochrome b (Mr=23500) and the iron sulfur protein (Mr=20000), which was in agreement with the previous reports, labeling with an arylazido-plastoquinone derivative (53). The fact that neither of these labels caused a decrease in the enzymatic activity was distressing, as covalent attachment of the plastoquinone label would be expected to cause a loss of activity. It was for this reason that these experiments were attempted on the lipid and plastoquinone deficient complex. The results, given in Chapter III, indicate the necessity of removing the bound lipid and quinone for true labeling of the plastoquinone binding site. The azido-group is a highly reactive species once activated by light, and will react with whatever is in close proximity. That is why it is extremely important to demonstrate that the quinone derivative is occupying the quinone binding site, prior to illumination.

The notion of two distinct plastoquinone binding sites, discussed previously, comes into play again here. The cytochrome b_6-f complex showed no significant incorporation of the labeled plastoquinone derivative in the cytochrome b, whereas the cytochrome b incorporates the label in the mitochondrial cytochrome b-c_1 complex (35), as well as the R.sphaeroides cytochrome b-c_1 complex (47) and the yeast mitochondrial ubiquinol-cytochrome c reductase (36). If, as mentioned previously, only one type of plastoquinone binding site has been depleted of plastoquinone, then a second site on the cytochrome b may exist. This second site may still be occupied by

plastoquinone, making it inaccessible to the photoaffinity label.

In order to test this hypothesis of a second plastoquinone binding site, it will be necessary to remove the remaining bound plastoquinone. This must be done without destroying the reconstitutive nature of the enzyme. This task may prove difficult to do, but some possible methods include: more extensive washing on the calcium phosphate column; repeated ammonium sulfate fractionation; or sucrose gradient ultracentrifugation. Further analysis of the lipid remaining bound to the enzyme may also aid in the choice of a procedure, as the remaining lipid may be unique to the plant system.

Electron Paramagnetic Resonance

The goal of the EPR studies performed here was to 1) compare and contrast the general features to the cytochrome b-c_1 complexes from photosynthetic bacteria and mitochondria; 2) determine the effect of lipid and plastoquinone in a defined system and their correlation to enzymatic activity; and 3) examine the plastosemiquinone radical signal in detail. The general features of the cytochrome b_6-f complex have been detailed previously (92,107), both indicate the possibility of two different cytochrome b species. Salerno et. al. (92) infer a second cytochrome b based on the similarities of the spectra with that of lipid-extracted succinate cytochrome c reductase cytochrome b_{562} and b_{566} which showed a single asymmetric peak. They suggest that the cytochrome b_{563} of the cytochrome b_6-f complex may be substantially modified during isolation. The data presented here would indicate that the single asymmetric peak is due to lipid and

plastoquinone depletion, which is associated with a loss of enzymatic activity. Reconstitution to the active form does indeed cause the appearance of a second, spectrally distinct cytochrome b.

Another study by Bergstrom et. al. on the EPR spectrum of cytochrome b_{563} on the cytochrome b_6-f complex combined optical and EPR studies to show that the two cytochromes b_{563} in the isolated cytochrome b_6-f complex are both in the low spin state with g_z about 3.5 (107). Two spectrally distinct b-cytochromes, with different redox potentially have been demonstrated. Further study by Nitschke and Hauska (109), showed that the high spin form of the cytochrome b at g=6 is apparently due to inactivation or denaturation of the cytochrome b and that the active cytochrome b is indeed in the low spin state. Their results show that this inactive, high spin form of the cytochrome b increases with time after isolation of the cytochrome b_6-f complex by their method. This indicates that their method of isolation may be partially delipidating their enzyme, and the soybean lecithin that is used to retain activity on sucrose gradients may be insufficient to prevent reversible denaturation.

A comparison of the EPR spectra of the cytochrome b_6-f complex from spinach chloroplasts with those from the mitochondrial and photosynthetic bacterial cytochrome b-c_1 complexes shows that the basic characteristics for theses complexes are the same. All three systems show a cytochrome f (c_1) signal at g-values of approximately 3.5. They show three iron sulfur signals in the g-value range of 1.8-2.1, with the appearance of a semiquinone radical under appropriate conditions.

Some differences in the EPR characteristics of the cytochrome

b_6-f complex from spinach chloroplasts and the cytochrome b-c_1 complex from R.sphaeroides are readily evident. Figure 16 shows a comparison of the fully oxidized EPR spectra of the cytochrome b_6-f complex with the fully oxidized spectra from the R.sphaeroides cytochrome b-c_1 complex. The first aspect that catches the eye is the size of the cytochrome f signal. The significance of this peak height can not be readily assessed as different enzyme concentrations were used for the two enzymes. The cytochrome f signal does appear to be a much sharper signal than that of its bacterial counterpart, and has a slightly higher g-value (g=3.50) than the cytochrome c_1 (g=3.40). The bacterial spectra shows a signal at g=3.75 which is not observed in the cytochrome b_6-f spectra. This signal at g=3.75 has been attributed to cytochrome b_{566} (106).

Figure 17 shows a comparison of the EPR spectra of the bacterial cytochrome b-c_1 and the lipid and plastoquinone reconstituted chloroplast cytochrome b_6-f complex in the ascorbate reduced state. The most obvious difference in the two spectra is the distance between the two b-cytochrome signals. The bacterial b-cytochromes are completely distinguishable, while the chloroplast b-cytochromes are overlapping. Potentiometric titrations of the bacterial b-cytochromes indicate that the b_{561} (g=3.4 signal) has a midpoint potential of +50 mV and the b_{566} (g=3.75) has a midpoint potential of -50 mV. The cytochrome b_6-f complex has a high-potential cytochrome b_{563} (g=3.45) with a midpoint potential of approximately +70 mV and a low potential b_{563} (g=3.53) with a midpoint potential of approximately -50 mV (92, 107).

Figure 18 is a comparison of the iron sulfur signals of the

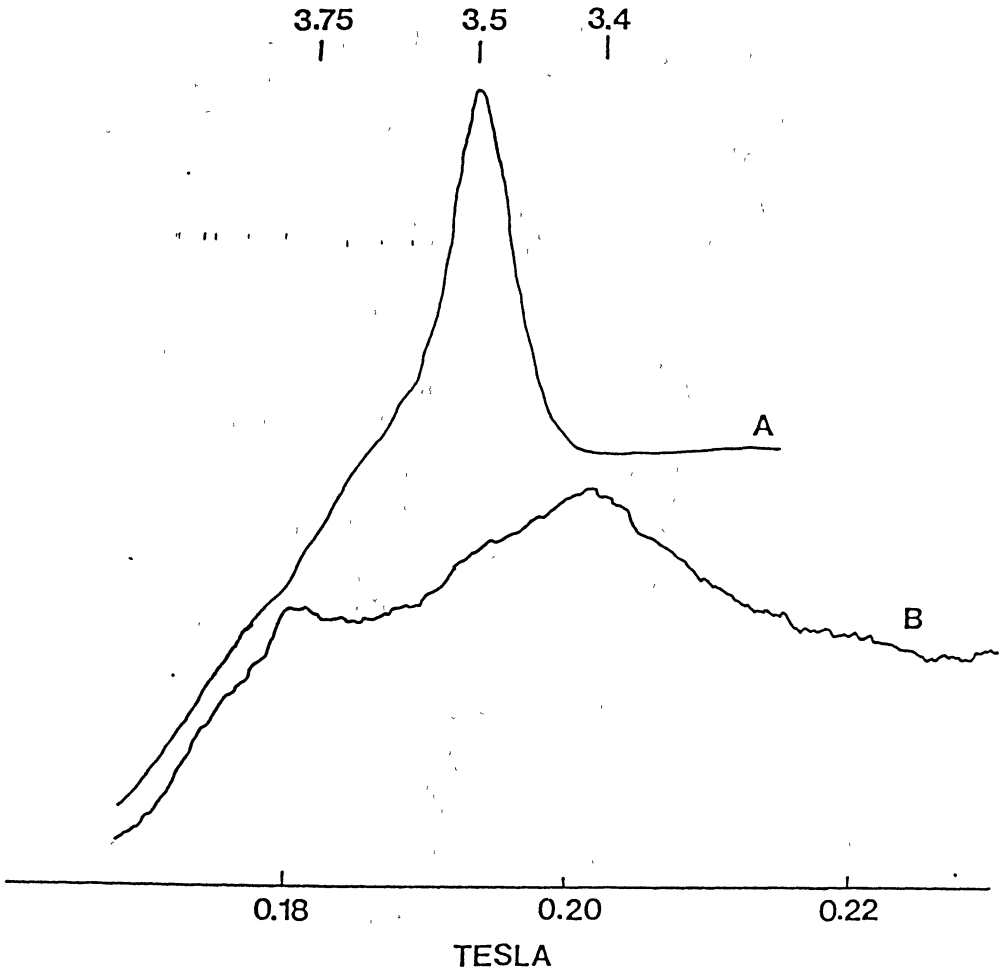

Figure 16: A comparison of the Fully Oxidized EPR spectra of the Cytochrome b_6-f Complex from Chloroplasts and the Cytochrome b-c_1 Complex from R.sphaeroides. The upper graph (A) represents the reconstituted bytochrome b_6-f complex with machine settings as in Figure 10. The lower graph (B) represents the cytochrome b-c_1 complex at a microwave power of 20 mW and a sample temperature of 15°K.

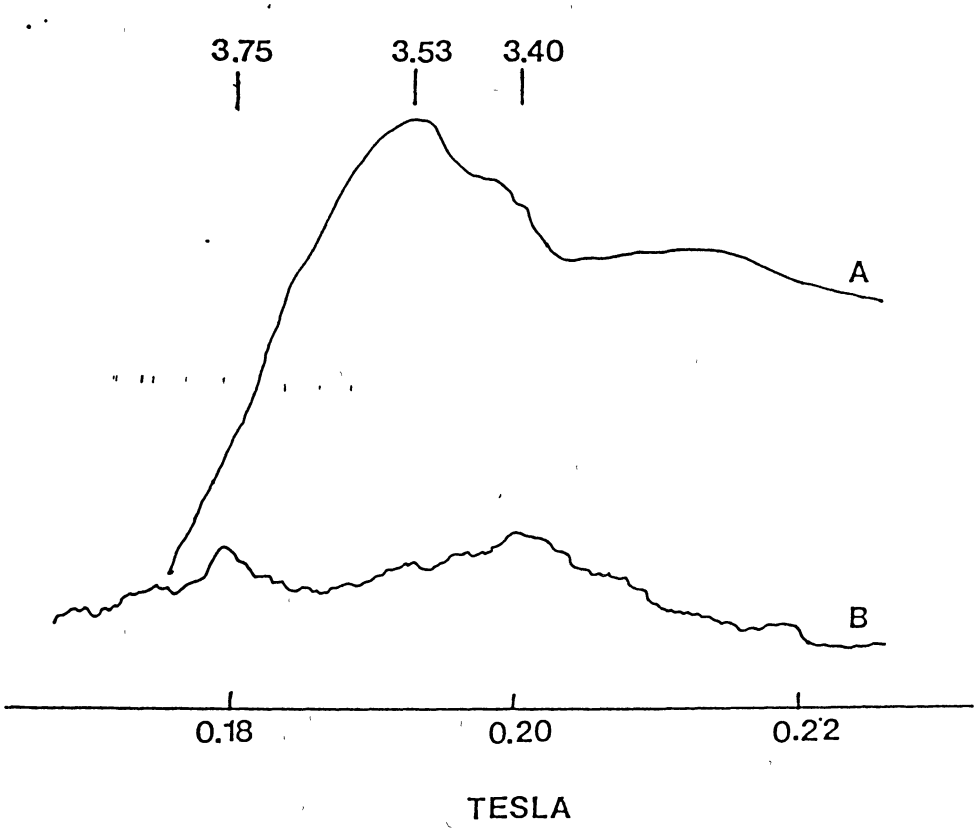

Figure 17: A Comparison of Ascorbate Reduced EPR Spectra of the Cytochrome b_6-f Complex from Chloroplasts and the Cytochrome b-c_1 Complex from R.sphaeroides. Spectrum A is the reconstituted cytochrome b_6-f complex. Spectrum B is the cytochrome b-c_1 complex.

bacterial and chloroplast cytochrome b-c_1 (f) complexes. The most striking features are the broadness of the g_z signal in the cytochrome b_6-f complex compared to the cytochrome b-c_1 complex, and the presence of the g=2.005 signal in the cytochrome b_6-f complex corresponding to a plastosemiquinone radical, which is absent in the bacterial complex. The plastosemiquinone signal was found to be sensitive to certain electron transfer inhibitors. The exact nature of their inhibition and its affect on the plastoquinone signal remains to be investigated. The argument put forth previously of a second plastoquinone binding site that was not depleted during isolation may play an important role in these inhibition studies.

Summary and View for the Future

The isolation and characterization of the cytochrome b_6-f complex from spinach chloroplasts has illustrated that, although differences do exist, this complex shows a great deal of similarity to the mitochondrial and bacterial cytochrome b-c_1 complexes. These integral membrane protein complexes have a requirement for lipid,although the exact requirement appears to vary according to the source of enzyme. The chloroplast cytochrome b_6-f complex appears to have a greater requirement for glycolipids than for phospholipid. Bound quinone appears to be required for electron transport through the enzyme complexes. A low molecular weight protein (Mr=15000 to 17500) functions as a quinone-binding protein. The cytochrome b_6-f complex does not show any involvement of the cytochrome b in quinone binding, whereas the cytochrome b from mitochondrial and bacterial

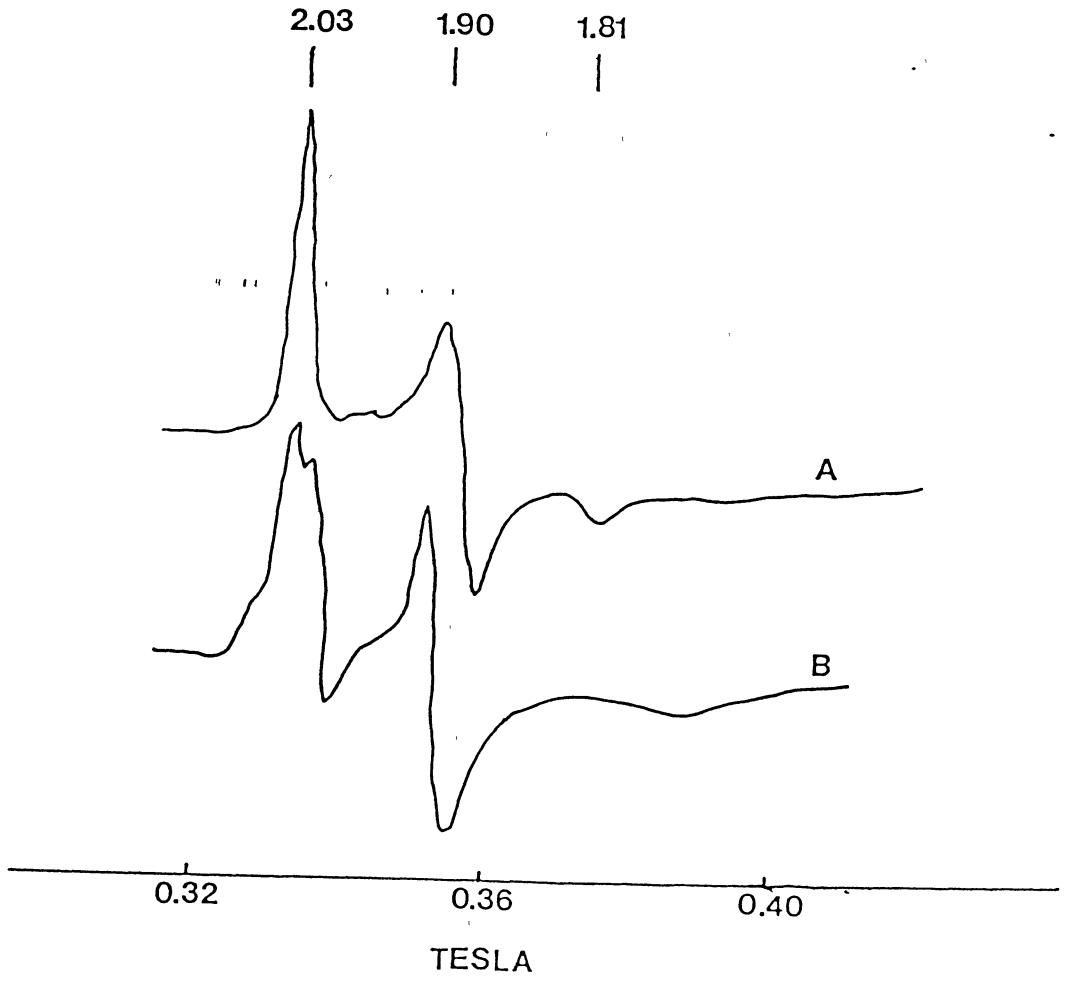

Figure 18: A Comparison of the EPR Spectra of the Iron Sulfur Proteins from the Cytochrome b_6-f Complex from chloroplasts and the Cytochrome b-c_1 Complex from R.sphaeroides. Spectrum A is the cytochrome b-c_1 complex with the microwave power at 2 mW. Spectrum B is the cytochrome b_6-f complex.

cytochrome b-c_1 complexes does appear to play a role in quinone binding. The EPR characteristics of the reconstituted cytochrome b_6-f complex are similar to those described for the mitochondrial and bacterial enzymes, with the only major difference being in the b-cytochromes. The cytochrome b_6-f complex shows a plastosemiquinone signal characteristic of a quinone-protein interaction.

Future experiments on this system should center around the theory of two plastoquinone binding sites. Total extraction of the plastoquinone from the complex would allow for further studies into the possibility of two plastoquinone binding sites and determining the location of the second site. The exact nature of the lipid required for reconstitutive activity could yield useful structural information. The coupling of enzymatic and circular dichroism spectra would allow insight into the alteration in structure of the individual components of the enzyme complex upon reconstitution. These experiments would require a lipid preparation that is totally free of any chlorophyll or caratenoids.

Another line of experimentation would explore the ability to isolate the individual components of the cytochrome b_6-f complex in such a manner that would allow for reconstitution of the activity. An important component to isolate would be the Mr=17000 protein responsible for plastoquinone binding. Proteolytic digests could yield information as to the portion of the protein responsible for plastoquinone binding. Also, the generation of monoclonal antibodies to these components could yield useful information on the structure function relationships of the constituents.

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