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The characterization and localization of DCCD binding to cytochrome *b* from the *b*-c_1-complex of yeast mitochondria

DeLoskey, Richard James, Ph.D.

City University of New York, 1990

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300 N. Zeeb Rd.
Ann Arbor, MI 48106

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THE CHARACTERIZATION AND LOCALIZATION OF DCCD BINDING TO CYTOCHROME b FROM THE b-c_1-COMPLEX OF YEAST MITOCHONDRIA

by

Richard DeLoskey

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Executive Officer

Arthur Cederbaum

Heng-Chung Li

Carolyn Slayman
Supervisory Committee

The City University of New York

Abstract

THE CHARACTERIZATION AND LOCALIZATION OF DCCD BINDING TO CYTOCHROME b FROM THE b-c_1-COMPLEX OF YEAST MITOCHONDRIA

by

Richard DeLoskey

Advisor: Diana S. Beattie, Ph.D.

Dicyclohexylcarbodiimide (DCCD) binds to cytochrome b resulting in the inhibition of H^+-translocation in yeast b-c_1 complex without significantly inhibiting electron transfer activity.

The approach to determining which carboxy amino acid of cytochrome b binds DCCD was to cleave cytochrome b into peptide fragments, purify these peptides, and determine the amino acid sequence of the peptide fragments with bound [^{14}C] DCCD. Finally the amino acid sequencing results could be compared with the sequence of cytochrome b deduced from the DNA sequence.

Three methods of isolating cytochrome b from b-c_1 complex were used: preparative 12.5% SDS PAGE, molecular sieve HPLC, and chromatography on phenyl-sepharose CL-4B. The latter two methods did not resolve cytochrome b from all the other subunits of the b-c_1 complex. In contrast, cytochrome b could be resolved from the other b-c_1 complex subunits by preparative 12.5% SDS PAGE.

Cytochrome b labeled with [^{14}C] DCCD extracted from preparative gels was cleaved at methionine residues using CNBr. Two methods were used to purify the resultant fragments of cytochrome b: C_{18} reverse phase HPLC and SDS-urea (SUDS) PAGE.

C_{18} reverse phase chromatography did not resolve the radioactive CNBr fragments of cytochrome b. Also the recovery of total radioactive counts loaded onto the column was at best 41%. When CNBr fragments of [^{14}C] DCCD labeled cytochrome b were chromatographed by SUDS PAGE, two radioactive bands migrating with apparent molecular weights of 10.7 kDa and 5.2 kDa were observed.

The effects of different b-c_1 complex inhibitors and ubiquinone analogues have on the binding of DCCD to cytochrome b were investigated. Antimycin, HQNO, BAL, DBH_2, and Q_6H_2 clearly inhibit [^{14}C] DCCD binding to cytochrome b. n-HNQ, EFA, DNFB, and DQH_2 all stimulate [^{14}C] DCCD binding. Myxothiazol may result in a slight inhibition of DCCD binding.

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I. INTRODUCTION

A. THE MITOCHONDRIAL ELECTRON TRANSPORT CHAIN AND OXIDATIVE PHOSPHORYLATION SYSTEM

Mitochondria are rod-like organelles found in the cytoplasm of eukaryotic cells. The major function of these organelles is to perform the final oxidation of carbohydrates and lipids to produce adenosine triphosphate (ATP)[1] as a readily availible energy source for the cell. The transduction of the energy released by these oxidative reactions to the phosphate bond of ATP is called oxidative phosphorylation. This process involves electron transfer by the respiratory chain resulting in a vectorial translocation of protons across the inner mitochondrial membrane. The protonic energy is then used to phosphorylate adenosine diphosphate (ADP) to form ATP (Boyer et. al., 1977). The protonmotive force is also utilized for ion translocation and protein import (Hatefi, 1985).

The respiratory chain has been fractionated into four distinct protein-lipid enzyme complexes: NADH-ubiquinone oxidoreductase (complex I), succinate-ubiquinone oxidoreductase (complex II), ubiquinol-cytochrome c oxidoreductase (complex III), and ferrocytochrome c-oxygen oxidoreductase (complex IV). ATP synthase or complex V is responsible for coupling of the electron transport chain to ATP synthesis

(Stiggall et. al., 1978 & 1979). The functional interaction of the five enzyme complexes of the mitochondrial oxidative phosphorylation system is shown in Fig. 1. The respiratory chain consists of three regions of quasi-equipotential as indicated by the E_m values at the top of Fig. 1. Each of the quasi-equipotential regions has an energy change of less than 100 mV, but the energy difference between these regions appears to be greater than 100 mV (Hatefi, 1985). This figure also shows that energy of the oxidation-reduction reactions is conserved via coupled vectorial proton translocation in complexes I, III, and IV.

NADH-ubiquinone oxidoreductase (complex I) catalyzes the oxidation of NADH and the reduction of ubiquinone with concomitant vectorial translocation of protons coupled to electron transfer (Hatefi et. al., 1976; Ragan & Hinkle, 1975; and Hatefi & Stiggall, 1976). Both activites, electron transfer and proton translocation, are inhibited by rotenone. Complex I consists of approximately 25 different polypeptide subunits (Hatefi et. al., 1979; Heron et. al., 1979; Earley & Ragan, 1981; and Hare & Hodges, 1982). The nonprotein components of complex I are noncovalently-bound FMN, nonheme iron (Fe), acid-labile sulfide (S), ubiquinone, and phospholipids. The ratio of FMN: Fe: S in complex I is 1: 22-24: 22-24. The non-heme iron and the acid-labile sulfide are the components of the eight iron-sulfur clusters in complex I (five binuclear [2Fe-2S] and three tetranuclear

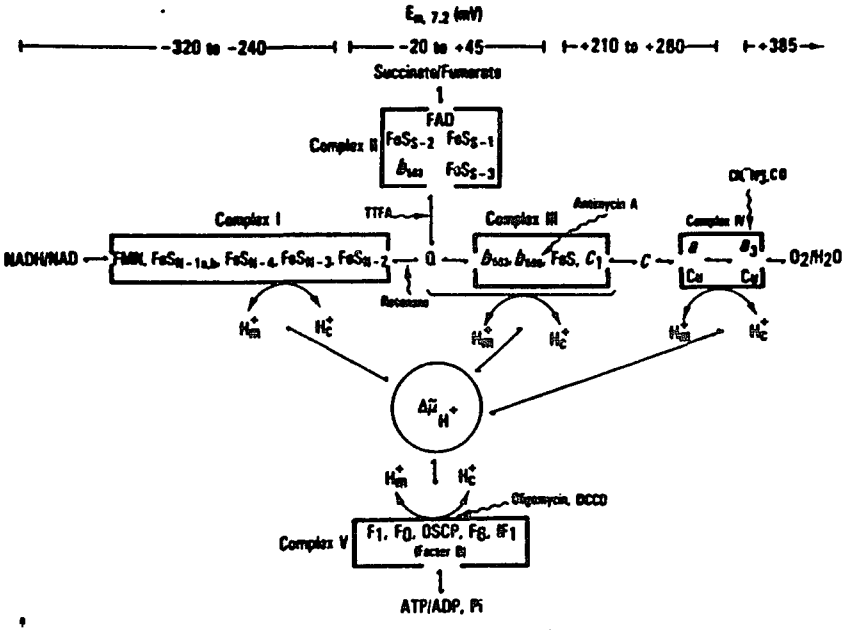


Figure 1 Profile of the mitochondrial electron transport-oxidative phosphorylation system, showing the well-characterized components of complexes I, II, III, IV, and V, and energy communication by way of $\Delta\tilde{\mu}_{H^+}$ among the energy transducing complexes I, III, IV, and V. FeS, iron-sulfur cluster [only the ESR-detectable clusters are shown; FeS subscripts denote individual FeS clusters according to the nomenclature of Ohnishi, ref. (65)]; *a*, *b*, and *c*, cytochromes *a*, *b*, and *c*, respectively (subscripts on *b* cytochromes denote position of their α peaks at ambient temperatures); H_m^+ and H_c^+, protons on the matrix and cystolic sides of the mitochondrial inner membrane; TTFA, thenoyltrifluoroacetone. For other notations, see abbreviations list and text. The E_m scale is applicable to all components of the respiratory chain, except FeS_{N-1a} (apparent $E_m \leq -400$ mV), FeS_{S-2} (apparent $E_m \sim -400$ mV), and b_{560} (apparent $E_m < -100$ mV).

Figure 1. The mitochondrial oxidative phosphorylation system. Figure 1 of Hatefi, Y. (1985) Ann. Rev. Biochem. 54, 1015-1069.

[4Fe-4S]). The reported molecular weight of complex I ranges from 700,000 to 900,000 depending on the preparation of purified complex.

Succinate-ubiquinone oxidoreductase (complex II) catalyzes the oxidation of succinate and the reduction of ubiquinone. The enzymatic activity of Complex II is inhibited by 2-thenoyltrifluoroacetone. Complex II contains succinate dehydrogenase and a low potential cytochrome b (b_{560}), consisting of two low molecular weight proteins, 15.5 and 13.5 kDa (Hatefi and Galante, 1980). Succinate dehydrogenase (SDH) is composed of equimolar amounts of two subunits: a large subunit of 70 kDa and a small subunit of 27 kDa. (Hatefi & Stigall, 1976; Hatefi & Hanstein, 1974; and Davis & Hatefi, 1971). SDH also contains one covalently bound FAD, 7-8 iron, and 7-8 acid-labile sulfides. Results from ESR studies suggest that iron and sulfide form FeS clusters of two types: two binuclear and one tetranuclear (Ohnishi & Salerno, 1982; Beinert & Albracht, 1982; and Ohnishi, 1979). The cytochrome b_{560} found in complex II is a cytoribosomal product, unlike the cytochrome b found in complex III which is encoded by the mitochondrial genome (Weiss & Kolb, 1979). Hatefi and Galante (1980) purified cytochrome b from an intact preparation of complex II isolated from beef heart mitochondria. The cytochrome b isolated from this complex II could be reconstitiuted with succinate dehydrogenase to obtain succinate-ubiquinone oxidoreductase activity.

Ubiquinol-cytochrome c oxidoreductase, complex III, catalyzes the reduction of cytochrome c by ubiquinol coupled to the vectorial translocation of protons (Leung & Hinkle, 1975). Complex III will be described in detail in the following section of this chapter.

Ferrocytochrome c-oxygen oxidoreductase, complex IV, catalyzes the oxidation of reduced cytochrome c by molecular oxygen with vectorial proton translocation coupled to electron transfer (Wikstrom & Krab, 1979). The enzymatic activity of complex IV is inhibited by carbon monoxide, cyanide, and azide. Complex IV preparations from bovine heart, rat liver, human placenta, S. cerevisiae, and N. crassa all contain 7-8 subunits of comparable size (Azzi, 1980). Complex IV purified from yeast mitochondria contains seven polypeptide subunits: three large subunits (I, 40,000; II, 33,000; and III, 22,000) are synthesized in the mitochondria whereas the four small subunits (IV, 14,500; V, 13,700; VI, 12,500; and VII, 4,600) are synthesized in the cytoplasm (Azzi, 1980; Poyton & Schatz, 1975; and Tzagoloff et al., 1979). The non-protein components of complex IV consist of two hemes, a and a_3, each of which is associated with an atom of copper. The heme a-copper complex (Cu_a) oxidizes cytochrome c and the heme a_3-copper complex (Cu_{a3}) reduces molecular oxygen to form water (Wilson et. al., 1975; Greenwood et. al., 1976; Beinert et. al., 1976; Chance et. al., 1975; and Chance et. al., 1978).

Cu_a and Cu_{a3} are associated with subunits I and II of complex IV (Winter et. al., 1980). The molecular weight of a monomer of complex IV is a minimum of 140,000 (Tzagoloff, 1979).

Lastly, ATP synthase or complex V catalyzes the synthesis of ATP from ADP and inorganic phosphate by utilizing the protonmotive force derived from the translocation of protons by the respiratory complexes I, III, and IV. Protons are transported by the respiratory complexes from the matrix to the cyosolic side of the inner mitochondrial membrane during electron transfer down the respiratory chain. These protons are then translocated back across the membrane through the ATP synthase. The energy of the proton gradient is thus coupled to ATP synthesis. Complex V can also hydrolyze ATP which results in the translocation of protons from the matrix to the cytosolic side of the inner mitochondrial membrane. Both the synthetic and hydrolytic activites of this enzyme complex are inhibited by oligomycin, rutamycin, and dicyclohexylcarbodiimide (DCCD).

Mitochondrial ATP synthase is composed of three sectors: the F_1 or catalytic sector, which can be isolated as a soluble active ATPase; the stalk region, which binds F_1 to the membrane; and the F_0 or membrane sector, which is responsible for proton translocation. ATP synthase isolated from yeast mitochondria consists of 9-13 subunits (Hartzell et. al., 1978 and Ryrie & Gallagher 1979). The F_1 sector is

comprised of five subunits with the following molecular weights: 1 (ã), 52,000; 2 (ß), 48,000; 3 (Ÿ), 31,000; 4 (õ), 14,500; and 5 (Ë), 10,700 (the Greek letters are the corresponding subunits found in mammalians). The 23,000 M_r subunit is the oligomycin-sensitivity-conferring-protein and forms the stalk region. The F_0 sector consists of five subunits with molecular weights of 28,500, 24,500, 21,500, 16,700, 12,700, 9000, and 7000 (Ryrie & Gallagher, 1979 and Ludwig et. al., 1980). The 9000 da and the 7000 da protein subunits correspond to the DCCD binding protein and the ATPase inhibitor protein respectively (Ludwig et. al., 1980 and Bowman & Bowman, 1986). Oligomycin also binds to the 9000 Da subunit of the F_0 membrane sector (Kiehl & Hatefi, 1980). The F_1 subunits are all coded by the nuclear genome whereas several of the F_0 subunits, including the DCCD-binding protein in yeast, are encoded by the mitochondrial genome (Bowman & Bowman, 1986). The stoichiometry of F_1 is $ã_3ß_3ŸõË$, but the stoichiometry for F_0 is not certain.

B. THE $\underline{b}$-$\underline{c}_1$ COMPLEX

The $\underline{b}$-$\underline{c}_1$ complex, ubiquinol-cytochrome $\underline{c}$ oxidoreductase or complex III, catalyzes the reduction of cytochrome $\underline{c}$ by ubiquinol. The $\underline{b}$-$\underline{c}_1$ complex isolated from yeast mitochondria contains at least nine subunits with the following molecular weights: I (core I), 49,000; II (core II), 39,000; III (cytochrome $\underline{b}$), 30,000; IV (cytochrome $\underline{c}_1$), 29,000; V (iron-sulfur protein), 22,400; VI (the proposed Q-binding protein), 13,400; VII, 11,100; VIII, 10,000; and IX, 9,000 (Sidhu & Beattie, 1982 and Beattie et. al., 1984). The redox centers of the $\underline{b}$-$\underline{c}_1$ complex include cytochromes $\underline{b}_{562}$, $\underline{b}_{566}$, and $\underline{c}_1$ and a binuclear [2Fe-2S] iron-sulfur cluster. In addition, the $\underline{b}$-$\underline{c}_1$ complex contains a lipid soluble electron carrier ubiquinone. The subunit stoichiometry varies depending upon the source of the isolated complex. The stoichiometry of the complex isolated from yeast has been reported to be two copies of core I (subunit I) and one copy of the remaining subunits (II-VII) (Sidhu and Beattie, 1982). In contrast, the subunit stoichiometry for the complex isolated from bovine and N. crassa has been reported to be 1:1:2:1:1:1:2:2 and 1:2:2:1:1:1:1:1, in decreasing order of molecular weight, respectively (Marres & Slater, 1977 and Weiss & Kolb, 1979).

The molecular weight of the $\underline{b}$-$\underline{c}_1$ complex is 200,000-250,000 as calculated from the heme content and the subunit stoichiometries (Marres & Slater, 1977; Katan et. al., 1976;

and von Jagow *et. al.*, 1978). Interestingly, the molecular weight of the *b*-c_1 complex isolated from yeast mitochondria as determined by molecular seive chromatography (Ultrogel AcA34) was approximately 450,000 (Sidhu & Beattie, 1982) suggesting that the *b*-c_1 complex may exist as a dimer in the membrane. Similar conclusions were drawn for the *b*-c_1 complex isolated from beef heart and *N. crassa* mitochondria (Hauska *et. al.*, 1983). Nalecz *et. al.* (1985) recently reported the isolation of monomeric and dimeric forms of the *b*-c_1 complex from beef heart mitochondria. The conversion of the monomer to the dimer required the addition of 50 mM KCl and was reversible by merely removing the salt. The two forms were both enzymatically active and sensitive to enzymatic inhibitors, but the dimeric form had a higher enzymatic activity than the monomeric form (Nalecz & Azzi, 1985).

Cytochrome *b*. The most extensively studied component of the *b*-c_1 complex is cytochrome *b*. It was first described and defined by Keilin and Hartree (1939) as having the following characteristics: it has (1) the lowest redox potential of any of the mitochondrial cytochromes, 2) an absoption spectrum with an ã-band around 560-564 nm, and 3) a lack of reactivity with CN^- or CO. Similar *b*-type cytochromes have been found in chloroplasts, bacterial membranes, and other non-mitochondrial organelles. The

respiratory chain in mitochondria contain three spectrally different cytochrome b's with ã-absorption bands of 558, 562, and 566 nm. The cytochrome b_{558} is attributed to cytochrome b found in succinate-ubiquinone oxidoreductase, while the other two b-type cytochromes correspond to the two b cytochromes found in the b-c_1 complex (Subik et. al., 1981). The redox midpoint potentials of cytochromes b_{562} and b_{566} in yeast mitochondria are +65 and -20 mV respectively (T'sai and Palmer, 1982); in beef heart mitochondria +93 and -34 mV (Nelson and Gellerfors, 1974); and in rat liver mitochondria +35 and -53 mV (Wilson and Dutton, 1970. The midpoint potentials for both forms of cytochrome b, in mitochondria and isolated b-c_1 complexes, are pH dependent. The potentials are approximately 40 mV more negative at pH 8 than at pH 7. This result suggests that cytochrome b may be involved in proton translocation during electron flow through the b-c_1 complex.

The two different forms of cytochrome b can also be distinguished by using specific inhibitors that cause different spectral shifts. Antimycin, the classical inhibitor of electron flow through the b-c_1 complex, binds to the isolated complex and results in a "red shift" of the ã-band of cytochrome b_{562} from 562 to 564 nm. The spectrum of cytochrome b_{566} is not altered by antimycin. Interestingly, the amount of antimycin bound to the b-c_1 complex is equimolar to cytochrome c_1. Hence, antimycin only binds to

half of the total cytochrome b in the b-c_1 complex (Dutton et. al., 1972).

Myxothiazol, an antibiotic produced by the myxobacterium Myxococcus fulvus, binds to cytochrome b_{566} resulting in a "red shift" of about 2 nm (von Jagow and Engel, 1981; von Jagow et. al., 1984). The spectral changes induced by myxothiazol are different and independent of the spectral shifts induced by antimycin. Therefore, the two inhibitors intereact with different forms of cytochrome b, myxothiazol with b_{566} and antimycin with b_{562}.

Despite the evidence for two different forms of cytochrome b in the b-c_1 complex of mitochondria, detergent solubilization of the b-c_1 complex resulted in the isolation of one species of cytochrome b having an ã-absorption maximum of 561- 562 nm (Lin and Beattie, 1978; T'sai and Palmer, 1982). Similar results were obtained for b-c_1 complex isolated from beef heart (Goldberger et. al.,1961; von Jagow et. al., 1978) and from N. crassa (Weiss and Ziganke, 1976). Recently, a study showed that removal of lipid from the isolated b-c_1 complex resulted in the disappearance of the spectral differences between the two b-hemes, similar to the ã-absorption spectra of purified cytochrome b (Salerno et. al., 1986).

The isolation of only one species of cytochrome b suggested that the two spectrally different forms of cytochrome b present in the b-c_1 complex may result from two copies of

the same or similar proteins in which the b-hemes are in different environments. The controversy of whether cytochrome b exists as a hetero- or a homo- dimer was resolved by the discovery of only one gene for cytochrome b in the mitochondrial genome of every species examined to date (Nobrega and Tzagoloff, 1980; Anderson et. al., 1981; Anderson et. al., 1982; Waring et. al., 1981; Mahler and Perlman, 1984). The amino acid sequence deduced from the DNA sequence predicts that the molecular weight of cytochrome b is 42,000. A comparison of the amino acid sequences from a number of species indicated a considerable amount of homology (Widger et. al., 1984). One interesting homology in the sequence of cytochrome b is the presence of four conserved histidine residues which are predicted using hydropathic plots to be localized within membrane spanning regions of the protein. These four histidine residues may be responsible for binding two heme ligands (Widger et. al., 1984). A single cytochrome b containing two hemes was isolated from the yeast b-c_1 complex (T'sai and Palmer, 1982). Amino acid analysis of this protein was comparable to that deduced from the DNA sequence (Nobrega and Tzagoloff, 1980). Cytochrome b apparently exists in the b-c_1 complex of mitochondria as a monomer containing the two b-hemes, b_{562} and b_{566}. Another prediction using hydropathic plots of cytochrome b is the presence of eight to nine membrane spanning regions (Saraste, 1984).

Cytochrome $\underline{c}_1$. The other major hemoprotein present in the $\underline{b}$-$\underline{c}_1$ complex is cytochrome $\underline{c}_1$. It contains a covalently attached heme group that is not autoxidizable and does not react with CO (Keilin and Hartree, 1955). Cytochrome $\underline{c}_1$ can be distinguished from cytochrome $\underline{c}$ in submitochondrial particles by spectral analysis at -190° C. At this temperature cytochrome $\underline{c}_1$ has an absorption maximum at 551-552 nm and cytochrome $\underline{c}$ has an asorption maximum at 548 nm. The midpoint potential of cytochrome $\underline{c}_1$ present in the mitochondrial membrane has been calculated to be 240 mV (Erecinska et. al., 1976), which is higher than the midpoint potential of either of the two $\underline{b}$ cytochromes.

Recently, cytochrome $\underline{c}_1$ was purified from beef heart mitochondria and sequenced (Wakabayashi et. al., 1982). The protein contains 241 amino acids and has a calculated molecular weight of 27,294. The heme group is linked via two cysteine residues, 37 and 40. The protein contains a cluster of hydrophobic amino acids near the carboxy terminus which may act to anchor the protein to the membrane (Wakabayashi et. al., 1982 and Li et. al., 1981). Amino acid analysis of cytochrome $\underline{c}_1$ from beef heart mitochondria revealed a high content of acidic amino acids (Wakabayashi, 1980).

The gene for cytochrome $\underline{c}_1$ has been sequenced from yeast

(Sadler et. al., 1984). The calculated molecular weight of the yeast protein is 27,419 and the deduced amino acid sequence contains both a hydrophilic and a hydrophobic region, similar to the beef heart protein. The hydrophilic domain contains the proposed heme binding sites. The hydrophobic domain, near the carboxy terminus, consists of 15 consecutive hydrophobic residues flanked on either side by lysine residues and has been proposed to anchor cytochrome $\underline{c}_1$ to the membrane.

Reiske iron-sulfur protein. The first suggestion that the $\underline{b}$-$\underline{c}_1$ complex may contain an iron sulfur protein was made by Hatefi et. al. (1962). They reported that the $\underline{b}$-$\underline{c}_1$ complex purified from beef heart mitochondria contained 10-13 nmol of non-heme iron/mg of protein as compared to 4.1-4.4 nmol of cytochrome $\underline{c}_1$/mg of protein in the same preparation of complex. Subsequently, Reiske et. al. (1964a) demonstrated that the $\underline{b}$-$\underline{c}_1$ complex had an epr signal with an absorption band at g = 1.90. The epr signal cofractionated with the non-heme iron after treatment of the complex with bile salts and ammoniun sulfate suggesting that the epr signal was due to the non-heme iron. In submitochondrial particles or in the isolated $\underline{b}$-$\underline{c}_1$ complex, the epr signal g = 1.90 appears upon the reduction of the complex with either ubiqunol-2 or ascorbate. The epr signal at g = 1.90 dissappears when the $\underline{b}$-$\underline{c}_1$ complex is oxidized with ferricyanide, cytochrome $\underline{c}$

alone, or cytochrome c oxidase plus cytochrome c (Reiske et. al., 1964b). Eventually, the iron sulfur protein was purified from the b-c_1 complex and the purified protein shown to have an epr signal at g = 1.90 (Rieske J.S. et. al., 1964b; Rieske J.S., 1967). This result provided conclusive evidence for the existence of a non-heme iron protein in the b-c_1 complex which undergoes both reduction and oxidation during respiration (Trumpower and Edwards, 1979).

The importance of the iron-sulfur protein for enzymatic activity of the b-c_1 complex was shown by Trumpower and Edwards in 1979. They isolated a reconstitutively active form of the iron-sulfur protein which restored succinate-cytochrome c reductase activity when added to iron-sulfur protein depleted b-c_1 complex. The purified protein had a g = 1.90 signal and migrated on SDS polyacrylamide gels with an apparent molecular weight of 24,500.

The evidence obtained to date suggests that the iron-sulfur protein present in the b-c_1 complex is a monomeric protein with one binuclear iron sulfur cluster (2Fe-2S) and has a midpoint potential of about 280 mV (Reiske, 1976; Trumpower, 1981).

Recently, the gene for the iron-sulfur protein has been isolated and sequenced from N. crassa (Harnisch et. al., 1985) and from yeast (Beckmann et. al., 1986). The gene sequence of yeast codes for a protein of 180 amino acid

residues. The molecular weight of the protein is calculated from the gene sequence to be 21,946. This value compares well with the molecular weight of 21,800 as determined by SDS PAGE (Beattie et. al., 1984).

The core proteins. The two largest proteins of the $\underline{b}$-$\underline{c}_1$ complex have been called core proteins I and II (Baum et. al. 1967). The molecular weights of these proteins were found to be 50 and 47 kDa. respectively in the $\underline{b}$-$\underline{c}_1$ complex isolated from beef heart mitochondria (Reiske, 1976) and 47 and 39 kDa. from yeast mitochondria (Sidhu and Beattie, 1982).

The importance of these two proteins for the catalytic activities of the $\underline{b}$-$\underline{c}_1$ complex is still not understood. Antibodies raised against the core proteins from both beef heart (Mendel-Hartwig and Nelson, 1981) and yeast (Sidhu and Beattie, 1982) $\underline{b}$-$\underline{c}_1$ complexes did not inhibit electron transport in either submitochondrial particles, mitoplasts or the isolated complex. These results could be interpreted in two different ways; 1) the core proteins are not directly involved in the catalytic activity of the complex or 2) the core proteins are inaccessible to the antibodies. Evidence that the core proteins are not directly involved in catalytic activity was reported by Mendel-Hartwig and Nelson (1983). They subjected submitochondrial particles to proteolytic digestion which removed 50% of the core proteins

from the matrix side of the membrane but did not result in the loss of NADH oxidation.

The core proteins, however, are necessary for a functional b-c_1 complex as demonstrated by Linke and Weiss (1986). They separated the b-c_1 complex from N. crassa into three fractions, a b-c_1 subcomplex, a fraction containing the two core proteins, and the iron sulfur protein. Reconstitution of ubiquinol-cytochrome c oxidoreductase activity required all three subcomplexes.

Interestingly, the b-c_1 complexes from chloroplasts or P. denitrificans do not contain any proteins corresponding to the core proteins found in mitochondrial complexes, but catalyze the same reactions as the b-c_1 complexes in mitochondria. Therefore the functional role of the core proteins in mitochondrial b-c_1 complexes is not known, but these proteins are essential for a catalytically active b-c_1 complex.

Q-binding Protein. The Q-binding protein was initially described by Yu and Yu (1982) in a b-c_1 complex isolated from beef heart mitochondria. Photoaffinity aryl azidoubiquinones were synthesized and shown to restore succinate-cytochrome c reductase activity in a lipid depleted b-c_1 complex. After illumination, the [^{3}H]-aryl azidoubiquinone derivative was bound to two proteins having molecular weights of 34,000 (cytochrome b) and 15-17,000 (Yu and Yu,

1982 a,b).

Similar studies were performed on the $\underline{b}$-$\underline{c}_1$ complex of yeast and the labeled ubiquinone bound to both cytochrome $\underline{b}$ and to a 14,000 Da. protein called the Q-binding protein (Yu et. al., 1986).

The so-called Q-binding protein has been isolated from beef heart mitochondria and sequenced. It contains 110 amino acid residues and has a calculated molecular weight of 13,389 (Wakabayashi et. al., 1985). The protein from beef heart is very similar to the 14,500 Da. subunit of the yeast $\underline{b}$-$\underline{c}_1$ complex as deduced from the gene sequence (de Haan et. al., 1984).

The proposed function of the Q-binding protein is to stabilize the semiquinone radical during electron transfer to the specific redox components of the $\underline{b}$-$\underline{c}_1$ complex (Mitchell, 1976; Ohnishi and Trumpower, 1980; and Yu and Yu, 1981).

Hinge Protein. Most preparations of cytochrome $\underline{c}_1$ contain a smaller protein which copurifies with it. This smaller protein has recently been purified from a preparation of cytochrome $\underline{c}_1$ by Wakabayashi et. al. (1982). The protein was first reported to have a molecular weight of 11,000 Da. but this value has subsequently been revised to 9,200 (Schagger et. al., 1986). The amino acid composition of this protein is unusual in that it does not contain methio-

nine, isoleucine, tyrosine, or tryptophan. However, it does contain a high quanitity of glutamate, 27% of the total amino acid residues or 21 glutamates out of 78 residues. Protein sequencing revealed eight consecutive glutamate residues near the amino terminus. It has been suggested that these acidic residues may intereact with basic regions of cytochromes c and c_1. In fact, a cytochrome c-c_1 complex containing equimolar amounts of the two cytochromes and the hinge protein has been isolated (Kim and King, 1983). Kim and King found that the hinge protein is required for the association of cytochromes c and c_1.

The DNA sequence of a 17,000 molecular weight protein from yeast b-c_1 complex codes for a protein containing 25 consecutive glutamate residues (van Loon et. al., 1984). This protein has considerable homology with the 9.2 kDa. protein from the beef heart complex.

Low molecular weight proteins found in beef heart but not in yeast b-c_1 complex. In the past several years the group of von Jagow has systematically purified the low molecular weight proteins of the b-c_1 complex from beef heart mitochondria (Schagger et. al., 1986). In short, the purification of all eleven subunits of the b-c_1 complex isolated from beef heart was accomplished by using hydroxapatite chromatography and various concentrations of salts, Triton

X-100, guanidine, and urea. Intially the $\underline{b}$-$\underline{c}_1$ complex was cleaved into three fractions: a 6.4 kDa protein, the "Rieske" iron-sulfur protein, and a $\underline{b}$-$\underline{c}_1$ subcomplex as shown in Fig. 2. The $\underline{b}$-$\underline{c}_1$ subcomplex can be processed further to yield a $\underline{c}_1$-subcomplex and a subcomplex containing the core proteins, cytochrome $\underline{b}$, 13.4 kDa, 11 kDa, and 8 kDa proteins. The individual proteins can be isolated from these subcomplexes as shown in Fig. 2.

There are four proteins found in the $\underline{b}$-$\underline{c}_1$ complex from beef heart which have no counterparts found in the yeast complex. These proteins have the molecular weights of 9.5, 8, 7.2, and 6.4 kDa. The 9.5 kDa protein has been isolated from the $\underline{b}$-$\underline{c}_1$ complex of beef heart mitochondria and sequenced by Bochart *et. al.* (1986). This protein consists of 81 amino acids : 14 basic, 5 acidic, and 33% hydrophobic. The molecular weight was calculated to be 9507. The functional role of this protein in the complex is not known, but it is associated with the core proteins during the purification of the $\underline{b}$-$\underline{c}_1$ complex into the three subcomplexes.

The 8 kDa polypeptide is the low molecular weight DCCD binding protein in the beef heart $\underline{b}$-$\underline{c}_1$ complex (Bochart *et. al.*, 1985). It consists of 78 amino acid residues, but does not have any tryptophan, isoleucine, or histidine residues and contains one of each of the following amino acids: lysine, phenylalanine, tyrosine, methionine, and cysteine.

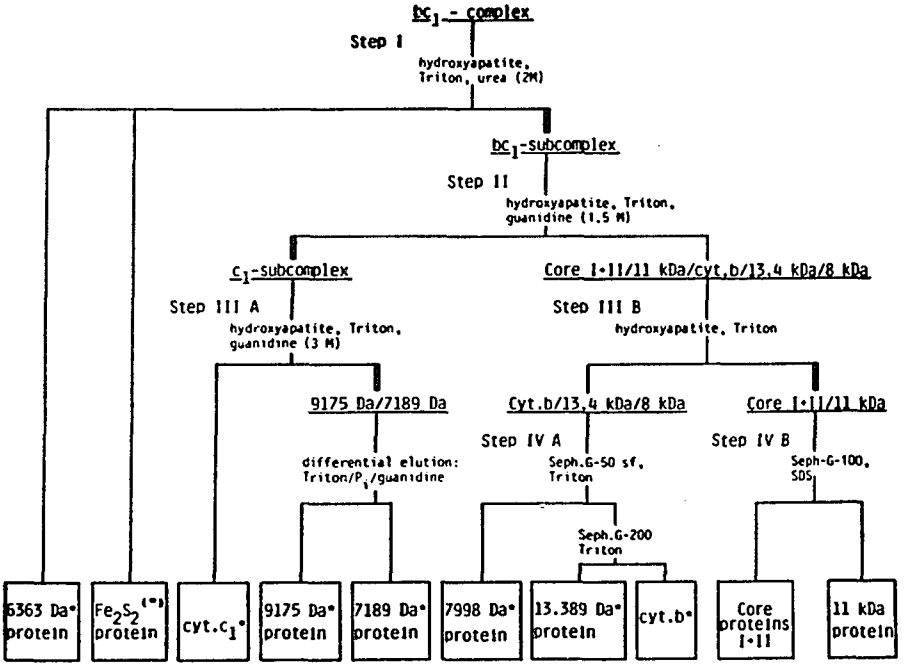


FIG. 1. Path of preparation of the 11 protein subunits of the bc_1 complex from beef heart mitochondria. Heavy lines indicate fractions bound to the hydroxyapatite column. The amino acid sequences of proteins marked by an asterisk have been elucidated.[3,16-21] The amino acid sequence of the iron-sulfur protein from *N. crassa* has been derived from the DNA sequence.[22] (*Note added in proof:* The amino acid sequence of the former 11 kDa protein has been determined (U. Borchart *et al.*, *FEBS Lett.*, submitted). The molecular mass is 9507 Da.)

Figure 2. Purification of the eleven subunits of the b-c_1 complex from beef heart mitochondria. Figure 1 of Schagger, H. et. al. (1986) Methods Enzymol. **126**, 224-237.

The calculated molecular weight is 7998. The predicted secondary structure contains one ã-helical region 26 amino acids long and a region consisting of 22 amino acids in a ß-sheet configuration. The DCCD binding site is not localized in the membrane spanning ã-helical region of the protein unlike other proteins which are associated with proton channels.

A protein with a molecular weight of 7,000 Da was isolated from a subcomplex of beef heart $\underline{b}$-$\underline{c}_1$ complex by von Jagow et. al. (1978). This subcomplex consisted of cytochrome $\underline{c}_1$, a 11,000 Da polypeptide, and the 7000 Da protein. Subsequently the molecular weight was recalculated to be 7189 (Schagger et. al., 1986). This protein of 62 amino acids contained a series of basic amino acids at the carboxy terminus and did not contain cysteine or methionine residues. Thirty four percent of the amino acids are aliphatic hydrophobic residues, but there are no hyrophobic segments long enough to traverse the membrane (Schagger et. al., 1983). The actual function of this protein in the $\underline{b}$-$\underline{c}_1$ complex is not known, but it has been suggested by Schagger et. al. (1983) that the basic amino acids at the carboxy-terminus may intereact with the glutamate residues in the amino-terminus of the hinge protein.

Lastly, the 6.4 kDa protein was isolated and sequenced by Schagger et. al. (1985) and consists of 56 amino acids. Thirty three percent of the amino acid residues are hydro-

phobic and there are eight basic residues but no cysteine or histidine residues. The N-terminus contains four arginines and the carboxy terminus is also hydrophilic. Amino acids 16-36 are hydrophobic, but this segment is not long enough to span the mitochondrial membrane. The funtional role of the this protein is unknown, but it does copurify with the "Reiske" iron-sulfur protein during isolation of the iron-sulfur protein from beef heart mitochondria $\underline{b}$-$\underline{c}_1$ complex.

C. The Topography of the $\underline{b}$-$\underline{c}_1$ Complex in the Mitochondrial Membrane

The three dimensional structure of the $\underline{b}$-$\underline{c}_1$ complex has been studied using crystals of the complex isolated from both N. crassa (Leonard et. al., 1981) and beef heart mitochondria (Ozawa et. al., 1983). Electron microscopy of the negatively stained crystals with a resolution of 3nm revealed that the $\underline{b}$-$\underline{c}_1$ complex in the membrane exists as a dimer. The two mononmers span the membrane and are oriented with the elongated dimension perpendicular to the membrane (Leonard et. al., 1981). The protein is unequally distributed on both sides of the membrane: 50% of the protein mass extends 7nm from the membrane, 20% protrudes 3nm from the opposite side of the membrane, and 30% is localized within the membrane. Acccording to this model, the two monomers contact each other partly in the large peripheral section and partly in the membrane (Leonard et. al., 1981).

Crystals of a $\underline{b}$-$\underline{c}_1$ subcomplex, which lacks the two core proteins and the iron-sulfur protein, were prepared from N. crassa. The isolated $\underline{b}$-$\underline{c}_1$ subcomplex is also a dimer. consisting of two lobes which correspond both in size and shape to the smaller peripheral section of the dimeric $\underline{b}$-$\underline{c}_1$ complex. The remainder of the subcomplex corresponds to the membrane section of the enzyme (Karlson et. al., 1983). The structure of the $\underline{b}$-$\underline{c}_1$ subcomplex has no part corresponding

to the large peripheral section observed in crystals of the whole enzyme complex. This observation plus the hydrophilic nature of the fraction containing the core proteins lead the authors to suggest that the large peripheral section which extends 7nm from the membrane must be the core proteins.

Weiss et. al. (1985) reported that the large peripheral section consisting of the two core proteins protrudes from the matrix side of the mitochondrial inner membrane. This conclusion was based on evidence that cytochrome c_1 must face the cytosolic side of the membrane in order to reduce cytochrome c, which is known to bind to the outer surface of the inner membrane.

A current model proposed by Weiss et. al. (1983) of the topography of the subunits of the b-c_1 complex is shown in Fig. 3.

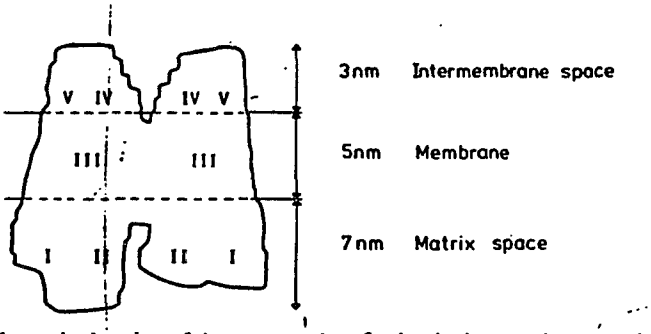


FIG. 4. Schematic drawing of the topography of subunits in cytochrome reductase from *Neurospora crassa* mitochondria. The roman numbers I and II refer to the subunits I and II, III to cytochrome *b*, and IV and V to the catalytic domain of cytochrome c_1 and the iron–sulfur subunit. The smaller subunits have not been included in the scheme because information about their location in the structure of the *Neurospora* enzyme is not available. (From Weiss *et al.*, 1983, with permission.)

Figure 3. Topography of the subunits of the b-c1 complex in the mitochondrial membrane. Figure 4 of Weiss, H. (1987) Curr. Top. Bioenerg. **15**, 67-90.

D. Electron Transfer and Proton Translocation in the b-c_1 Complex

Models for electron transfer and proton translocation in the b-c_1 complex. The pathway of electron transfer through the b-c_1 complex is a controversial issue. Part of this debate involves whether the b-c_1 complex functions as a monomer or as a dimer. Regardless of which form is considered the functional unit of the b-c_1 complex, the model must account for the following generally accepted properties: 1) cytochrome b can be reduced by substrate via two distinct pathways, antimycin sensitive and myxothiazol sensitive pathways; 2) four protons are translocated from the matrix-side to the cytosolic side of the inner mitochondrial membrane for every pair of electrons transferred through the b-c_1 complex; and 3) there are two different species of semiquinone which can be detected by EPR (Konstantinov and Ruuge, 1977; De Vries et. al., 1981).

The first property is based on several experiments using different inhibitors of electron transfer activity through the b-c_1 complex (Chance, 1958; Deul and Thorn, 1962; Clejan and Beattie, 1983). Initially electron transfer was thought to occur by electrons reducing consecutive components of the respiratory chain in a linear fashion, but the following experimental data could not be explained by this linear model. Deul and Thorn (1962) reported that cytochrome b

could be reduced by substrate in the presence of either antimycin or BAL (2,3-dimercaptopropanol) but not in the presence of both inhibitors. Another result which could not be explained by a linear model was the extra reduction of cytochrome b by the oxidation of both the iron-sulfur protein and cytochrome c_1 (Chance, 1958). This oxidant induced reduction of cytochrome b occurred in the presence of antimycin, an inhibitor of electron transport in the b-c_1 complex (Clejan and Beattie, 1983). These results suggested that electrons from substrate are transferred to cytochrome b via two different pathways.

Basically there are two current models for both electron transfer and proton translocation activites in the b-c_1 complex functioning as a monomer, the Q-cycle and the b-cycle. The Q-cycle was originally proposed by Mitchell (1975) and is shown in Fig. 4. Ubihydroquinone is intially formed by a one electron transfer from cytochrome b_{562} to quinone to form the semiquinone anion. This electron transfer occurs at the Q_i-site as indicated in the figure and is inhibited by antimycin. Subsequently the semiquinone anion is fully reduced, on the matix side of the membrane, to ubihydroquinol by protonation of the anionic semiquinone and an electron transfer from one of the dehydrogenase complexes. The ubihydroquinol then migrates to the Q_o-site where it tranfers one electron to the higher

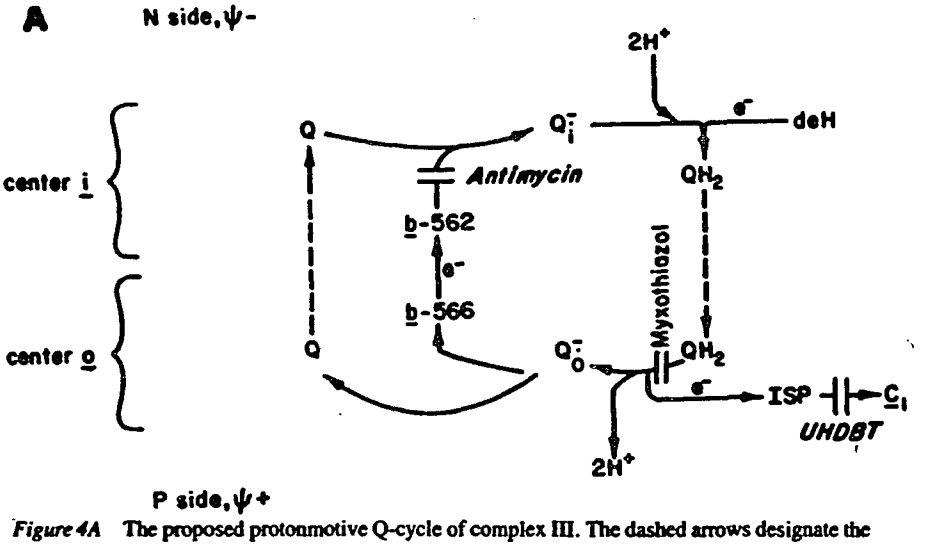


Figure 4A The proposed protonmotive Q-cycle of complex III. The dashed arrows designate the limited mobilities of ubiquinone and ubiquinol. The subscripts i and o designate specific species of ubisemiquinone anion, which react at the electronegative N (matrix) side (center i) or electropositive P (cytosolic) side (center o) of the inner mitochondrial membrane. ISP, iron-sulfur protein; deH, dehydrogenase. From (167) and (236).

Figure 4. The proposed Q-cycle of the b-c1 complex. Figure 4 of Hatefi, Y. (1985) Ann. Rev. Biochem. 54, 1015-1069.

potential branch of the $\underline{b}$-$\underline{c}_1$ complex, the iron-sulfur protein and cytochrome $\underline{c}_1$ forming a semiquinone anion. The other electron reduces the lower potential branch, cytochromes $\underline{b}_{566}$ and $\underline{b}_{562}$. The transfer of electrons from ubihydroquinone to the iron-sulfur protein is inhibited by both myxothiazol and BAL (2,3 mercaptopropanol). Two protons are released to the cytoplasmic side of the inner mitochondrial membrane during the transfer of electrons at the Q_o-site. Since only one of two electrons is transferred from the dehydrogenase complex by each cycle, four protons would be tranlocated across the membrane by the $\underline{b}$-$\underline{c}_1$ complex for every pair of electrons transferred down the respiratory chain. According to the Q-cycle model, there is a strict stoichiometry of 2 H^+'s translocated per electron transferred through the $\underline{b}$-$\underline{c}_1$ complex.

Another model of electron transfer and proton translocation in the $\underline{b}$-$\underline{c}_1$ complex is the b-cycle. This model was initially proposed by Wikstom and Krab (1980) and recently modified by the same authors in 1986 (Fig. 5A). The semiquinone formed at the Q_o-site does not necessarily reduce cytochrome $\underline{b}_{566}$ as in the Q-cycle, but can be reduced to ubihydroquinone by cytochrome $\underline{b}_{562}$. Also the b-cycle model does not stipulate the number of protons translocated per electron transfered nor does it imply that ubiquinone is the only proton carrier in the $\underline{b}$-$\underline{c}_1$ complex. In fact, the model was designed with the idea that the $\underline{b}$-$\underline{c}_1$ complex may

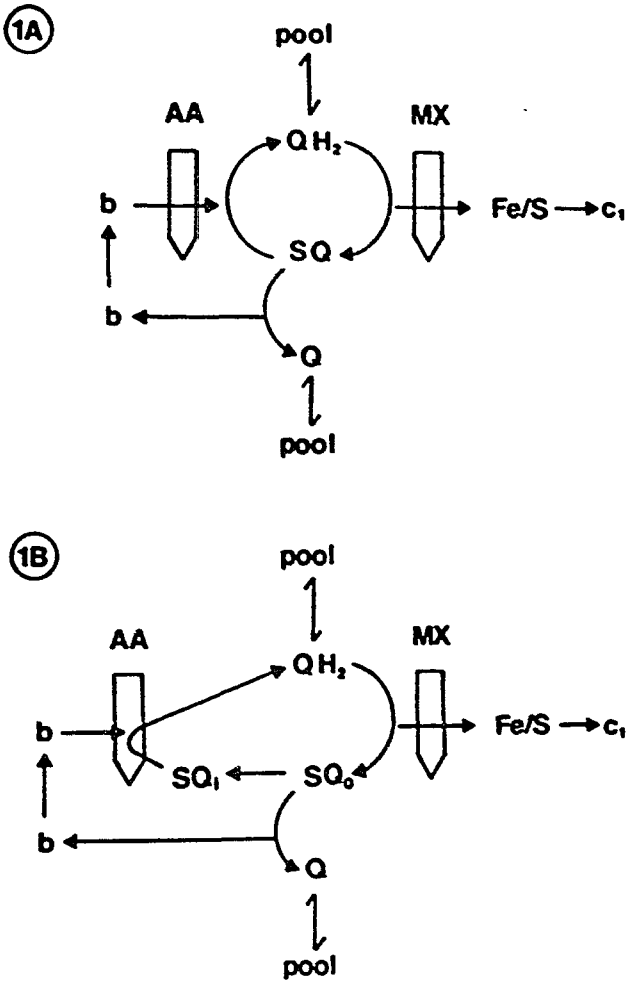


Fig. 1. A: The electron transfer scheme of the *b* cycle (slightly modified from Fig. 4 in Wikström and Krab, 1980). AA, antimycin; MX, myxothiazol; SQ, semiquinone. The *b*-type hemes are both indicated with a "*b*", of which the lower and the upper one correspond to *b*-566 and *b*-562, respectively. "Pool" indicates the membranous pool of ubiquinone. Black-headed arrows denote one-electron transfer reactions. B: Electron transfer scheme of the *b*-cycle with explicit separation of o and i domains, and showing the corresponding interaction of SQ with these domains. Such separation has not been drawn for Q or QH_2 for simplicity, and due to the postulated rapid equilibration of these protein-associated species with the membranous pool. Black-headed arrows (except the transition of SQ_o to SQ_i) denote one-electron transfer steps.

Figure 5. The proposed b-cycle and SQ-cycle models of electron transfer in the b-c1 complex. Figures 5A and 5B correspond to Figures 1A and 1B of Wikstrom, M. and Krab, K. (1986) J. Bioenerg. Biomembr. **18**, 181-193. A. The proposed b-cycle. B. The SQ-cycle.

be a proton pump or H^+-channel. As mentioned before, Wikstrom and Krab (1986) recently proposed a modified b-cycle which they call the SQ-cycle (Fig. 5B). The SQ-cycle model is an intermediate between the b and Q cycles. As proposed in the orginal b-cycle, the semiquinone formed upon the reduction of the iron-sulfur cluster can serve as an electron acceptor from cytochrome $\underline{b}_{562}$. The SQ-cycle is similar to the Q-cycle in that there are two proposed semiquinone binding sites and that ubiquinone acts as the only proton carrier in the $\underline{b}$-$\underline{c}_1$ complex thus eliminating the possibility that the $\underline{b}$-$\underline{c}_1$ complex is a proton pump.

Recently a model for the transfer of electrons through the $\underline{b}$-$\underline{c}_1$ complex functioning as a dimer was proposed by de Vries *et. al.* (1982). The model called the double Q-cycle, Fig. 6, is essentially the same as the Q-cycle proposed for the monomeric enzyme except that Q_i^- is reduced by the second cytochrome $\underline{b}_{562}$ instead of one of the dehydrogenase complexes. The model does not show nor do the authors discuss where the electrons from the dehydrogenases are transferred into the $\underline{b}$-$\underline{c}_1$ complex. This model was proposed to explain the biphasic kenetics of the inhibition of cytochrome $\underline{b}_{562}$ reduction by antimycin. The authors suggest that a high potential form of cytochrome $\underline{b}_{562}$ is more sensitive to antimycin and results in a rapid reduction of half the iron-sulfur clusters and cytochrome $\underline{c}_1$. The slower reduction of the remaining iron-sulfur clusters and

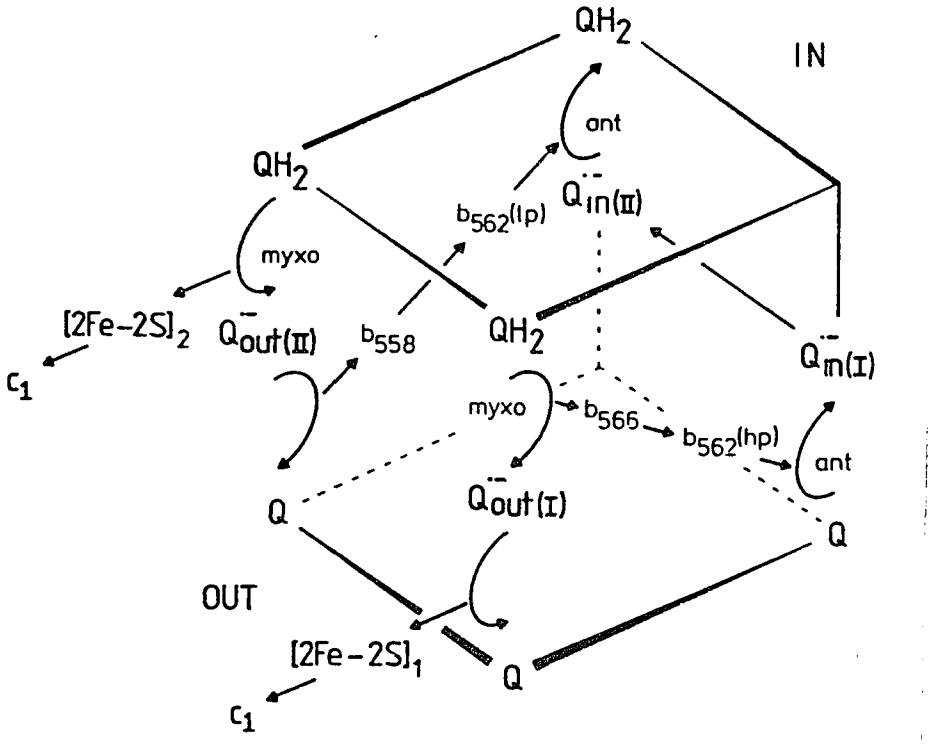


Fig. 5. Diagram showing a three-dimensional representation of a double Q cycle that describes electron transfer in the dimeric QH_2 : cytochrome *c* oxidoreductase. The meaning of the various symbols is the same as in Fig. 1. The front and posterior face, carrying the cytochrome *b* polypeptides, represent the membrane fractions of protomers I and II, respectively. Indices I or II indicate that Q^{-} is located in protomers I and II, respectively. Cytochrome *b*-566 is placed arbitrarily in protomer I, and cytochrome *b*-558 in protomer II. It is equally possible that cytochrome *b*-558 is in protomer I and cytochrome *b*-566 in protomer II, or that a single type of cytochrome *b*-566/558 is present in both protomer I and protomer II (see also text). Also, the Fe-S cluster called cluster 1 on the basis of the EPR spectrum is not necessarily the Fe-S cluster located in protomer I. The same uncertainty holds for Fe-S cluster 2. In contrast to the previous proposals (de Vries *et al.*, 1982a, 1983; Slater, 1983), the oxidation of QH_2 in protomer I is not inhibited by antimycin.

Figure 6. The proposed double Q-cycle model of electron transfer in the dimeric b-c1 complex. Figure 5 of deVries, S. (1986) J. Bioenerg. Biomembr. **18**, 195-224.

cytochrome $\underline{c}_1$ is attributed to the lower potential $\underline{b}_{562}$ in the dimeric form of the $\underline{b}$-$\underline{c}_1$ complex.

Inhibitors of electron transfer activity in the $\underline{b}$-$\underline{c}_1$ complex. The use of inhibitors of electron transfer in the $\underline{b}$-$\underline{c}_1$ complex provided strong evidence for the existence of two different sites for the reduction and oxidation of ubiquinone. Most of the $\underline{b}$-$\underline{c}_1$ complex inhibitors can be characterized into three groups according to the site of interaction of the inhibitor with the complex. The Q-cycle model (Fig. 4) will be used to describe the sites of interaction of the different inhibitors.

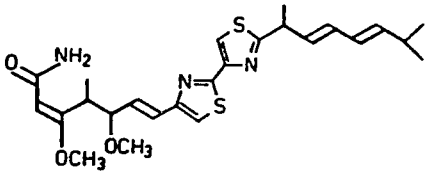
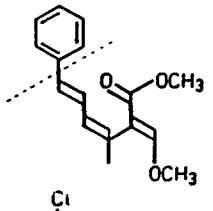
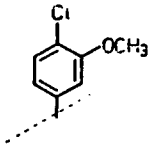
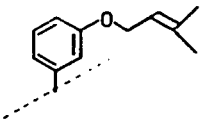
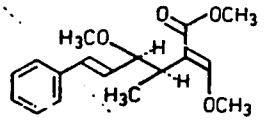
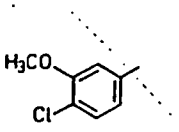
Group I inhibitors. This group of inhibitors all contain an Ë-ß-methoxyacrylate group, which resembles part of the structure of ubiquinone (von Jagow and Link, 1986). These inhibitors block the reduction of the iron-sulfur protein and cytochrome $\underline{b}_{566}$ at the Q_o-site, but permit the reduction of cytochrome $\underline{b}_{562}$ via an antimycin (a Group III inhibitor) sensitive pathway. The most commonly used Group I inhibitor is myxothiazol (Table I). The binding of myxothiazol to the $\underline{b}$-$\underline{c}_1$ complex results in a 2 nm red shift of the absorption maximum of cytochrome $\underline{b}_{566}$ and has an estimated K_d of less than 10^{-6} M (von Jagow and Engel, 1981).

Group II inhibitors. This group of inhibitors also have structural similarities to ubiquinone by containing a

TABLE I

Group I inhibitors of the b-c1 complex. Table I of von Jagow and Link (1986) Methods Enzymol. **126**, 253-271.

TABLE I
GROUP I: INHIBITORS OF UQH_2-OXIDATION
(IRON–SULFUR CENTER/HEME b_L REDUCTION)[a]

Inhibitor	Structural formula	Molecular formula
Myxothiazol[11,12]		$C_{25}H_{33}N_3O_3S_2$
Strobilurin A[14–16] (mucidin[17,18])		$C_{16}H_{18}O_3$
Strobilurin B[19]		$C_{17}H_{19}ClO_4$
Strobilurin C[19]		$C_{21}H_{26}O_4$
Oudemansin A[20]		$C_{17}H_{22}O_4$
Oudemansin B[19]		$C_{18}H_{23}ClO_5$

[a] References 11–20 quote the first publication on the respective inhibitor and publications elucidating the structural formula.

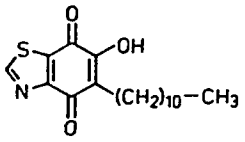
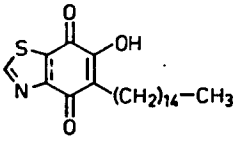
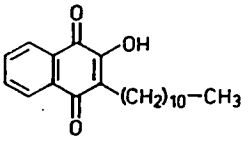
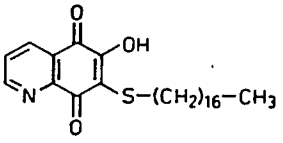
6-hydroxyquinone with different substitutions at position 2 and 3 (Table II). In addition, a heterocyclic ring structure fused to the benzoquinone ring enhances the inhibitory activity. Group II inhibitors completely block electron transfer between the iron-sulfur center and cytochrome $\underline{c}_1$. Unlike group I inhibitors, they do not cause a red shift of either of the two $\underline{b}$ cytochromes (Bowyer et. al., 1980) suggesting that they do not bind in the vicinity of the $\underline{b}$ hemes. Moreover, the titer of inhibition of these inhibitors is proportional to the iron-sulfur protein content and the K_d is dependent upon the redox state of the iron-sulfur center (Bowyer et. al., 1982; Zhu et. al., 1982). These observations suggest that these inhibitors bind to the iron-sulfur protein and displace the native ubiquinol. The group II inhibitor used in experiments described in this dissertation is 3-nonyl 2-hydroxy 1,4 naphthoquinone (nonyl-HNQ), which is similar to UHNQ in Table II.

Group III inhibitors. Unlike the other groups of inhibitors, group III inhibitors are not structurally similar to one another (Table III), but they all block the flow of electrons from cytochrome $\underline{b}_{562}$ to oxidized ubiquinone at the Q_i-site. Group III inhibitors compete with each other, but not with either group I or II inhibitors. The addition of both a group III inhibitor plus a group I or II inhibitor completely blocks the reduction of cytochrome $\underline{b}$

TABLE II

Group II inhibitors of the b-c$_1$ complex. Table III of von Jagow and Link (1986) Methods Enzymol. **126**, 253-271.

TABLE III
GROUP II: INHIBITORS OF REOXIDATION OF THE IRON–SULFUR CENTER AND OF REDUCTION OF b_l[a]

Inhibitor	Structural formula	Molecular formula
UHDBT[23,24] (undecylhydroxydioxobenzothiazole)		$C_{18}H_{25}NO_3S$
PHDBT (pentadecylhydroxydioxobenzothiazole)		$C_{22}H_{33}NO_3S$
UHNQ[25,26] (undecylhydroxynaphthoquinone)		$C_{21}H_{28}O_3$
HMHQQ[27] (heptadecylmercaptohydroxyquinoline quinone)		$C_{26}H_{39}NO_3S$

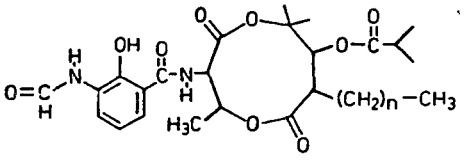
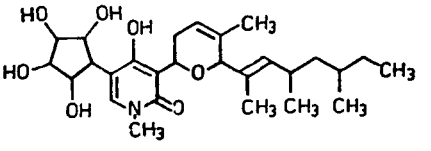
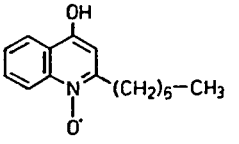
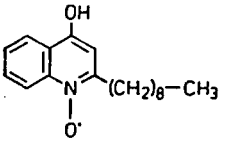
[a] References 23–27 quote the first publication on the respective inhibitor and publications elucidating the structural formula.

TABLE III

Group III inhibitors of the b-c$_1$ complex. Table IV of von Jagow and Link (1986) Methods Enzymol. **126**, 253-271.

TABLE IV

GROUP III: INHIBITORS OF UBIQUINONE REDUCTION (OXIDATION OF THE b_h CENTER)[a]

Inhibitor	Structural formula	Molecular formula
Antimycin[28,29]		$C_{28}H_{40}N_2O_9$[b]
Funiculosin[30,31]		$C_{27}H_{41}NO_7$
HQNO[32] (heptylhydroxyquinoline-*N*-oxide)		$C_{16}H_{21}NO_2$
NQNO (nonylhydroxyquinoline-*N*-oxide)		$C_{18}H_{25}NO_2$

[a] References 28–32 quote the first publication on the respective inhibitor and publications elucidating the structural formula.

[b] Natural antimycin A is a mixture of various homologs with different lengths of the alkyl side chain (n = 3–6); the main component (n = 5) is shown here.

from either the Q_i or the Q_o-sites.

The most commonly used group III inhbitor is antimycin which is an antibiotic produced by various species of Streptomyces (Slater, 1973). Antimycin binds with a high affinity (K_d= 3.2 x 10^{-11} M) to the b-c_1 complex stoichiometrically to the cytochrome c_1 content indicating that anitmycin binds to the complex in a mole to mole ratio. The binding of antimycin to the b-c_1 complex results in a red shift in the ã absorption band of cytochrome b_{562} from 562 to 564 nm suggesting that antimycin binds in the vicinity of cytochrome b_{562} (Berden and Opperdoes, 1972).

Another Group III inhibitor used in this study was heptylhydroxyquinoline N-oxide (HQNO) which binds to the b-c_1 complex with an affinity three orders of magnitude less than antimycin (K_d= 6.4 x 10^{-8} M) and results in a minute red shift of cytochrome b_{562}.

Miscellaneous inhibitors. There are several other inhibitors of electron transfer in the b-c_1 complex which do not affect cytochrome b. The first group of inhibitors include sulfhydryl reagents, British Anti-Lewisite (BAL: 2,3-dimercaptopropanol) and DTNB (5,5'-dithiobis 2-nitrobenzoic acid), which interact with the iron-sulfur centers of the "Reiske" iron-sulfur protein.

Initially, Slater (1949) observed that the combination of BAL and oxygen irreversibly blocked electron flow by the iron-sulfur protein. Subsequently Slater and de Vries

(1980) demonstrated that treatment of the $\underline{b}$-$\underline{c}_1$ complex with BAL results in the destruction of the iron sulfur centers in the $\underline{b}$-$\underline{c}_1$ complex.

DTNB also binds to the iron-sulfur protein but does not result in the destruction of the iron sulfur cluster in the same manner as BAL (Marres et. al.,1982). However, the binding of DTNB to the $\underline{b}$-$\underline{c}_1$ complex does result in the broadening of the g_x component of the epr spectra of the "Reiske" iron-sulfur protein. DTNB blocks electron transfer from duroquinol (an enzymatically functional ubiquinol analogue) to cytochrome $\underline{c}$ but does not inhibit the reduction of cytochrome $\underline{b}$ (Marres et. al., 1982).

The second group of miscellaneous inhibitors include two reagents which bind to the core proteins. The first of these inhibitors is ethoxyformic anhydride (EFA). The inhibition of ubiquinol-cytochrome $\underline{c}$ oxidoreductase activity by EFA is readily reversed by hydroxylamine (Yagi et. al., 1982). Yagi et. al. reported that EFA blocks electron tranfer at a site between cytochrome $\underline{b}_{562}$ and cytochrome $\underline{c}_1$, but EFA inhibition does not result in any spectral changes of either cytochrome $\underline{b}_{562}$ or $\underline{b}_{566}$. Recently, Lorusso et. al. (1987) reported that EFA strongly inhibited 1) the antimycin-insenstive reduction of $\underline{b}$ cytochromes, 2) the antimycin-promoted oxidant-induced reduction of the $\underline{b}$ cytochromes, and 3) the oxidation of pre-reduced $\underline{b}$ cytochromes. EFA appeared to cause a chemical modification of a

histidine residue in core protein II. Interestingly, EFA modification of core protein II decreased the sensitivity of the iron-sulfur protein to the thiol modifing reagent DACM (N-(7-dimethylamino-4-methylcoumarinil)maleimide) suggesting that EFA modification of core protein II causes a perturbation of the iron-sulfur protein.

The second inhibitor which binds to a core protein is 2,4-dinitrofluorobenzene (DNFB), a reagent known to modify cysteine and histidine residues (Lorusso et. al., 1986). Chemical labelling studies of the $\underline{b}$-$\underline{c}_1$ complex revealed that [^{3}H] DNFB binds to core protein I, the apoprotein of cytochrome $\underline{b}$, and the 12 kDa subunit of the $\underline{b}$-$\underline{c}_1$ complex of beef heart mitochondria. DNFB blocks the flow of electrons between cytochromes $\underline{b}_{566}$ and $\underline{b}_{562}$ and apparently binds to cytochrome $\underline{b}_{562}$ since DNFB binding results in a loss of antimycin binding to $\underline{b}_{562}$ (Lorusso et. al., 1986).

The studies of the mechanism of the inhibitors discussed above have provided most of the information of the pathway of electron transfer through the $\underline{b}$-$\underline{c}_1$ complex. The inhibitors which modify amino acids of the subunits of the $\underline{b}$-$\underline{c}_1$ complex have just begun and studying these types of inhibitors may provide more significant information on both electron transport and subunit interaction in the $\underline{b}$-$\underline{c}_1$ complex.

Pathways of proton translocation in the $\underline{b}$-$\underline{c}_1$ complex. The

mechanism of proton translocation across the mitochondrial membrane by the $\underline{b}$-$\underline{c}_1$ complex is not known. It is generally accepted that four protons are transported across the membrane concurrent with the passage of two electrons through the $\underline{b}$-$\underline{c}_1$ complex.

The Q-cycle model proposes that ubiquinone is the only proton carrier in the $\underline{b}$-$\underline{c}_1$ complex. In contrast, the b-cycle model proposes that the cytochromes in the $\underline{b}$-$\underline{c}_1$ complex are the carriers of protons. What is the evidence supporting both models for electron transport and proton translocation?

An important experimental result supporting the Q-cycle was the identification of two different species of ubisemiquinone in the $\underline{b}$-$\underline{c}_1$ complex. Ohnishi and Trumpower (1980) identified an antimycin-sensitive semiquinone anion corresponding to the quinone at center i in the Q-cycle. Subsequently, an antimycin-insensitive species of semiquinone anion was detected in the $\underline{b}$-$\underline{c}_1$ complex in submitochondrial particles under conditions of the oxidant-induced extrareduction of cytochrome $\underline{b}$ (de Vries *et. al.*, 1981). This ubisemiquinone was sensitive to the inhibitor BAL (which reacts with the iron-sulfur center) and corresponds to Q_o or center o in the Q-cycle model. In fact, subsequent studies by Zhu *et. al.* (1982) revealed that the Q-analogue inhibitor 7-(n-heptadecyl) mercapto-6-hydroxy 5,8-quinolinequinone (HMHQQ) bound to the $\underline{b}$-$\underline{c}_1$ complex with

two different affinities. The high affinity site was shown to be responsible for electron transfer near the iron-sulfur cluster, while the low affinity site was apparently similar to the antimycin-binding site.

Although two different species of ubisemiquinone have been observed in the $\underline{b}$-$\underline{c}_1$ complex, they have not been detected simultaneously. Hence, it is possible that the two different EPR spectra of the two species of ubisemiquinone may actually represent one ubisemiquinone bound in different environments and not two distinct sites.

The evidence supporting the b-cycle include the pH dependency of the redox midpoint potential of cytochrome $\underline{b}$ and, more importantly, the inhibition of proton translocation in the $\underline{b}$-$\underline{c}_1$ complex by dicyclohexylcarbodiimide (DCCD), a well known carboxyl modifying reagent.

DCCD has been used to study the mechanism of proton translocation in various enzyme complexes. Initially, DCCD was shown to inhibit proton translocation in F_1-F_0 ATPases by covalently binding to a single, specific glutamyl or aspartyl residue (Fillingame, 1980). Subsequently, DCCD has been reported to inhibit proton translocation in cytochrome $\underline{c}$ oxidase (Casey et. al., 1980), H^+-ATPases of the plasma membrane (Sussman and Slayman, 1983), the mitochondrial transhydrogenase (Pennington and Fisher, 1981; Phelps and Hatefi, 1981), NADH-ubiquinone oxidoreductase (Honkakoski and Hassinen, 1986), the Ca^{2+}/H^+-ATPase of the sarcoplasmic

reticulum (Pick and Racker, 1979; Murphy, 1981), and the mitochondrial K^+/H^+ antiporter (Martin et. al., 1986). Interestingly, DCCD also inhibits the Na^+, K^+-ATPase (Gorga, 1985) which does not translocate protons accross a membrane.

Beattie and Villalobo (1982) reported that DCCD inhibited proton translocation in yeast b-c_1 complex reconstituted into liposomes without significantly inhibiting electron tranfer activity. Treatment of the reconstituted b-c_1 complex in liposomes with DCCD resulted in a decrease of the $H^+/2e^-$ ratio from 3.81 to 2.41. The cytochrome c reductase activity (measured as ferricyanide reduction) actually increased upon DCCD treatment from, 1.02 to 1.38 moles min^{-1} mg^{-1} and was sensitive to antimycin. Carbonyl cyanide m-chlorophenylhydrazone (CCCP), a proton ionophore, also decreased the $H^+/2e^-$ ratio of b-c_1 complex reconstituted in liposomes from 3.81 to 2.0. The two protons released in the presence of CCCP result from the release of two scalar protons from the oxidation of the quinol substrate. CCCP treatment also results in a 1.21-fold increase of cytochrome c reductase activity of DCCD treated b-c_1 complex proteo-liposomes and a 3-fold increase in the rate of control proteoliposomes not treated with DCCD. Since the effects of DCCD and CCCP on proton translocation are similar, it is possible that DCCD like CCCP increases the permeability of the membrane to protons. However, measurements of proton conductance indicated that DCCD treated b-c_1 complex had a

lower proton conductance than control complex similarly embedded into liposomes (Beattie and Villalobo, 1982). Therefore DCCD treatment does not result in an increased permeability of membranes to protons. DCCD inhibits proton translocation with minimal effects on electron transfer activity. Similarly, DCCD was reported to inhibit electrogenic proton translocation without significant effects on the rate of cytochrome c reductase activity in mitochondria from beef heart (Esposti et. al., 1982; Esposti et. al., 1983) or rat liver (Price and Brand, 1983; Clejan et. al., 1984). Subsequently, Lorusso et. al. (1983) observed that DCCD inhibited proton movement across the membrane and actually enhanced cytochrome c reductase activity of the b-c_1 complex from beef heart mitochondria reconstituted into liposomes.

In contrast, Nalecz et. al. (1983) observed in studies on isolated and reconstituted b-c_1 complex in liposomes from beef heart mitochondria that DCCD inhibited both proton translocation and cytochrome c reductase activity. The observation of Nalecz et. al. (1983) that DCCD inhibits both activites may be due to incubating the b-c_1 complex with DCCD at 35°C for various times. These same conditions were observed to result in a significant loss of cytochrome c reductase activity for the b-c_1 complex from yeast mitochondria with or without DCCD treatment (Beattie et. al., 1984).

The inhibitory effects of DCCD on proton translocation were both time, concentration, pH dependent, and temperature dependent (Clejan et. al. 1984; Beattie et. al. 1984). Uncoupling of proton translocation and electron transfer activity by DCCD is contradictory to all proposed mechanisms of the Q-cycle in which an obligatory coupling between the two processes is depicted. The uncoupling of proton translocation from electron transfer through the $\underline{b}$-$\underline{c}_1$ complex by DCCD suggests another proton carrier other than ubiquinol may be involved in proton translocation.

Binding site of DCCD in the $\underline{b}$-$\underline{c}_1$ complex. Initially radioactive DCCD was shown to selectively bind to cytochrome $\underline{b}$ in yeast $\underline{b}$-$\underline{c}_1$ complex suggesting that this protein maybe involved in proton translocation in complex III (Beattie and Clejan, 1982; Beattie et. al.,1984). In contrast, Nalecz et. al., (1983) reported that DCCD preferentially bound to cytochrome $\underline{b}$ but also clearly labeled other subunits of the $\underline{b}$-$\underline{c}_1$ complex from beef heart mitochondria reconstituted into liposomes. In fact, crosslinking was observed between subunits V (the iron-sulfur protein) and VII after DCCD treatment. They observed a correlation between crosslinking of the two subunits and loss of both proton translocational and cytochrome $\underline{c}$ reductase activities.

Subsequently, Lorusso et. al. (1983) reported that DCCD preferentially bound to the 8 kDa subunit of isolated $\underline{b}$-$\underline{c}_1$

complex from beef heart mitochondria with crosslinking of both the 8 kDa and 12 kDa subunits to the iron-sulfur protein. Esposti et. al. (1983) also reported DCCD binding to the 8 kDa subunit, but observed no crosslinking unless the concentration of DCCD was greater than 200 mol DCCD/mol of cytochrome $\underline{c}_1$. They also correlated the binding of DCCD to the 8 kDa subunit with the selective inhibition of electrogenic proton translocation. Recently, Clejan et. al., (1984) reported that DCCD binds to both cytochrome $\underline{b}$ and the 8 kDa subunit of $\underline{b}$-$\underline{c}_1$ complex from beef heart mitochondria. No crosslinking was observed after DCCD treatment unless the $\underline{b}$-$\underline{c}_1$ complex was reisolated by precipitation with ammonium sulfate (Clejan et. al., 1984; Beattie et. al., 1985). Nalecz et. al., (1983) and Lorusso et. al. (1983) both reisolated the $\underline{b}$-$\underline{c}_1$ complex by ammonium sulfate precipitation after [^{14}C] DCCD incubation.

These results demonstrate that DCCD clearly binds to cytochrome $\underline{b}$ in the $\underline{b}$-$\underline{c}_1$ complex from yeast mitochondria and to both the 8 kDa subunit and to cytochrome $\underline{b}$ in the beef heart complex. The 8 kDa protein has not been predicted to span the mitochondrial membrane and this subunit in conjuction with cytochrome $\underline{b}$ and other $\underline{b}$-$\underline{c}_1$ complex subunit(s) possibly form the proton channel of the $\underline{b}$-$\underline{c}_1$ complex. Since DCCD strongly binds to cytochrome $\underline{b}$ of the $\underline{b}$-$\underline{c}_1$ complex in yeast mitochondria and to cytochrome $\underline{b}$ in the beef heart complex, cytochrome $\underline{b}$ most likely has a major role in proton

translocation in the $\underline{b}$-$\underline{c}_1$ complex.

DCCD binding site in other enzyme complexes. As mentioned before, DCCD is a well known carboxy modifying reagent. When DCCD reacts with a carboxy residue, an unstable O-acylisourea intermediate is initially formed, Fig. 7(I). This adduct can rearrange to form a stable N-acylurea resulting in the incorporation of radiolabelled DCCD into the protein at the reactive carboxyl residue, Fig. 7(II). Alternatively, the O-acylisourea can react with a nucleophile. If the nucleophile is water the protein is not modified, Fig. 7(IV), but if the nucleophile is an amino group of amino acid, an inter- or intra- molecular crosslink is formed Fig. 7(III). Notice that the nucleophilic reactions do not result in the labeling of the protein with [^{14}C] DCCD. Therefore it is possible that crosslinking may explain the action of DCCD. Crosslinking between subunits of an enzyme complex can usually be detected by SDS PAGE, but intramolecular crosslinking is not easily detectable.

The reactions outlined in Fig. 7 are not specific for DCCD, but are common to all carbodiimides. A unique and extremely useful property of DCCD is the hydrophobic nature of the carbodiimide enabling it to react with carboxyl residues in hydrophobic environments (Solioz, 1984). In fact, when DCCD is added to an aqueous suspension of protein or protein containing membranes, it will partition

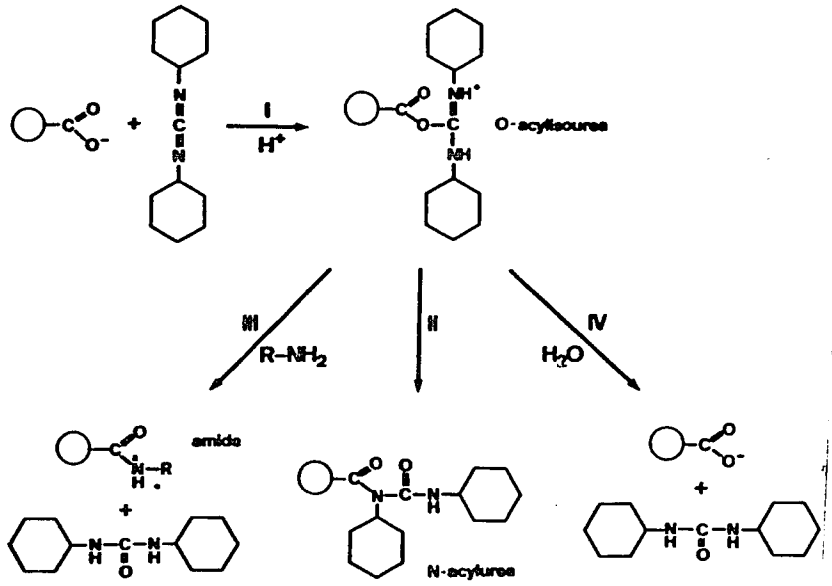


Fig. 2. Possible routes of the interaction of DCCD with a carboxyl residue. When DCCD reacts with a carboxyl residue, an unstable O-acylisourea adduct is first formed (I), which can then rearrange to a stable N-acylurea (II). Only this reaction sequence results in the incorporation of radioactivity when radiolabeled DCCD is employed. The O-acylisourea adduct can also interact with a nucleophile, such as a nearby ε-amino group of a lysine residue, to form an amide bond and dicyclohexylurea (III). If the nucleophile is water, the free carboxyl residue is restored (IV).

Figure 7. Possible reactions of DCCD with carboxyl residues. Figure 2 of Solioz, M. (1984) Trends Biochem. Sci. 9, 309-312.

into hydrophobic regions of proteins and lipids. For this reason, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC),a water soluble carbodiimide does not react with the same carboxyl residues that are specifically labelled with DCCD (Solioz, 1984).

The binding site of DCCD has been determined for a number of enzyme complexes. Initially DCCD was shown to covalently bind and label a single, specific glutamyl or aspartyl residue in the 8 kDa proteolipid subunit of F_0F_1 ATPases (Fillingame, 1980). Subsequently the specific binding sites have been determined for cytochrome c oxidase subunit III (Prochaska, et. al., 1987).

Interestingly, a single carboxyl residue in the plasma membrane H^+-ATPase, the F_0F_1-ATPase, and cytochrome c_1 oxidase was found to react covalently with DCCD. This reactive glutamate or aspartate is located in a very hydrophobic segment of the protein and predicted by hydropathy plots to be localized within a membrane.

One of the aims of this research was to determine the DCCD binding site(s) in cytochrome b from yeast mitochondria b-c_1 complex. The amino acid sequence surrounding the reactive residue in cytochrome b can be compared to the known DCCD binding sites found in other proton translocating enzymes. If the DCCD reactive site of cytochrome b is similar to the reactive sites of F_0F_1-ATPase, plasma membrane H^+-ATPase, and cytochrome c oxidase; then cytochrome b may have a major

role in forming a proton channel in the $\underline{b}$-$\underline{c}_1$ complex.

The inhibitory effects of antimycin and DCCD have on the binding of each other to cytochrome $\underline{b}$. Antimycin is an inhibitor which binds to cytochrome $\underline{b}$ near or at center $\underline{i}$ of cytochrome $\underline{b}$, in the terminology of the Q cycle, and results in a red shift of the ã-band of cytochrome $\underline{b}_{562}$. Incubating the isolated cytochrome $\underline{b}$-$\underline{c}_1$ complex with antimycin prior to labeling with DCCD was previously reported to result in a 90% decrease in the amount of DCCD bound to cytochrome $\underline{b}$ (Beattie et. al., 1984). The converse was also true, as preincubation of the $\underline{b}$-$\underline{c}_1$ complex with DCCD decreased the binding of antimycin as determined by both fluorescence quenching and the absence of the red shift induced by antimycin (Clejan and Beattie, 1983). Preincubating either isolated $\underline{b}$-$\underline{c}_1$ complex or proteoliposomes with $\underline{b}$-$\underline{c}_1$ complex with DCCD also decreased the sensitivity of the enzymatic activity to antimycin. These results suggested that antimycin and DCCD binding probably change the conformation of cytochrome $\underline{b}$ affecting the binding of the other compound.

The $\underline{b}$-$\underline{c}_1$ complex has 8-9 subunits that appear essential for enzymatic activity in yeast mitochondria. Of some interest is the role that the different subunits have in proton translocation. For example, how does the conformation of these subunits affect the binding of DCCD to cytochrome $\underline{b}$? One approach to investigate this is to

examine the effects of other inhibitors and substrates of the enzymatic activity of the $\underline{b}$-$\underline{c}_1$ complex have on the binding of DCCD to cytochrome $\underline{b}$. Studies using this approach are described in this dissertation.

II. METHODS AND MATERIALS

A. PURIFICATION OF CYTOCHROME $\underline{b}$-$\underline{c}_1$ COMPLEX

Isolation of Yeast Mitochondria Using a Dyno-Mill Disintegrator: Mitochondria were isolated from 5 lbs. of commercial fresh-pressed bakers yeast (Red Star) as described by Clejan and Beattie (1986). The yeast cells were crumbled into approxiamately 5 mm pieces, suspended in KPE buffer (0.1% KCl, 50 mM K_2HPO_4, pH 7.6, 1 mM Na_2EDTA) in a ratio of 450 ml of buffer per lb. of yeast, and incubated at 20°C overnight with constant aeration. The yeast cell suspension was then cooled in an ice bath and 0.5 mM PMSF was added. The cells were broken by using a Dyno-Mill Type KDL Disintegrator equipped with a continous flow chamber pre-cooled to -20°C and filled with 500 ml of 0.45-0.5 mm glass beads. The agitator speed was set to 3000 rpm and the cells were pumped through the chamber at a flow rate of 7 L per h. All subsequent steps were performed at 4°C. The broken cells were diluted 1:1 with KPEP buffer (KPE buffer + 0.5 mM PMSF), and centrifuged at 2600 g for 10 min. The supernatant was brought back to the original volume and recentrifuged twice at 2600 g for 10 min. The subsequent supernatant was then centrifuged at 16000 g for 25 min. The mitochondrial pellets were washed twice with KPEP buffer and the final pellet was resuspended to 40-50 mg/ml and stored

at -20°C. Typical yields were 4-6 g of mitochondria per lb. of fresh pressed yeast.

Preparation of Submitochondrial Particles and Dodecylmaltoside Extraction: All steps in the following procedures were performed at 4°C. Seven grams of mitochondria were diluted to 14 mg/ml with KPEP buffer prior to sonication. Sonication was performed on 50 ml batches at 4°C with a Q Horn Flat tip, in a Rossette cup, for 3 X 45 sec at 40% output with 45 sec pauses. The sonicated mitochondria were centrifuged twice at 8000 g for 10 min, and then at 100,000 g for 60 min to obtain submitochondrial particles (SMP's). The SMP's were washed by resuspending the pellet with Buffer A (50 mM Tris-HCl, 1 mM $MgSO_4$, 1 mM PMSF, 1 mM DFP, pH 8.0) to 30 mg/ml, readjusting the pH to 8.0, and then centrifuging the SMP's at 100,000 g for 90 min as described by Ljungdahl et. al. (1986). The resultant pellet was resuspended to 10 mg/ml with Buffer A and the membranes were solubilized by the addition of dodecylmaltoside to a ratio of 0.8 g DM per g of SMP's using a stock solution of 100 mg/ml . This mixture was stirred for 30 min and then centrifuged at 100,000 g for 90 min. To the resultant supernatant, 4 M NaCl was added to a final concentration of 100 mM and this was stirred for 60 min.

DEAE BioGel A Ion Exchange Chromatography: The above mixture was loaded onto a BioGel A column (2.5 X 15 cm), pre-equilibrated at 4°C with Buffer B (50 mM Tris-HCl, 1 mM

$MgSO_4$, 0.1 mg/ml DM, pH 8.0) containing 100 mM NaCl as described by Ljungdahl *et. al.* (1986). The flow rate was established at 30 ml/h; absorbance at 280 nm was monitored; and 10 ml fractions were collected. The column was washed with the above buffer until the absorbance at 280 nm returned to baseline. The *b*-c_1 complex was eluted off the column by using a 300 ml linear gradient of 100-300 mM NaCl in Buffer B. Fractions which had a red tint were analyzed spectrally to determine the content of cytochromes *b*, c_1, and cytochrome aa_3. Fractions containing maximal amounts of cytochromes *b* and c_1 and minimal amounts of cytochromes aa_3 were pooled and the ubiquinol-cytochrome *c* oxidoreductase activity was determined.

DEAE Sepharose CL-6B Ion Exchange Chromatography: The pooled fractions from the DEAE BioGel A column were loaded onto a DEAE Sepharose column (2.5 X 15 cm), that was pre-equilibrated with Buffer B containing 100 mM NaCl at 4°C. The flow rate during the enire procedure was 30 ml/h. The column was washed with Buffer B containing 100 mM NaCl until the A_{280} returned to baseline and then the *b*-c_1 complex was eluted by a 300 ml linear gradient of 100-400 mM NaCl in Buffer B. The fractions were analyzed spectrally for maximal cytochromes *b* and c_1 content and for minimal cytochrome aa_3 content and were pooled. The ubiquinol-cytochrome *c* oxidoreductase activity was then determined.

Amicon Concentration and Glycerol Dilution of Purified *b*-c_1 Complex: The pooled fractions from the DEAE Sepharose CL-6B were concentrated to approximately 1 ml using an Amicon Diaflo PM30 membrane, diluted 1:1 with glycerol, and then stored at -70°C. A typical preparation had an ubiquinol-cytochrome *c* oxidoreductase activity of 4-6 moles cytochrome *c* reduced/min/mg.

B. DETERMINATION OF CYTOCHROME OXIDASE AND CYTOCHROME $\underline{b}$-$\underline{c}_1$ COMPLEX CONTENT

The amount of cytochrome $\underline{aa}_3$ and cytochromes $\underline{b}$ and $\underline{c}_1$ were used to estimate the content of both cytochrome oxidase and $\underline{b}$-$\underline{c}_1$ complex respectively. Difference spectroscopy (reduced minus oxidized) was used for this determination with ferricyanide as the oxidizing agent for the reference sample and sodium hydrosulfite as the reducing agent for the experimental cuvette. Prior to the addition of the above reagents, a baseline was determined to compensate for the differeces in absorption between the reference and experimental cuvettes. The wavelengths and extinction coefficients (mM^{-1}) for cytochromes $\underline{aa}_3$ were 603-630 nm, 37.4; cytochrome $\underline{b}$: 560-575 nm, 25.6; and cytochrome $\underline{c}_1$: 553-539 nm, 20.9 as described by van Gelder (1978).

C. ENZYMATIC ASSAYS

Ubiquinol-cytochrome c oxidoreductase activity was measured in 2.5 ml of assay medium containing 250 mM sucrose, 1 mM EDTA, 50 mM Tris-HCl, pH 7.4, 2 mM KCN, and 20 M cytochrome c. 20μM 2,3-dimethoxy-5-methyl-6-n-decyl-1,4 benzoquinone (DBH_2) was used as the substrate and was added to start the reaction. The reaction was allowed to proceed for at least 20 s and then 2 μM antimycin was added to compensate for non-specific reduction of cytochrome c. The diffrence in absorption of 550 minus 539 nm was measured to determine the rate of cytochrome c reduction using 20 mM^{-1} as the extinction coefficient.

D. THE BINDING OF DCCD TO CYTOCHROME b IN CYTOCHROME b-c_1 COMPLEX

Approximately 4 mg (13-16 nmol cytochrome b) of b-c_1 complex was pipetted into a glass test tube and a buffer containing 100 mM KCl, 5 mM HEPES, pH 7.2 was added at a volume 10-fold that of the b-c_1 complex. The complex was labeled by adding a methanolic solution containing 4 mM [^{14}C] DCCD to a final concentration of 50 nmol DCCD/ nmol of cytochrome b. This mixture was incubated at 12°C for 1 h with shaking according to the method of Beattie et. al. (1984). The free [^{14}C] DCCD was separated from the bound ligand by pelleting the complex through 9 volumes of 10% sucrose at 100,000 g, 4°C for 3 h.

E. TREATMENT OF CYTOCHROME b-c_1 COMPLEX WITH ENZYMATIC INHIBITORS, REDUCING AGENTS, OXIDIZING AGENTS, AND UBIQUINOL ANALOGUES

The cytochrome b-c_1 complex (200 μM, 0.67 nmol cytochrome b) was added to 1 ml of 100 mM KCl, 5 mM HEPES, pH 7.2. Various compounds were added to the above mixture (in different experiments) at final concentrations of 10 μM antimycin, 10 μM myxothiazol, 20 mM 2,3 dimercaptopropanol (BAL), 60 μM 2-n-heptyl 4-hydroxyquinoline N-oxide (HQNO), 100 μM 3-nonyl 2-hydroxy 1,4 naphthoquinone (nonyl-HNQ), 2 mM KCN, 10 mM ascorbate (pH 6.0), 50 M $K_3Fe(CN)_6$, 100 μM 2,3-dimethoxy-5-methyl-6- decyl-1,4-benzoquinol(DBH_2), 50 μM Q_0H_2, 100 μM DQH_2, 100 μM menadiol (K_3H_2), and 20 μM Q_6H_2. The quinols were prepared by reduction of the corresponding quinones with potassium borohydride and neutralized with HCl just prior to their addition. The above compounds were incubated with the b-c_1 complex at 12°C for 10 min. The following reagents were incubated with b-c_1 complex under different conditions: 5 mM ethoxyformic anhydride (EFA) at 4°C for 10 min, 360 mM hydroxylamine (4°C, 10 min), and 10 mM 2,4-dinitrofluorobenzene (DNFB) at room temperature for 10 min.

F. POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-Polyacrylamide Gel Electrophoresis (SDS PAGE): SDS PAGE was performed on analytical slab gels (16 cm long, 1.2 mm thick) using the discontinous SDS-glycine buffer system of Laemmli (1970). The running gels were 15% and the stacking gels were 5% acrylamide. A calibration kit (Pharmacia) containing the proteins phosphorylase b (94,000), bovine serum albumin (67,000), ovalalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and ã-lactalbumin (14,400) was used for molecular weight standards. Samples were dissociated overnight at room temperature in a buffer containing 50 mM Tris, pH 6.8, 10% glycerol, 2 mM EDTA, 5% SDS, and 5% 2-mercaptoethanol. The gels were stained for 20 min with a 0.25% Coomassie Brilliant Blue R, 45% methanol, and 9.2 % glacial acetic acid solution, according to Weber and Osborn (1969). The gels were destained using a solution containing 8% glacial acetic acid and 5% methanol and Kimwipes were added to absorb the excess dye. The gels were usually destained in 1-2 days.

Some gels were silver stained by the method of Wray et. al. (1981) with the modified wash procedure of Wedrychowski et. al. (1986). Immediately after the electrophoresis was complete, the gels were soaked on a rocking platform in 10%

acetic acid for 12 h with three changes of solution. The gels were then washed extensively on a rocking platform by alternating washes of distilled water for 30 min and 50% methanol for 3 h repeating this three times. After the last methanol wash, the gels were then soaked for 1-2 min with distilled water. Gels were stained for 15 min in a solution containing 0.8% $AgNO_4$, 0.00756% NaOH, and 0.21 M NH_4OH; washed in distilled water 5 min with two changes; and developed for 10-15 min with a solution containing 0.005% citric acid, 0.019% formaldehyde, and 5-10% methanol (decreased the rate of development). The gel was then washed with distilled water and stored in either 50% methanol or 45% methanol/ 10% acetic acid.

Stacking Urea-Dodecyl Sulfate (SUDS) Polyacrylamide Gel Electrophoresis: SUDS gels were utilized for the separation of fragments of cytochrome b obtained by treatment with cyanogen bromide according to the method of Kyte and Rodriguez (1983). The gel system contained 8 M urea and a discontinous buffer system to obtain high resolution for the separation of peptides ranging from 25-250 residues in length. The stacking gel was 4.3% acrylamide, 368 mM HCl, 450 mM pyridine, pH 6.8, 8 M urea, 0.1% SDS, 0.32% TEMED (v/v), and 0.13% ammoniun persulfate. The running gel was 20% acrylamide, 323 mM Tris-HCl, pH 9.0, 8 M urea, 0.1% SDS, 0.17% TEMED (v/v), and 0.01% ammoniun persulfate. The upper reservoir buffer was 213 mM MES, pH 6.8, 269 mM pyridine,

and 0.1% SDS and the lower reservior buffer was 323 mM Tris-HCl, pH 9.0. Samples were dissolved in a buffer containing 370 mM HCL, 450 mM pyridine, pH 6.8, 8 M urea, 1% SDS, 10% glycerol, and 1% 2-mercaptoethanol and dissociated overnight at room temperature. Cytochrome c dissolved in dissociation buffer was used as a tracker dye. A calibration kit (Pharmacia) containing myoglobin partially digested with cyanogen bromide resulting in fragments with molecular weights of 17.2 kDa, 14.6 kDa, 8.24 kDa, 6.38 kDa, and 2.58 kDa was used as standards. The gel was run at 70 V until cytochrome c had migrated one-third of the the way through the running gel. The proteins were then visualized by silver staining.

Fluorography: Gels that were used to make autoradiograms were treated with sodium salicylate as an enhancer, according to the method of Chamberlain (1979). In order to prevent the precipitation of salicylate by acetic acid, the gels were first washed with water for 30 min, and then soaked in 1 M salicylate for 30 min. The gels were then dried and exposed to Kodak X-Omat AR film at -70°C.

G. ISOLATION OF CYTOCROME b FROM b-c_1 COMPLEX

Preparative SDS Polyacryamide Electrophoresis: Preparative gel electrophoresis was performed using 12.5% acrylamide preparative slab gels (24 cm long X 16 cm wide X 4 mm thick). The upper reservoir buffer included 0.1 mM sodium thioglycolate to prevent the destruction of tryptophan, histidine, and methionine side chains by free radicals or oxidants in the trapped gel matrix, as suggested by Hunkapiller *et. al.* (1983). Each sample lane was loaded with 1 mg of purified b-c_1 complex previously labeled with [^{14}C] DCCD. The molecular weight standards were the same as for the analytical gels. The gel was run at 75 mA until the tracker dye (bromphenol blue) migrated off the running gel. The subunits of b-c_1 complex had the folowing apparent molecular weights; Core I, 49,000; Core II 39,000; cytochrome b, 34,000; cytochrome c_1, 30,000; iron sulfur protein, 24,500; and low molecular weight proteins 17,500, 15,500, and 11,000.

The protein band corresponding to cytochrome b (as determined by molecular weight and radioactivity) was excised from the gel and was then destained by soaking the gel slices in 10 ml of dimethylsulfoxide (DMSO) at 37°C according to the method of Tal *et. al.* (1985). The DMSO destaining procedure was usually started at night. The next

morning the DMSO solution was changed and subsequent solution changes were made every 2 h until all the visible dye was removed from the gel slices.

Electroelution of Cytocrome *b*: Cytochrome *b* was electro-eluted from the gel slices by placing the slices into dialysis tubing (M.W. cutoff 2000) and putting the tubing into a BioRad Trans-Blot apparatus filled with elution buffer (0.1 M NH_4HCO_3, 0.1% SDS) acording to Hankapillar *et. al.* (1983). The electroelution was done at 400 mA during the day and 200 mA at night for the time period indicated in results.

SDS Extraction of *b*: For most experiments, cytochrome *b* was extracted from the gel slices by the method of Mendel-Hartvig and Nelson (1981). The gel pieces were minced and extracted using a solution containing 0.1% SDS, 0.1 M NH_4HCO_3 at 50°C. The gel slurry was centrifuged every hour and resuspended with same buffer until no more radioactivity was extracted. The extract was then lyophilized.

Purification of Cytochrome *b* by High Pressure Liquid Chromatography: Several attempts were made to purify cytochrome *b* from *b*-c_1 complex labeled with [^{14}C] DCCD using a HPLC molecular sieve column, Waters Protein Pak 300sw (7.5 mm X 30 cm) as described by Lorusso *et. al.* (1987) for the beef-heart *b*-c_1 complex. The running buffer was 200 mM sodium phosphate, pH 7.0, and 0.1% SDS; the flow rate was 0.2 ml/ min; the absorbance at 238 nm was monitored; and

aliquots of the collected fractions were counted.

H. CYANOGEN BROMIDE FRAGMENTATION OF CYTOCHROME b

Purified cytochrome b was cleaved at methionine residues with cyanogen bromide according to the procedures of Kyte and Rodriguez (1983) and Kyte et. al. (1983). Cytochrome b was dialyzed against 0.03 M NH_4HCO_3, 0.15% SDS to lower the SDS concentration. The protein was then reduced with dithiothreitol (DTT) by overnight dialysis under nitrogen using a solution containing 0.01 M DTT, 0.2 M Na borate, pH 9.4, 0.1% SDS. Subsequently the protein was denatured with 6 M urea and 5% 2-mercaptoethanol (v/v) for 2-3 h at 37°C under N_2. Cytochrome b was then alkylated with 5 mM sodium iodoacetate for 1 h at 37°C, Kyte et. al. (1983), dialyzed overnight against a solution containing 5 mM Na_2HPO_4,pH 7.0, and 0.05% SDS, and lyophilized to dryness. The protein was dissolved in 70% formic acid containing a 20-fold molar excess of cyanogen bromide and incubated for 24 h at room temperature under nitrogen.

I. PURIFICATION OF PEPTIDE FRAGMENTS OF Cytochrome b

C_{18} Reverse Phase HPLC: After cleavage with cyanogen bromide, the peptides were separated on a Waters C_{18} Bondapak reverse phase column (3.9 mm X 30 cm) using trifluoroacetic acid and organic solvents as suggested by Mahoney and Hermodson (1980) and Heinemann and Ozols (1982). HPLC was performed on a system with the following components from Waters: two 510 pumps, a U6K injector, 481 variable wavelength dectector, and a 680 gradient controller. All the solvent systems used for the chromatography contained 0.1% TFA and were either 1) water and acetonitrile, 2) water and 20% acetonitrile/ 80% 2-propanol, or 3) 20% 2-propanol and 20% acetonitrile/ 80% 2-propanol.

Sephadex LH-60 Chromatography: One attempt was made to partially separate the cyanogen bromide fragments of cytochrome b by running the peptides on a Sephadex LH-60 column prior to reverse phase HPLC as described by Gerber et. al. (1979). A sample (1.8 ml) containing 8600 cpm was loaded onto a column (1 X 35 cm, 27.5 ml) pre-equilibrated with the running buffer containing 88% formic acid in ethanol at a ratio of 3:7 and the flow rate was 4 ml/h.

III. RESULTS AND DISCUSSION

A. LABELING OF THE b-c_1 COMPLEX WITH [^{14}C] DCCD

The b-c_1 complex from yeast mitochondria consists of nine subunits which migrate with the apparent molecular weights shown in Table IV. Cytochrome b migrates anamalously on gels and its apparent molecular weight on analytical gels varies from 30,000 Da on 12.5% SDS PAGE gels to 34,000 Da on 15% gels. The other subunits of the b-c_1 complex migrate with essentially the same apparent molecular weight regardless of the percent acrylamide in the gel. A typical 12.5% gel is shown in Fig. 8 (lanes 1-3). The apparent molecular weights for the b-c_1 complex subunits for this particular gel are core I, 48,000 Da; core II, 37,500 Da; cytochrome b, 31.000 Da; cytochrome c_1, 30,000 Da; and the iron sulfur protein, 24,000 Da; however the low molecular weight subunits of the b-c_1 complex are not well resolved in this gel.

When the b-c_1 complex was first incubated with [^{14}C] DCCD and then an analytical 12.5% SDS PAGE performed, cytochrome b was consistently labeled with [^{14}C] DCCD as shown in Fig. 8 (lane 4). Notice that DCCD teatment did not result in any apparent crosslinking of the b-c_1 complex subunits. This confirmed the results obtained by Beattie and Clejan (1982). The dark diffuse spot at the bottom of

TABLE IV

Subunit composition of the b-c_1 complex

The values for the apparent molecular weights were from: for 12.5% acrylamide gels, Sidhu and Beattie (1983) and for 15% acrylamide gels, Beattie *et. al.* (1984).

Subunit number	Subunit	percent acrylamide 15%	12.5%
		M_r	M_r
I	Core protein I	49,000	47,000
II	Core protein II	39,000	39,000
III	Cytochrome b	34,000	31,000
IV	Cytochrome c_1	30,000	30,000
V	Iron-sulfur protein	22,400	24,500
VI		13,400	17,700
VII		11,100	15,500
VIII		10,000	
IX		9,000	

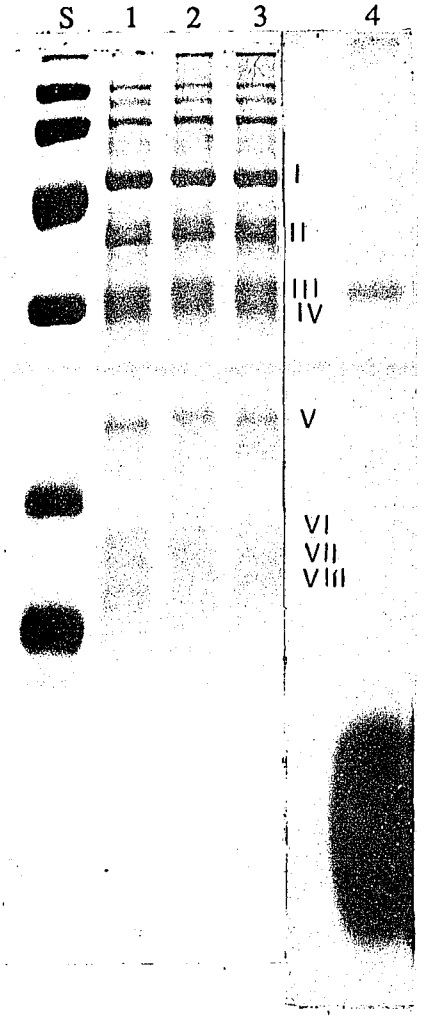


Figure 8. SDS PAGE of [^{14}C] DCCD Labeled b-c_1 complex. 40 ug of b-c_1 complex was labeled with [^{14}C] DCCD (50 nmol DCCD/ nmol cytochrome b at 12^{0}C as described in Methods. The labeled complex was centrifuged through 10% sucrose and 12.5% SDS PAGE was performed. Lanes S, 1-3 ; Coomassie Blue-stained 12.5% SDS PAGE. High-molecular-weight standards: 94,000, 67,000, 43,000, 30,000, 20,100, and 14,400 (lane S) and b-c_1 complex incubated with DCCD for 0, 30, and 60 min, respectively (lanes 1-3). Lane 4, fluorogram of the gel of b-c_1 complex treated with [^{14}C] DCCD for 60 min.

the autoradiograph is caused by the [^{14}C] DCCD bound to cardiolipin that migrates with the same mobility of cardiolipin as described by Beattie et. al. (1984).

B. PURIFICATION OF CYTOCHROME b

Preparative 12.5% SDS Polyacrylamide Electrophoresis: The subunits of the $\underline{b}$-$\underline{c}_1$ complex were separated using preparative gels as described in experimental methods. Cytochrome $\underline{b}$ migrated as a diffuse band with a molecular weight of 34,000 Da and was usually resolved from cytochrome $\underline{c}_1$ (30,000 Da.) and Core II protein (39,000 Da), but sometimes cytochrome $\underline{b}$ would comigrate with cytochrome $\underline{c}_1$ (data not shown). The anomalous migration of cytochrome $\underline{b}$ on polyacrylamide gels is not unusual for hydrophobic proteins. Cytochrome $\underline{b}$ was excised only from preparative gels with good resolution. Another problem encountered with the preparative gels was that the protein bands tended to curve upward at the outer edges of the gel lanes. In order to excise the protein band accurately it was necessary to stain the entire gel and then to destain with DMSO at 37°C.

Extraction of Cytochrome $\underline{b}$ from Gels: Cytochrome $\underline{b}$ was extracted from preparative gel slices either by electroelution or by SDS extraction as described in methods. The recovery of cytochrome $\underline{b}$ was determined by the amount of total [^{14}C] DCCD bound to cytochrome $\underline{b}$ electroeluted or extracted from the gel slices. Initially electroelution was used to extract cytochrome $\underline{b}$ from the excised gel slices, but the recovery was low and inconsistent (Table V). The

TABLE V

Efficiency of cytochrome b extraction from preparative 12.5% SDS PAGE.

Each experiment was performed with the specified conditions once.

Method	Percent Recovery	Conditions
Electroelution	Dialysate	Time
	17	72 h
	2	24 h
	9	80 h
0.1% SDS Extraction	Extract	Time, number of solution changes
	49	2h, 4
	11	1.5 h, 3
	8	4 h, 4
	2	3h, 3
	15	3h, 3

recovery varied from 2% after electroelution for 24 h to 17% after 72 h; however, the recovery was not necessarily time dependent. In one experiment only 9% of the cytochrome b was recovered even after 80 h of electroelution. Therefore, another method for extracting proteins from polyacrylamide gels was tried.

Mendel-Hartvig and Nelson (1981) described a method for extracting the core proteins I and II of the b-c_1 complex from gels using 0.1% SDS. The core protein bands were excised from the gels, the slices were then diced, and then extracted four times at 50°C for 2 h with a 0.1% SDS solution. This extraction procedure when applied to cytochrome b resulted in recoveries ranging from 2% to 49% as shown in Table V. However the majority of these extractions resulted in a yield of less than 20%.

It was concluded that the recovery of cytochrome b from preparative 12.5% SDS PAGE gels by electroelution or SDS extraction was too inefficient to obtain sufficient protein for the subsequent analysis of peptides. Either another method of extracting cytochrome b from gel bands should be developed or another method for separation of the subunits of the b-c_1 complex not involving cytochrome b extraction is required.

Discussion: Purification of transmembrane proteins has been a difficult task, but new techniques have recently been developed which may make possible the purification of these

proteins. In order to investigate which amino acid residue(s) of cytochrome b are important for the inhibition of proton movement by DCCD, cytochrome b must be purified so that amino acid analysis and sequencing can be performed. The results of the experiments described above suggested that purifing cytochrome b by preparative SDS PAGE and then extracting b from the gel may not be the method of choice. A major problem with this technique is that cytochrome b cannot be easily extracted from the gels.

Lorusso et. al. (1987) recently described a procedure to partially purify the subunits of b-c_1 complex from beef heart mitochondria using molecular sieve HPLC. These workers loaded 100 g of purified b-c_1 complex onto a 0.75 X 75 cm TSK G-3000 column purchased from Toyo Soda. Five protein peaks containing 1) the core proteins, 2) cytochrome b, 3) FeS protein and cytochrome c_1, 4) 14 kDa. protein, 5) the low molecular weight proteins were obtained. Attempts in our laboratory to purify cytochrome b from the b-c_1 complex of yeast mitochondria by molecular sieve HPLC failed to resolve cytochrome b from the other subunits.

Another procedure to purify the iron-sulfur protein, ubiquinone-binding protein (15,500 Da), cytochrome c_1, and cytochrome b from a b-c_1 complex isolated from beef heart mitochondria using phenyl-Sepharose CL-4B was described by Shimomura et. al. (1986). This procedure yielded cytochrome b that was apparently pure as determined by SDS PAGE. These

workers loaded approximately 100 mg of beef heart $\underline{b}$-$\underline{c}_1$ complex onto a phenyl-Sepharose CL-4B column (1.5 X 75 cm) pre-equilibrated with 25 mM Tris-HCl, pH 8.0, 0.25% deoxycholate, 0.2 M NaCl, 1 mM EDTA, 1 mM DTT, and 20% glycerol. The column was washed with the same buffer to remove phospholipids, and then the iron-sulfur protein was eluted by the same buffer containing 1% deoxycholate instead of 0.25%. Subunit VI, the proposed ubiquinol-binding protein, was eluted with 1.5 M guanidine hydrochloride dissolved in 25 mM Tris-HCl, pH 7.5, 1% cholate, 1 mM DTT, and 20% glycerol (Buffer A). The core proteins I and II were eluted by 3 M guanidine-HCl dissolved in Buffer A, followed by the elution of cytochrome $\underline{c}_1$ by using Buffer A containing 1% dodecyloctaethylene glycol monoether. Finally, the remaining protein was eluted off the column using Buffer A containing 2% SDS. When this last protein fraction was analyzed by analytical SDS PAGE, it consisted entirely of cytochrome $\underline{b}$. The percent yield of pure cytochrome $\underline{b}$ from this chromatography procedure was reported to be approximately 30%. Our laboratory has recently tried this method to purify cytochrome $\underline{b}$. The results obtained suggest that with small amounts of $\underline{b}$-$\underline{c}_1$ complex, cytochrome $\underline{b}$ can be separated completely from the other subunits; however, with preparative amounts of complex contaminating proteins are present in the cytochrome $\underline{b}$ fraction. Moreover, the SDS present in the sample appears to interfere in certain steps of the

cyanogen bromide cleavage and gel electrophoresis.

C. THE PURIFICATION OF CYANOGEN BROMIDE FRAGMENTS OF [^{14}C] DCCD LABELED CYTOCHROME *b*

The ultimate goal of this research is to determine which carboxy amino acid of cytochrome *b* binds DCCD. The easiest approach to accomplish this task was to cleave cytochrome *b* into peptide fragments, purify these peptides, determine the amino acid sequence of the peptide fragments which bound [^{14}C] DCCD, and finally to compare the sequencing results with the amino acid sequence of cytochrome *b* as predicted from the DNA sequence. Cyanogen bromide was chosen as the reagent to cleave cytochrome *b* into smaller peptide fragments since cyanogen bromide can be easily separated from the resultant peptides unlike proteolytic enzymes.

Cytochrome *b* was purified from a *b*-c_1 complex previously labeled with DCCD by preparative 12.5% SDS PAGE. The protein band corresponding to cytochrome *b* was excised and the protein was extracted from the gel slices by SDS extraction. The purified cytochrome *b* was cleaved using cyanogen bromide treatment as described in experimental methods.

There are sixteen methionine residues in cytochrome *b* predicted from the DNA sequence (Nobrega and Tzagoloff, 1980) shown in Table VI. If cyanogen bromide cleavage was complete, then ten of these fragments would have predicted

TABLE VI

CNBr Fragments of cytochrome b. The amino acid sequence is deduced from the DNA sequence of cytochrome b, Nobrega, F.G. and Tzagoloff, A. (1980) J. Biol. Chem. **255**, 9828-9837. The bold and underlined amino acid residues are the predicted residues which may bind DCCD. These amino acid residues are predicted to be localized in or near the membrane, Widger et al (1984). The aspartate of fragment 14 is also conserved in cytochrome b from many different species.

Fragment	Sequence
1	M
2	AFRKSNVYLSLVNSYIIDSPQPSSINYWWNM
3	GSLLGLCLVIQIVTGIFM
4	AM
5	HYSSNIELAFSSVEHIIRDVHNGYILRYLHANGASFFFM
6	VM
7	FM
8	HM
9	AKGLYYGSYRSPRVTLWNVGVIIFILTIATAFLGYCCVYGQM
10	SHWGATVITNFLSAIPFVGN**D**IVSWLWGGFSVSNPTIQRFFALHYL VPFIIAAM
11	VIM
12	HLM
13	ALHIHGSSNPLGITGNLDRIPM
14	HSYFIFK**D**LVTVFLFM
15	LILALFVFYSPNTLGHPDNYIPGNPLVTPASIDPEWYLLPFYAILRSI PDKLLGVITM
16	FAAILVLLVLPFTDRSVVRGNTFKVLSKFFFFIFVFNFVLLGQIGACH VEVPYVLM
17	GQIATFIYFAYFLIIVPVISTIENVLFYIGRVNK

molecular weights ranging from 2184 to 7519 Da (Table VII). The remaining seven fragments would consist of one to three amino acid residues ranging in molecular weights from 149 to 436. In order to investigate where DCCD was covalently bound to cytochrome b, it was necessary to purify the cyanogen bromide fragments so that the peptides containing radioactive label could be analyzed for amino acid composition and sequenced.

Purification of Cyanogen Bromide Fragments by Reverse Phase HPLC: Reverse phase HPLC on a C_{18} Bondapak column was used to purify the cyanogen bromide fragments of DCCD labeled cytochrome b as described in methods. Several solvent systems were utilized to accomplish this goal. The first solvent system employed 0.1% TFA in water as the aqueous mobile phase and 0.1% TFA in acetonitrile as the organic phase. The peptides were separated by using a three step linear gradient indicated in Fig. 9. Radioactive material was eluted from the column in 7-9 unresolved peaks where the gradient contained 44-49% acetonitrile. The peaks containing radioactive material were all in one region of the chromatograph and accounted for 36% of the total counts loaded onto the column.

One possible reason that the peaks containing the labeled region of cytochrome b were not resolved was that the fragments of cytochrome b obtained by cyanogen bromide aggregated because of their hydrophobicity. Hence,

TABLE VII

Predicted amino acid composition and molecular weights of fragmented cytochrome b obtained by cyanogen bromide treatment

The values reported are the percent composition of the amino acid for each fragment and were calculated from the predicted amino acid sequence reported by Nobrega and Tzagoloff (1980). The fragments are those shown in Table VI and are numbered in the same manner.

A.A.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
A		3.2		50	7.7				7.1	9.3			4.6		5.1	5.4	5.9
R		3.2			5.1				4.8	1.9			4.6		1.7	3.6	2.9
N		13			7.7				2.4	5.6			9.1		5.1	3.6	5.9
D		3.2			2.6					1.9			4.6	6.3	5.1	1.8	
C			5.6						4.8							1.8	
Q		3.2	5.6						2.4	1.9					1.7	1.8	2.9
E					5.1										1.7	1.8	2.9
G			17		5.1				12	7.4			14		5.1	5.4	5.9
H					10			50		3.7		33	9.1	6.3		1.8	
I		9.7	17		10				9.5	11	33		13	6.3	10	5.4	21
L		6.5	22		7.7				9.5	7.4		33	14	13	17	14	5.9
K		3.2							2.4					6.3	1.7	3.6	
M	100	3.2	5.6	50	2.6	50	50	50	2.4	1.9	33	33	4.6	6.3	1.7	1.8	2.9
F		3.2	5.6		10		50		4.8	11				25	5.1	18	12
P		6.5							2.4	5.6			9.1		14	3.6	2.9
S		19	5.6		13				4.8	9.3			9.1	6.3	5.1	3.6	2.9
T			5.6						7.1	5.6			4.6	6.3	5.1	3.6	5.9
W		6.5							2.4	5.6					1.7		
Y		9.7			7.7				12	1.9				6.3	6.9	1.8	8.8
V		6.5	11		5.1	50			9.5	9.3	33			13	5.1	18	12
No. A.A.	1	31	18	2	39	2	2	2	42	54	3	3	22	16	58	56	34
M.W.	149	4235	2184	238	5242	266	314	304	5448	6966	398	436	2692	2278	7519	7376	4521

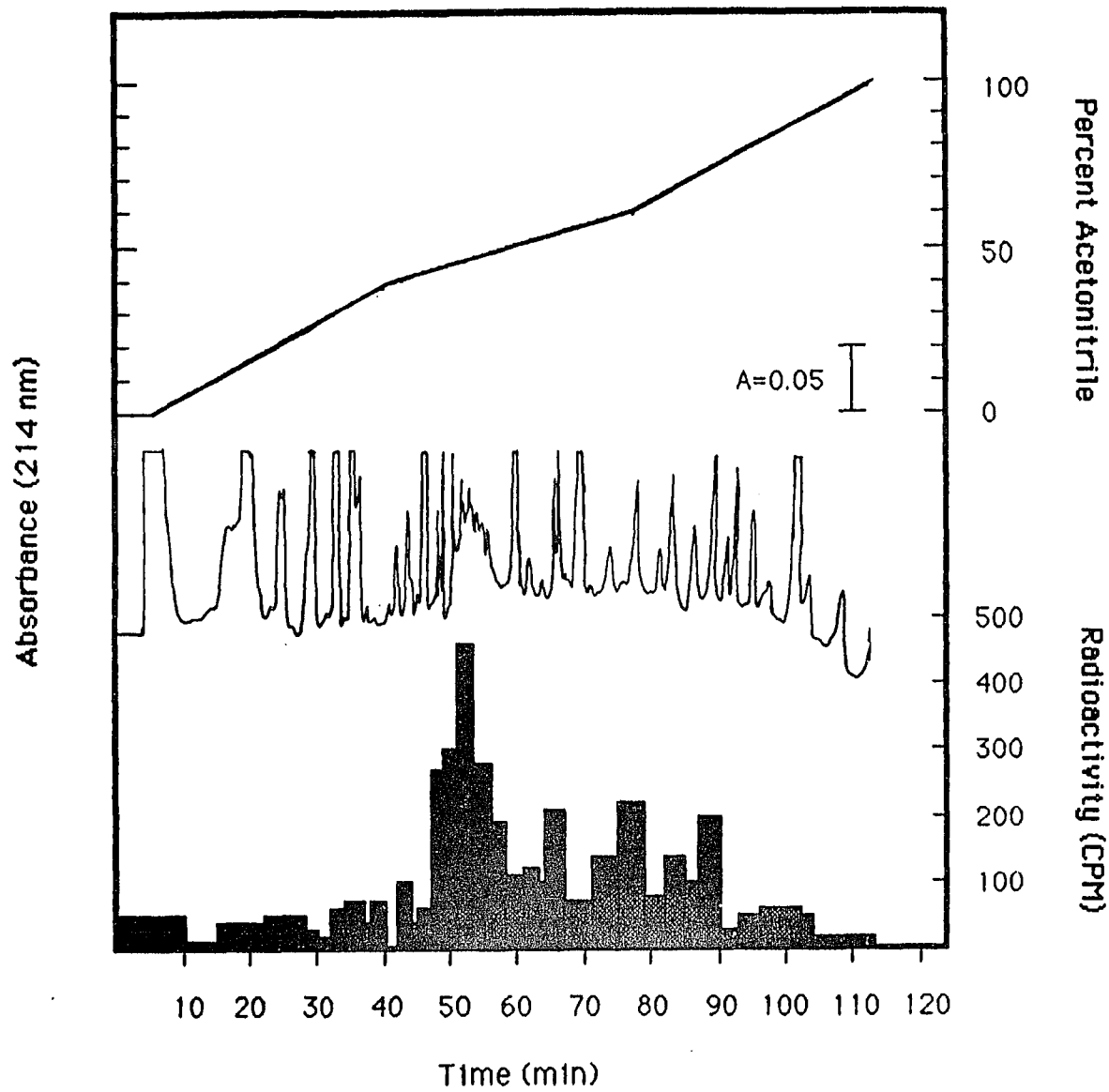


Figure 9. Purification of CNBr fragments of [^{14}C] DCCD labeled cytochrome b by Reverse Phase HPLC using 0.1% TFA, water and 0.1% TFA, acetonitrile as solvents. [^{14}C] DCCD labeled cytochrome b treated with CNBr (4,260 CPM) was chromatographed on a Waters C-18 Bondapak reverse phase column (3.9 mm X 30 cm). The solvents used were 0.1% TFA in water as the aqueous phase and 0.1% TFA in acetonitrile as the organic phase at a flow rate of 1 ml/min. The gradient shown in the upper graph was started using 0.1% TFA, water. Both the absorbance at 214 nm and radioactivity from the [^{14}C] DCCD label were monitored. The absorbance trace is the middle graph and the lower bar graph is the radioactivity (CPM).

another solvent system containing the same aqueous solvent but a more nonpolar organic solvent was used. This solvent contained 0.1% TFA in 80% 2-propanol/ 20% acetonitrile. The modified solvent system improved the resolution and decreased the number of unresolved radioactive peaks to about six, but it did not increase the recovery of the radioactivity (Fig. 10). Since 2-propanol may have increased the solubility of the cyanogen bromide fragments, it was reasoned that including 2-propanol in the starting solvent may further improve the chromatography of the peptide fragments. The last solvent system used in these experiments contained 0.1% TFA in 20% 2-propanol as the beginning solvent and 0.1% TFA in 80% 2-propanol/ 20% acetonitrile as the final solvent (Fig. 11). This system further improved the resolution of the various peaks; however there were still six peaks and 41% recovery of total radioacivity.

Sephadex LH-60 Chromatography: The cyanogen bromide fragments of cytochrome b are hydrophobic in nature and may tend to aggregate on reverse phase columns, which has been reported for other hydrophobic proteins (Tarr and Crabb, 1983; Gerber and Khorana, 1982). The fragments of cytochrome b that were expected to bind DCCD are hydrophobic in nature and have predicted molecular weights of 2278 or 6966 Da (Table VII). If the cyanogen bromide fragments were separated from the aggregated larger peptides, then maybe this would decrease the possibility of forming large

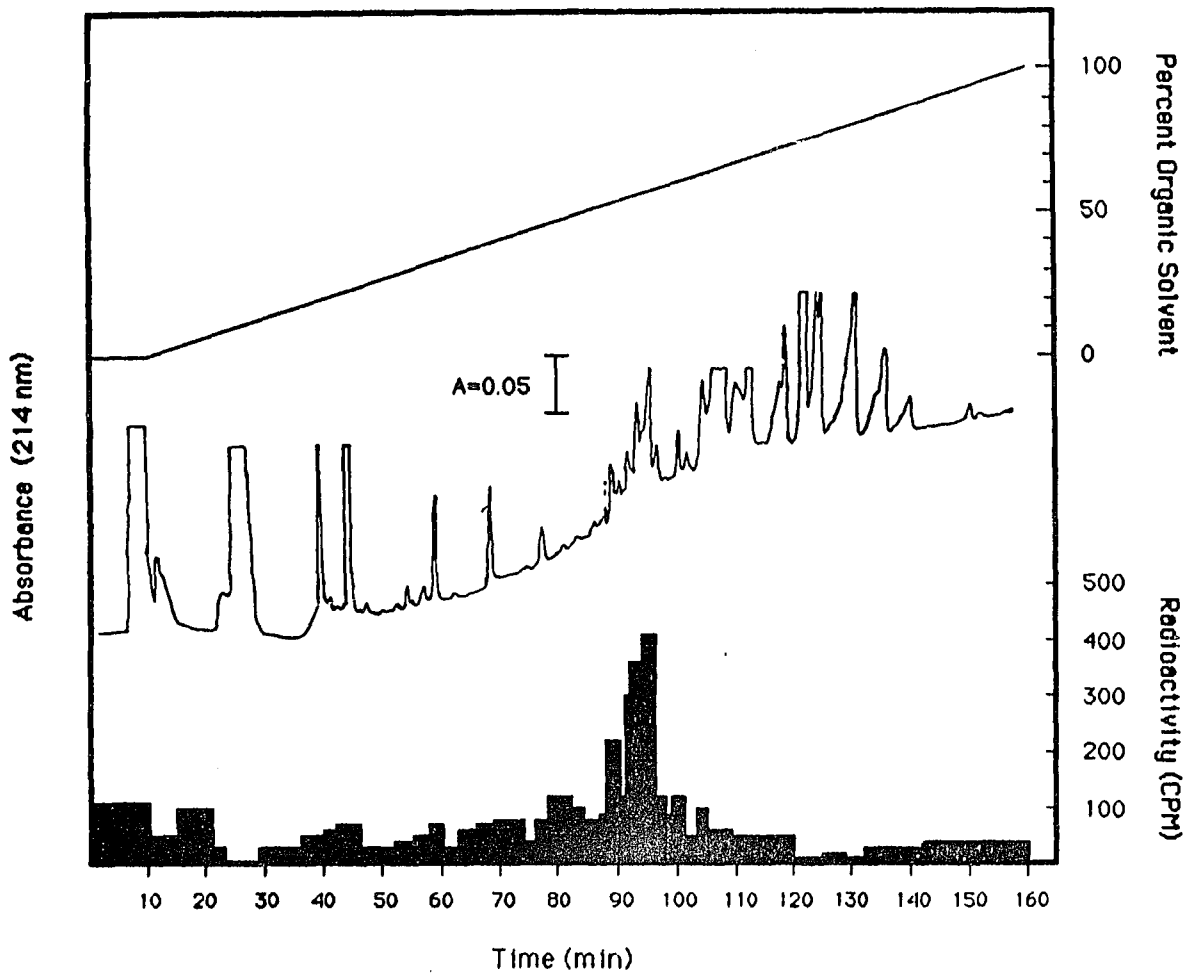


Figure 10. Purification of CNBr fragments of [^{14}C] DCCD labeled cytochrome b by Reverse Phase HPLC using 0.1% TFA in water and 0.1% TFA in 80%propanol/ 20% acetonitrile as solvents. [^{14}C] DCCD labeled cytochrome b treated with CNBr (3370 CPM) was chromatographed on a Waters C-18 Bondapak reverse phase column (3.9 mm X 30 cm). The solvents used were 0.1% TFA in water as the aqueous phase and 0.1% TFA in 80% propanol/ 20% acetonitrile as the organic phase at a flow rate of 0.5 ml/min. The gradient shown in the upper graph was started using 0.1% TFA, water. Both the absorbance at 214 nm and radioactivity from the [^{14}C] DCCD label were monitored. The absorbance trace is the middle graph and the lower bar graph is the radioactivity (CPM).

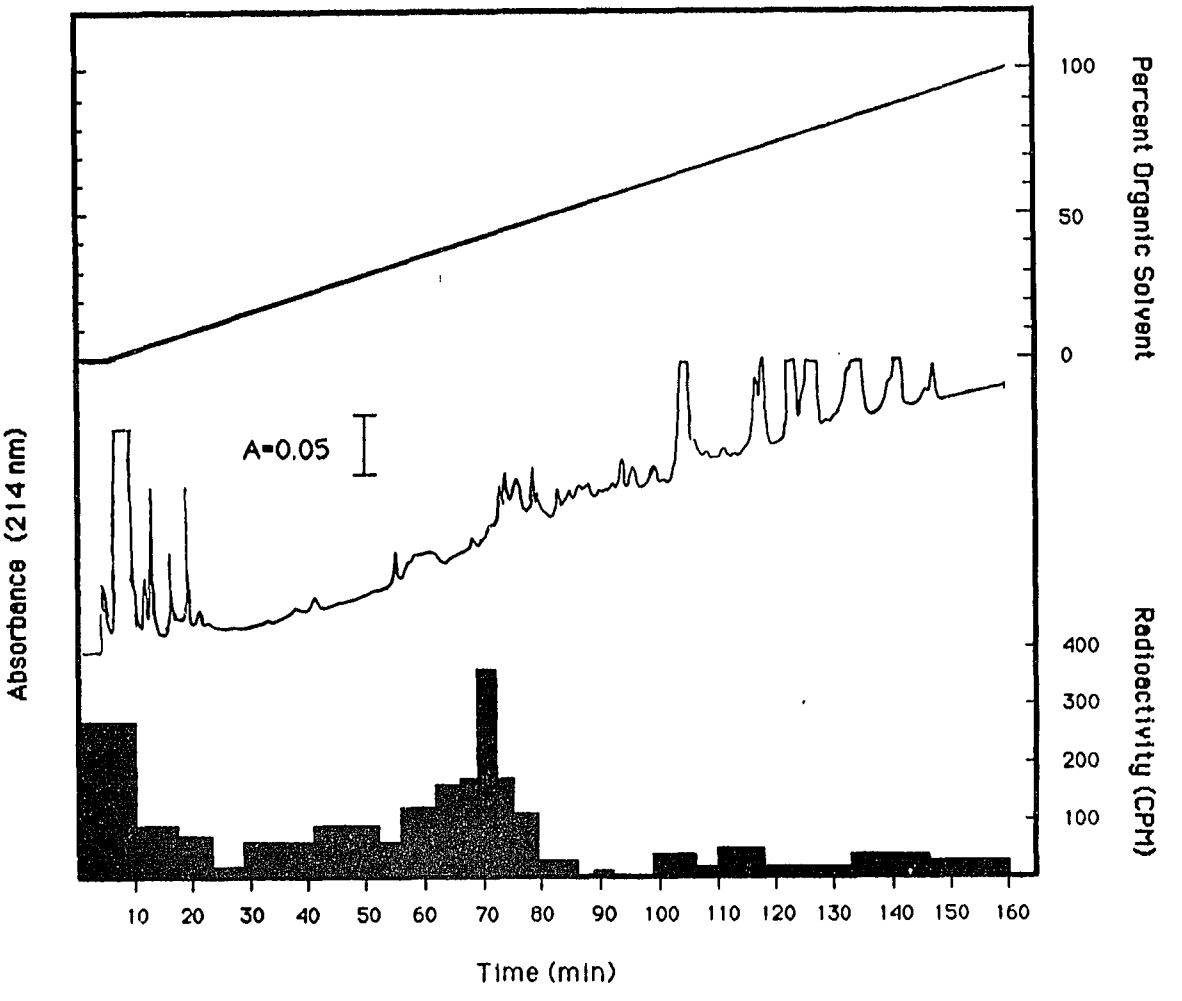


Figure 11. Purification of CNBr fragments of [^{14}C] DCCD labeled cytochrome b by Reverse Phase HPLC using 0.1% TFA in 20% propanol and 0.1% TFA in 80%propanol, 20% acetonitrile as solvents. [^{14}C] DCCD labeled cytochrome b treated with CNBr (2690 CPM) was chromatographed on a Waters C-18 Bondapak reverse phase column (3.9 mm X 30 cm). The solvents used were 0.1% TFA in 20% propanol as the initial mobile phase and 0.1% TFA in 80% propanol, 20% acetonitrile as the organic phase at a flow rate of 0.5 ml/min. The gradient is depicted in the upper graph. Both the absorbance at 214 nm and radioactivity from the [^{14}C] DCCD label were monitored. The absorbance trace is the middle graph and the lower bar graph is the radioactivity (CPM).

aggregates that could not migrate through a reverse phase column and increase the chance of isolating one peak with radioactiviy. Sephadex LH-60 chromatography was chosen as the method to accomplish this goal. The Sephadex LH-60 resin is composed of hydroxypropylated Sephadex G-50 resin which separates proteins not only by gel filtration, but also by adsorption and straight-phase partition chromatography. The cyanogen bromide fragments of cytochrome b dissolved in 70% formic acid were loaded onto a Sephadex LH-60 column as described in methods. The amount of radioactive material eluted off the column was monitored. Nearly 100% of the 8600 cpm's loaded onto the column were recovered (Fig. 12). The radioactive material was eluted from the column as a broad unresolved peak suggesting that the peptide fragments had aggregated. Consequently, this technique was not used in further experiments.

SUDS PAGE: The final method used to purify the cyanogen bromide fragments of cytochrome b was Stacking Urea-Dodecyl Sulfate (SUDS) PAGE (Kyte and Rodriguez, 1983). This method is useful in the separation of peptides containing from 25-250 amino acid residues and was used to purify the fragments of N-bromosuccinimide cleaved plasma membrane H^+-ATPase from N. crassa as reported by Sussman et. al. (1987). The sample used for this experiment was the pooled fractions obtained from the Sephadex LH-60 column as described above. As a control, BSA was fractionated with

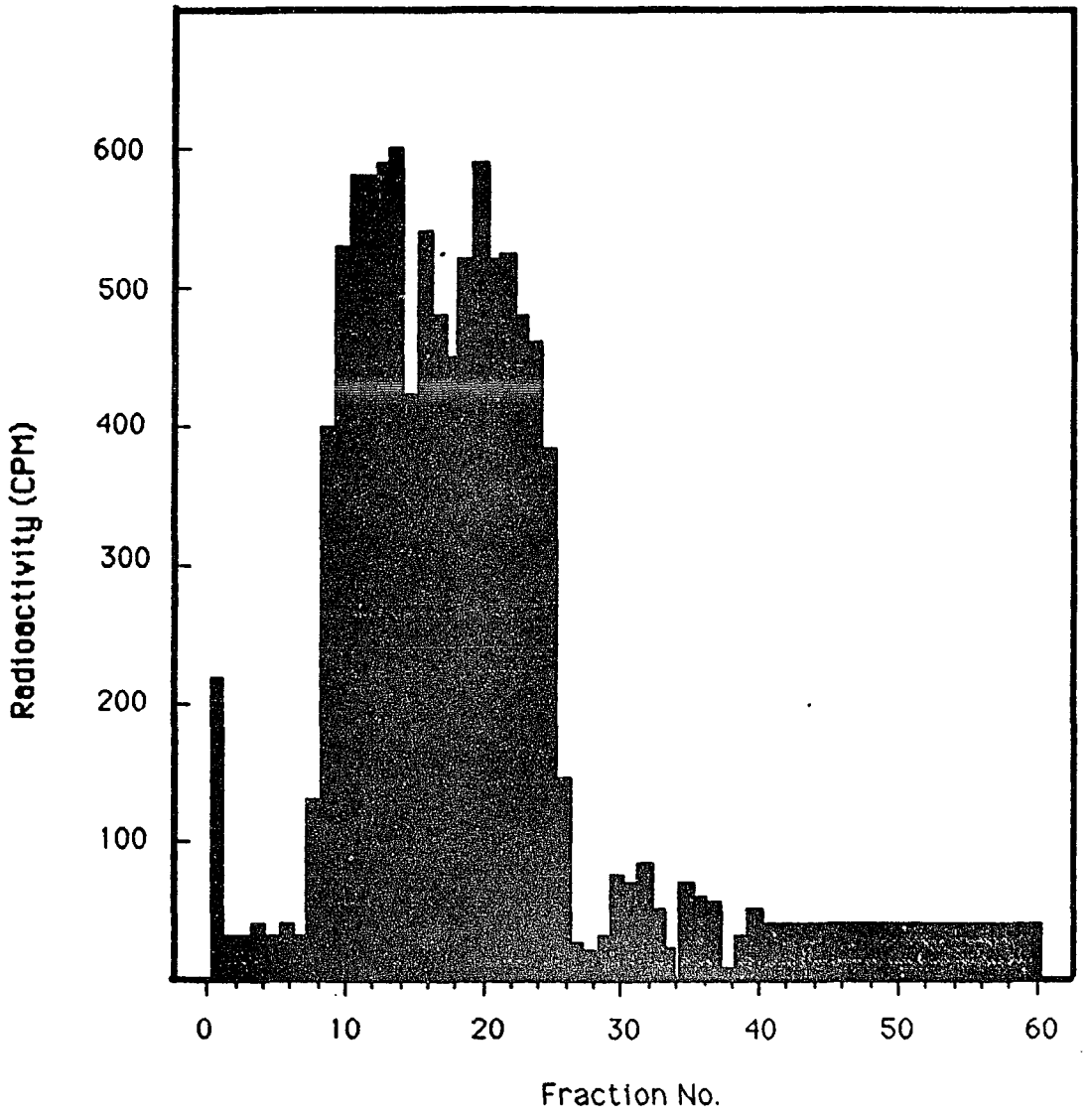


Figure 12. Purification of CNBr fragments of cytocrome b by Sephadex LH-60 chromatography. A sample of [^{14}C] DCCD labeled cytochrome b (8600 CPM) treated with CNBr was chromatographed on a Sephadex LH-60 column as described in Methods. The amount of radioactive label in each fraction collected from the column was measured.

cyanogen bromide treated identically as cytochrome b. BSA has 5 methionines and since the first amino acid is methionine, cleavage of BSA with cyanogen bromide should lead to the formation of 5 fragments detectable on a SUDS PAGE gel. This in fact was the result as seen in Fig. 13, lane 1. The amount of CNBr fragmented cytochrome b material loaded onto the gel contained 800 cpm, but there was insufficent protein to be detected even by silver staining (Fig. 13, lane 2). The autoradiogram of the gel revealed the presence of two labeled bands, a heavier labeled band with an apparent molecular weight of 5.2 kDa and a minor band with an apparent molecular weight of 10.7 kDa (Fig. 13, lane 3).

Amino Acid Analysis of Reverse Phase HPLC of Cytochrome b Fragments: Four samples were sent to Dr. John Hempel at the University of Pittsburgh for amino acid analysis. The samples were obtained from different C_{18} reverse phase HPLC experiments which utilized cytochrome b isolated from preparative SDS PAGE gels, extracted from gel slices via SDS extraction, and fragmented by cyanogen bromide treatment. Samples I-III were from chromatographs using the solvent system containing 0.1% TFA in 20% 2-propanol and 0.1% TFA in 80% 2-propanol/ 20% acetonitile. Sample IV was from an HPLC run using the first solvent system described: 0.1% TFA in water and 0.1% TFA in acetonitrile. A fraction with an absorption at baseline from a HPLC chromatograph served as the control or blank sample which would indicate the amount

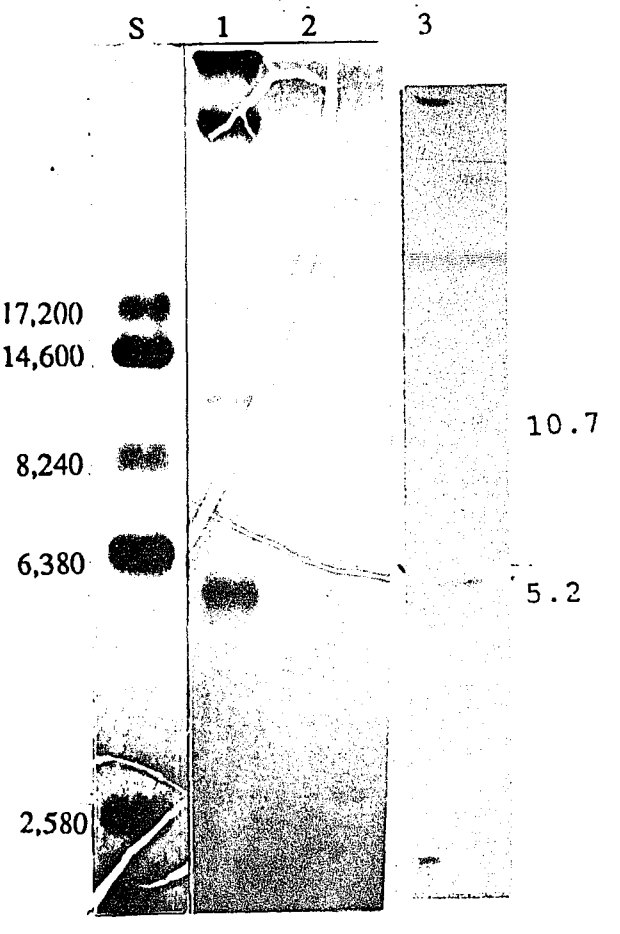


Figure 13. Purification of CNBr fragments of cytochrome b by SUDS Gel Electrophoresis. Pooled fractions of Sephadex LH-60 chromatography (fig. 14) were concentrated and used for SUDS Gel Electrophoresis. Lane S, 1-2; Silver-stained SUDS Gel. Low-molecular-weight standards: 17,200, 14,600, 8,240, 6,380, and 2,580 (lane S); 5 ug BSA CNBr fragments (lane 1); and 800 cpm of CNBr fragments of [^{14}C] DCCD labeled cytochrome b (lane 2). Lane 3, florograph of CNBr fragments of ^{14}C] DCCD labeled cytochrome b (lane 2).

of background or contaminating amino acids introduced by experimental error. The samples had the same amino acid composition as the blank (Table VIII).

Discussion: The purification and identification of the cyanogen bromide fragment(s) which binds DCCD was unsuccessful; but preliminary results obtained from SUDS PAGE analysis are promising and may eventually lead to the successful identification of the DCCD binding site(s) on cytochrome b. Two radioactive bands were observed on the autoradiogram of the SUDS gel. One band had an apparent molecular weight of 5.2 kDa while a second band with an apparent molecular weight of 10.7 kDa was lightly labeled as indicated by the intensity on the autoradiograph. The absence of other radioactive bands and the presence of one predominantly labeled band suggested that the cleavage with cyanogen bromide was essentially complete and that the problems with C_{18} reverse phase HPLC and Sephadex LH-60 chromatography were probably due to aggregation of the cyanogen bromide fragments.

The possible sites for the interaction of DCCD with cytochrome b are present in fragments 10 and 14. The predicted DCCD binding site on fragment 10 is aspartate 160 in the yeast mitochondria sequence (Fig. 14). This residue is predicted to be localized in the middle of the inner mitochondrial membrane and has surrounding hydrophobic amino acids. Several other species lack the aspartate at

TABLE VIII

Amino acid analysis of fragments of cytochrome b after cyanogen bromide treatment

The values reported are the percent composition for each amino acid in the analyzed sample.

	SAMPLE				
AMINO ACID	I	II	III	IV	Blank
A	8.3	6.7	9.6	10.1	8.0
R	ND	5.4	ND	6.1	ND
B (D & N)	9.9	9.4	11.5	11.8	8.6
C	1.7	2.7	ND	5.9	ND
Z (E & Q)	14.9	13.4	21.2	15.7	13.4
G	18.2	20.1	21.2	*	27.1
H	1.7	2.7	ND	2.5	1.4
I	5.0	4.0	3.8	5.1	3.4
L	9.1	7.4	7.7	8.1	6.6
K	5.0	4.0	ND	7.3	4.4
M	ND	ND	ND	0.2	ND
F	3.3	2.7	ND	3.7	3.3
P	3.3	4.0	ND	5.6	3.6
S	6.6	5.4	11.5	5.2	9.9
T	4.1	3.4	5.8	3.6	3.8
W	-	-	-	-	-
Y	2.5	2.7	ND	2.4	1.8
V	6.6	6.0	7.7	6.8	4.7
Total nmol	1.21	1.49	0.52	59.2	8.79

ND= Not detectable
*= sample had high value for glycine which indicated contamination.

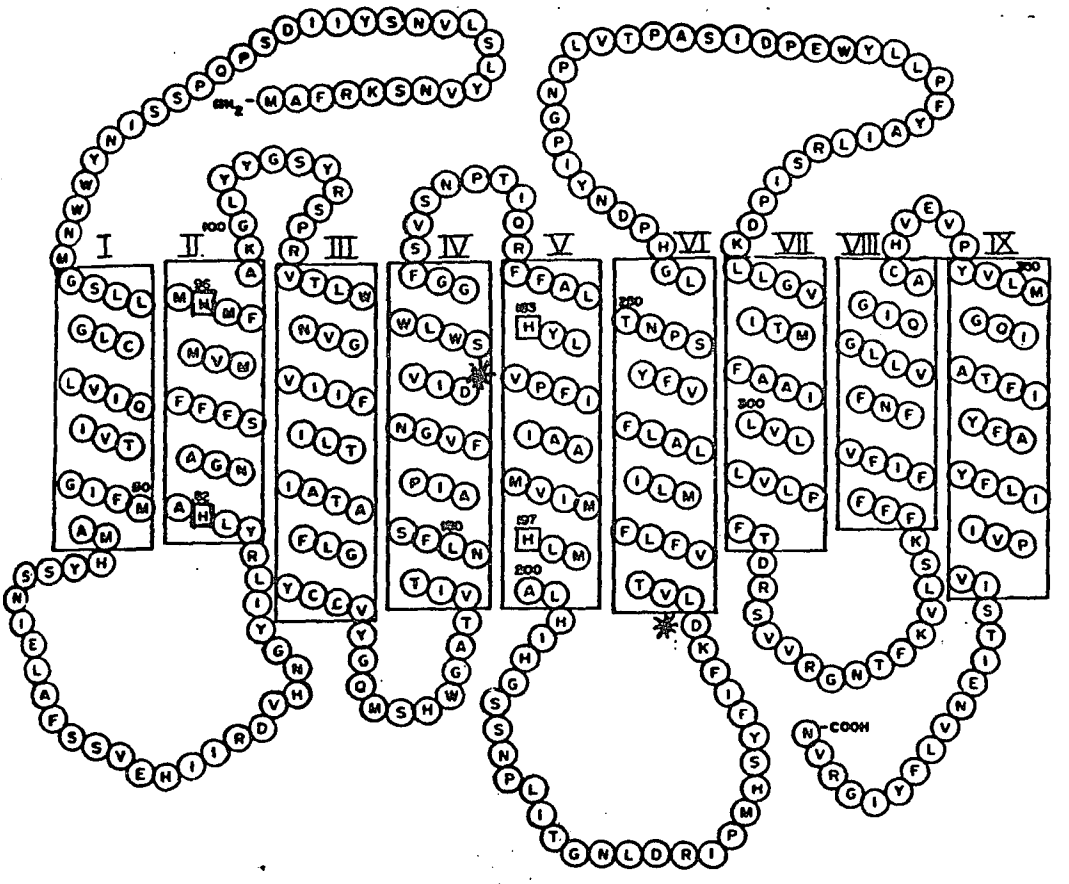


FIG. 3. Folding of the yeast mitochondrial cytochrome *b* in the inner mitochondrial membrane according to the hydropathy pattern of Fig. 2. Spanning segments are numbered by the same convention as in Fig. 2. Alternatives at positions 253 and 270 are Q and V (13).

Figure 14. Predicted topography of yeast mitochondrial cytochrome b from hydropathic plots. Figure 3 of Widger et. al. (1984) Proc. Natl. Acad. Sci. **81**, 674-678. The predicted DCCD-binding aspartate residues 160 and 229 are indicated by asterisks.

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160 but have a carboxy amino acid within three residues and thus in the same ã-helical region of the protein. The explanation of the two radioactive bands found on the autoradiograph from SUDS PAGE if aspartate 160 of fragment 10 binds DCCD, is much simpler than for fragment 14, aspartate 229. The molecular weight for fragment 10 is 6966 Da and if methionine 193 is not cleaved the resultant fragment (9 and 10) would be 12.4 kDa. Both fragments are hydrophobic and may migrate anomalously on gels and not at their true molecular weights. This possibility could explain the 5.2 and 10.7 kDa bands containing radioactive DCCD after SUDS PAGE analysis.

Residue 229 (aspartate) present on fragment 14 may also bind DCCD. Aspartate 229 of fragment 14 is conserved in cytochrome b from every species examined to date including those of bacteria. Hydropathy plots, however, do not predict that this amino acid is localized well within the membrane. Furthermore, aspartate 229 is not completely surrounded by hydrophobic amino acids. In fact, adjacent to aspartate 229 there is a positively charged lysine residue which is highly conserved. If aspartate 229 is found to bind DCCD, then it will be the first DCCD binding site described with an adjacent charged residue.

The data from the SUDS gel can also be explained if DCCD is bound to aspartate 229 (fragment 14). Fragment 14 has a predicted molecular weight of 2278, but if cyanogen bromide

does not completely cleave at methionines 199 and/or 221 the resultant fragments would be 4970 Da for fragments 13 and 14 and 5406 Da for fragments 12-14. Since these fragments would be hydrophobic in nature and have low molecular weights, they might show anamalous migration behavior on gels and not at their true molecular weight. This possibility could explain the existence of a 5.2 kDa band containing radioactive DCCD after SUDS PAGE analysis. The 10.7 kDa band might also be the result of an incomplete cleavage. If cyanogen bromide failed to cleave metionines 193, 196, 199, and 221 (fragments 10-14); methionines 221 and 237 (fragments 13-15); or methionine 237 (fragments 14 and 15), this would result in peptides of 12.8 kDa, 12.3 kDa., or 9.65 kDa respectively. Alternatively, the peptides consisting of fragments 12-14 or 13 and 14 which possibly migrate at 5.2 kDa might aggregate into a dimer thus leading to a doubling of the apparent molecular weight.

The 5.2 kDa band on the autoradiograph of the SUDS PAGE may also be explained by DCCD binding to fragment 5 (Table VII). Fragment 5 has a molecular weight of 5242 Da. and contains one aspartate (asp 70) and two glutamate (58 and 65) residues; however all of these residues are predicted by hydropathy plots to be localized outside of the membrane (Fig. 14). So far, the known DCCD binding sites (resulting in the inhibition of H^+-translocation upon binding DCCD) for the plasma membrane H^+-ATPases, the F_0F_1-H^+-ATPases, and

cytochrome oxidases from different species have all been predicted to reside well within the lipid bilayer of a membrane. In addition, the carboxy amino acid which binds DCCD is generally surrounded by hydrophobic residues (Table IX) as described by Sussman et. al.(1987). Another point is that only aspartate 70 is conserved throughout the different species, the two glutamate residues are not. Therefore, it seems unlikely that the amino acids with a free carboxy group in fragment 5 bind DCCD.

The fragments obtained by cyanogen bromide treatment of cytochrome b are obviously very hydrophobic and are not easily purified by reverse phase chromatography or by Sephadex LH-60 chromatography, although these techniques were used successfully to purify the fragments of cytochrome P-450, cytochrome b_5, plasma membrane H^+-ATPase, epoxide hydrolase, and bacteriorhodopsin.

Initially C_{18} reverse phase HPLC was used in this study to purify the cyanogen bromide fragments of cytochrome b, but resolving the radioactive fragments into one peak was not accomplished. The lack of resolution of the radioactive peaks could have been due to several reasons: 1) DCCD binds to more than one acidic amino acid residue on different fragments of cytochrome b obtained by cyanogen bromide and these fragments had similar hydrophobicities, 2) DCCD binds to a single site on cytochrome b but cleavage with cyanogen bromide was incomplete such that several fragments with

TABLE IX

Comparison of the amino acid sequence surrounding the DCCD-reactive glutamate or aspartate for different H^+-translocating proteins

The amino acid sequences for the cytochrome b were obtained from the predicted sequences as reported by Nobrega and Tzagoloff (1980). The remainder of the table was from Sussman et. al. (1987) fig. 5. The arrow indicates the carboxy amino acid which binds DCCD

Cyanogen bromide frag. of cyt. b	↓
Fragment 10 (asp 160)	-PHE VAL GLY ASN ASP ILE VAL SER TRP LEU TRP-
Fragment 14 (asp 229)	-PHE ILE PHE LYS ASP LEU VAL THR VAL PHE LEU-
Plasma Membrane H^+-ATPase	
N. crassa	-GLN PHE VAL MET GLU GLY ALA ALA VAL LEU ALA-
S. cerevisiae	-GLN PHE VAL MET GLU ALA ALA ALA ILE LEU ALA-
F_0F_1-H^+-ATPase	
N. crassa mito.	-PHE ALA PHE VAL GLU ALA ILE GLY LEU PHE ASP-
S. cerevisiae mito.	-PHE ALA LEU SER GLU ALA THR GLY LEU PHE CYS-
Bovine mito.	-PHE ALA LEU SER GLU ALA MET GLY LEU PHE CYS-
Spinach chloroplast	-LEU ALA PHE MET GLU ALA LEU THR ILE TYR GLY-
R. rubrum	-PHE ALA VAL THR GLU ALA ILE ALA LEU PHE ALA-
M. laminosus	-LEU ALA PHE MET GLU SER LEU THR ILE TYR GLY-
E. coli	-MET GLY LEU VAL ASP ALA ILE PRO MET ILE ALA-
A. calderius	-VAL ALA LEU VAL GLU ALA PHE PRO VAL ILE ALA-
PS-3	-VAL ALA LEU VAL GLU ALA LEU PRO ILE ILE GLY-
Cytochrome c Oxidase	
N. crassa mito.	-PHE ILE VAL SER GLU ALA LEU PHE PHE LEU ALA-
S. cerevisiae mito.	-PHE VAL LEU SER GLU VAL LEU ILE PHE ALA GLY-
A. nidulans mito.	-PHE ILE ILE SER GLU VAL PHE PHE PHE LEU ALA-
D. melanogaster mito.	-PHE ILE LEU SER GLU VAL LEU PHE PHE VAL SER-
Mouse mito.	-PHE ILE VAL SER GLU VAL PHE PHE PHE ALA GLY-
Bovine mito.	-PHE ILE ILE SER GLU VAL LEU PHE PHE THR GLY-
Human mito.	-PHE ILE THR SER GLU VAL PHE PHE PHE ALA GLY-

similar hydrophobicities were obtained, and/or 3) DCCD binds to one site but the one fragment obtained from cyanogen bromoide may tend to aggregate.

The first possibility that DCCD binds to more than one residue seems unlikely since the resolution of the eluted peaks containing radioactivity increased when both the organic solvent was made more nonpolar or hydrophobic and the "steepness" of the gradient was increased. If there were different DCCD binding fragments with similar hydrophobicities, then the resolution would decrease by increasing both the hydrophobic nature of the organic solvent and the rate of change in the gradient. The second possibilty that the cleavage with cyanogen bromide was incomplete also seems unlikely to be the only cause of the resolution problem, since this would mean that 1-8 consecutive methionines were sometimes not cleaved. The last possibilty that the fragments obtained after cyanogen bromide cleavage aggregated on the C_{18} column appears to be the most logical reason for the lack of resolution of the radioactive material. No matter which solvent system was used, the radioactive material eluted as a broad absorbance peak with multiple peaks, which is characteristic of aggregating peptides. This explanation is not surprising since cytochrome b as a whole protein tends to aggregate during purification.

Amino acid analysis of the isolated peptide containing

radioactive DCCD should be sufficient to determine the actual binding site of DCCD on cytochrome b assuming that DCCD binds to either aspartate 160 or aspartate 229. The amino acid composition of fragments 10 and 14, or any of the variations discussed, are so different that sequencing of the DCCD fragment would not provide much additional information, but would serve as another means of verification (Table VII).

The SUDS technique appears to be the most promising method for the evential isolation of fragments obtained by cyanogen bromide cleavage of cytochrome b previously labeled with [^{14}C] DCCD.

D. THE EFFECTS OF $\underline{b}$-$\underline{c}_1$ COMPLEX INHIBITORS AND SUBSTRATES HAVE UPON THE BINDING OF DCCD TO CYTOCHROME $\underline{b}$

As mentioned previously in the introduction of this dissertation, treatment of the $\underline{b}$-$\underline{c}_1$ complex with antimycin was reported to inhibit the binding of DCCD to cytochrome $\underline{b}$ Beattie *et. al.* (1984). The opposite is also true, as treatment of the $\underline{b}$-$\underline{c}_1$ complex with DCCD results in a change in the antimycin induced red shift of cytochrome $\underline{b}$ (Clejan and Beattie, 1983). These results suggested that the binding of antimycin or DCCD may change the conformation of cytochrome $\underline{b}$ affecting the binding of the other compound. Other inhibitors of the enzymatic activity of the $\underline{b}$-$\underline{c}_1$ complex were next examined to learn how their interaction with the complex affected the binding of DCCD to cytochrome $\underline{b}$.

Effects of Inhibitors on DCCD Binding: Antimycin, myxothiazol, 2-n-heptyl 4-hydoxyquinoline N-oxide (HQNO) and 3-nonyl 2-hydroxy 1,4 naphthoquinone (n-HNQ) all inhibit ubiquinol-cytochrome $\underline{c}$ oxidoreductase activity by inhibiting electron flow through the $\underline{b}$-$\underline{c}_1$ complex (von Jagow and Link, 1986). Antimycin and HQNO both bind to the Q_i site (Fig. 4), but antimycin binding causes a red shift of cytochrome $\underline{b}_{562}$ from 562 to 564 nm whereas HQNO causes a red shift of less than 1 nm (van Ark and Berden, 1977). Myxothiazol and n-HNQ both inhibit enzymatic activity via the center $\underline{o}$ site

by preventing the reduction of the iron sulfur center and cytochrome $\underline{b}_{566}$; however each inhibitor acts by a different mechanism (von Jagow and Engel, 1981; Thierbach and Reichenbach, 1981; and Matsuura et. al., 1983). Myxothiazol causes a red shift of cytochrome $\underline{b}_{566}$ from 566 to 568 nm, but n-HNQ does not.

Purified $\underline{b}$-$\underline{c}_1$ complex was incubated with the inhibitors at concentrations which fully inhibited ubiquinol-cytochrome c oxidoreductase activity as described in methods and then labeled with DCCD. Preincubating the $\underline{b}$-$\underline{c}_1$ complex with the Q_i inhibitors antimycin or HQNO resulted in a decrease of DCCD binding (Table X). Antimycin decreased the binding of DCCD to cytochrome $\underline{b}$ by 33-88% when compared to controls not preincubated with the inhibitors. HQNO decreased DCCD binding by 36-72%. The Q_o site inhibitor, n-HNQ may have stimulated the binding of DCCD to cytochrome $\underline{b}$. The other Q_0 inhibitor tested, myxothiazol, may have resulted in a decrease of DCCD binding to cytochrome $\underline{b}$, but clearly not as much as the Q_i inhibitors antimycin and HQNO. These results suggest that DCCD binds to cytochrome $\underline{b}$ at a site which is affected more by Q_i site inhibitors than by Q_o inhibitors; however, the results do not imply that DCCD necessarily binds closer to the Q_i site than to the Q_o site.

The effects of other inhibitors of ubiquinol-cytochrome $\underline{c}$ oxidoreductase upon the binding of DCCD to cytochrome $\underline{b}$ were also investigated. These inhibitors included

TABLE X

The effects of b-c_1 complex inhibitors and ubiquinol analogues have upon the binding of DCCD to cytochrome b

Purified b-c_1 complex (200 g) was pre-incubated with the reagent indicated and then was labeled with 50 nmoles [^{14}C] DCCD per nmole of cytochrome b as described in methods. SDS PAGE was then performed on the labelled b-c_1 complex and the relative amount of [^{14}C] DCCD bound to cytochrome b was determined by densitometric scanning of the silver stained gels and the autoradiographes. Negative values indicate that the reagent resulted in an inhibition of DCCD binding to cytochrome b vs. controls ([^{14}C] DCCD alone), whereas a positive value corresponds to a stimulation of DCCD binding.

Inhibitor or Q-analogue	Experiment		
	1	2	3
Antimycin	-88	-33	-47
HQNO	-72	-36	-49
Myxothiazol	-55	-1.3	-6.9
n-HNQ	+55	+4.5	
EFA	+455	+146	
DNFB	+161	-4.6	
BAL	-71	-54	-50
DBH_2	-79	-71	
Q_6H_2	-100	-54	
Q_0H_2	+224	-20	
K_3H_2	+49	-49	
DQH_2	+209	+21	
$Na_2S_2O_4$	+267	N.D.	
$K_3Fe(CN)_6$	+2.4	+56	

ethoxyformic anhydride (EFA), 2,4-dinitrofluorobenzene (DNFB), and 2,3-dimercaptopropanol (BAL). EFA apparently binds to a histidine residue of the Core II protein resulting in an inhibition mainly of the antimycin-insensitve pathway of cytochrome b reduction similar to that observed with myxothiazol. EFA also has a minor effect on the antimycin sensitive pathway (Lorusso et. al., 1987). DNFB has been shown to bind predominantly to Core I protein and to a lesser extent cytochrome b (Lorusso et. al., 1986). The inhibitory effects have been attributed to the blocking of electron flow between b_{566} and b_{562}. BAL detroys the iron sulfur cluster of the Reiske iron sulfur protein (Slater and de Vries, 1980). Preincubation of the isolated b-c_1 complex with EFA and DNFB resulted in an apparent stimulation of the binding of DCCD to cytochrome b; however, BAL decreased the binding of DCCD to cytochrome b by 50-70%. (Table X). The concentrations of the inhibitors used in the DCCD binding experiments inhibited ubiquinol-cytochrome c oxidoreductase activity by at least 80% (data not shown).

Ubiquinol Analogue Substrates: Different quinols were incubated with the isolated b-c_1 complex prior to labeling with DCCD in order to determine the effect of reduction of the complex on the subsequent binding of DCCD (Table X). Incubation with both DBH_2 and Q_6H_2 resulted in an inhibition of DCCD binding to cytochrome b by 75% and 54-100% respectively. Incubation with the analogues Q_oH_2 and K_3H_2 did not

result in a decrease of DCCD binding, while incubation with DQH_2 apparently increased the amount of DCCD bound to cytochrome b.

The effects of oxidizing or reducing the b-c_1 complex with artificial electron donors and acceptors on DCCD binding were also studied. The complex was reduced using sodium hydrosulfite and oxidized by potassium ferricyanide. Twice as much DCCD bound to cytochrome b when the complex was reduced than when it was oxidized. Therefore, the decrease in DCCD binding to cytochrome b in the presence of reduced Q_6H_2 and DBH_2 resulted from substrate binding to the complex rather than to the reduction of cytochrome b.

The variation of the values in Table X can be attributed many sources of error. A likely explanation is the non-uniformity of both silver staining SDS gels and exposure and developement of the film used for autoradiography. The incubation of the b-c_1 complex with ubiquinol-cytochrome c oxidoreductase inhibitors, ubiquinol analogues, and [^{14}C] DCCD for all experiments were performed under carefully controlled conditions for concentration of reagents, time, and temperature.

Discussion: The results from the inhibitor studies indicated that the DCCD binding site is affected by Q_i inhibitors (antimycin and HQNO) more than Q_o inhibitors (myxothiazol and n-HNQ). It is tempting to say that DCCD binds closer to cytochrome b_{562} than to b_{566}, but more

evidence is needed for this conclusion. How then is DCCD binding to cytochrome b affected by other ubiquinol-cytochrome c oxidoreductase inhibitors which bind b-c_1 complex subunits other than cytochrome b? EFA which binds to Core II protein stimulates DCCD binding to cytochrome b, however, EFA treatment (unlike antimycin) does not apparently result in a detectable conformational change of cytochrome b, since EFA inhibition does not result in any spectral changes of either cytochrome b_{562} or b_{566}. DNFB which binds to Core I and slightly to cytochrome b, does not affect DCCD binding even though it suppresses antimycin binding (Lorusso *et. al.*, 1986).

BAL was the only inhibitor tested that does not bind to cytochrome b and apparently causes a decrease of DCCD binding to cytochrome b. BAL has been shown to destroy the iron sulfur cluster of the Reiske iron sulfur protein, as determined by the disappearance of the EPR signal of this iron sulfur cluster. Treating the b-c_1 complex with BAL, however, did not result in any spectral changes in the heme groups of cytochrome b. The lack of spectral changes does not preclude that BAL treatment may cause conformational changes in cytochrome b; but BAL may also directly block the binding of DCCD to cytochrome b without causing any conformational change in cytochrome b.

The results obtained with inhibitors of the b-c_1 complex which bind to subunits other than cytochrome b suggest that

the conformation of the core protein II and the iron-sulfur protein may effect the conformation of cytochrome b. It is interesting that EFA stimulates DCCD binding to cytochrome b while BAL which binds the iron-sulfur protein is inhibitory. If the same carboxy amino acid is modified no matter which inhibitor is present, then the results indicate that the carboxy amino acid residue which binds DCCD can exist in different environments.

The results of the studies involving ubiquinol-analogues indicated that the high affinity ubiquinols such as Q_6H_2, the native ubiquinol in yeast mitochondria, and DBH_2 both decrease the binding of DCCD to cytochrome b. In contrast, the analogues with a lower affinity to the b-c_1 complex, Q_0H_2, K_3H_2, and DQH_2, either do not affect or actually stimulate DCCD binding. The high affinity ubiquinols are better substrates for ubiquinol-cytochrome c oxidoreductase activity than the lower affinity analogues, both when the b-c_1 complex is in a membrane and also in the isolated form, Zhu and Beattie (1988). The ubiquinols were incubated with isolated b-c_1 complex in the absence of electron acceptors, such as cytochrome c or ferricynanide, for these experiments. Therefore the binding of the high affinity ubiquinols to isolated b-c_1 complex may simulate conformational changes that occur to the subunits of the complex during the initial stages of electron flow. Under these conditions, it is possible that the amino acid residue which binds DCCD is

in a different environment after the ubquinol binds to the $\underline{b}$-$\underline{c}_1$ complex. If the DCCD binding amino acid of cytochrome $\underline{b}$ serves as a proton carrier and moves during proton translocation, then the movement of this amino acid residue may be part of the mechanism of proton translocation across a biological memebrane. But much more evidence is needed to make such a conclusion.

IV. CONCLUSION

One of the specific aims of this research was to determine which amino acid residue(s) of cytochrome b binds DCCD. The experimental approaches described in this dissertation provide information concerning methods which may be useful in the eventual success of determining the site to which DCCD binds cytochrome b. A major obstacle of this research is to isolate enough cytochrome b in a soluble form from [^{14}C] DCCD labelled yeast b-c_1 complex. Cytochrome b can be resolved from the other subunits of the b-c_1 complex by SDS PAGE, but extracting the purified cytochrome b from the polyacrylamide gel is a problem. Electroelution and SDS extraction resulted in poor recoveries of cytochrome b. The other methods that were used to isolate cytochrome b, molecular sieve HPLC and chromatography on phenyl-sepharose, failed to resolve cytochrome b from all of the other subunits of the b-c_1 complex. Therefore, gel electrophoresis is currently the best method for isolating cytochrome b from the other subunits of the b-c_1 complex. The problem to be solved is how to efficiently recover cytochrome b from an SDS polyacrylamide gel. A method involving direct recovery of protein as the gel runs is currently being used in our laboratory to isolate large quantities of cytochrome b.

Once a large amount of [^{14}C] DCCD labeled cytochrome b is obtained, the protein can then be cleaved with CNBr and the

resultant CNBr fragments can probably be resolved on a SUDS gel. The [^{14}C] DCCD labelled CNBr fragment(s) of cytochrome b can then be extracted from the polyacrylamide gel and analyzed for amino acid content and sequence.

Recently the results of studies by di Rago and Colson (1988) on diuron resistant mutants of S.cerevisiae suggested that the model for the folding of cytochrome b in the inner mitochondrial membrane proposed by Widger et. al. (1984) may have to be modified. di Rago and Colson found that a single change in amino acid residues isoleucine 17, arginine 31, or phenylalanine 225 conferred resistance of S. cerevisiae to diuron, a Q_i-site inhibitor of the b-c_1 complex. According to the cytochrome b folding model of Widger et. al. (1984), Fig. 14, isoleucine 17 and arginine 31 are on one side of the membrane and phenylalanine 225 is on the opposite side. The separation of the two Q_i-inhibitor resistant regions on opposite sides of the membrane seems to be inconsistent with diuron inhibition of the Q_i-site which supposedly is closer to the mitochondrial matrix side of the membrane.

Similar studies by di Rago et. al. (1989) on mxyothiazol, mucidin, and stigmatellin (Q_0-inhibitors) resistant mutants of S. cerevisiae were performed and point mutations which conferred resistance were identified. Mutations which resulted in single amino acid changes of glycine 137, asparagine 256, and leucine 275 resulted in resistance to both myxothiazol and mucidin. In addition, a change in

phenylalanine 129 also conferred myxothiazol resistance but not resistance to mucidin. A single point mutation changing isoleucine 147 to phenylalanine resulted in resistance to stigmatellin. According to the cytochrome b folding model of Widger et. al. (1984), amino acid residue phenylalanine 129 is localized within a membrane-spanning portion of the protein near the matrix side of the membrane and glycine 137 and isoleucine 147 are localized on the matrix side of the membrane. Amino acids asparagine 256 and leucine are localized on the outer side of the membrane. The localization of the two Q_o-inhibitor binding regions on opposite sides of the mitochondrial inner membrane is inconsistent with inhibition by the three Q_o-inhibtors. The Q_o-site is proposed to be closer to the outer side of the mitochondrial inner membrane.

In fact, calculations of the hydrophobicity index and hydrophobic moments by Brasseur (1988) indicated that helix IV of the Widger model probably does not span the inner mitochondrial membrane. Withdrawal of the fourth helix from the membrane results in the amino acid residues involved in diuron resistance to be on the same side of the membrane and the amino acids involved in myxothiazol, mucidin, and stigmatellin resistance to be on the other side of the membrane (Fig. 15).

The new model of di Rago and Colson also changes the

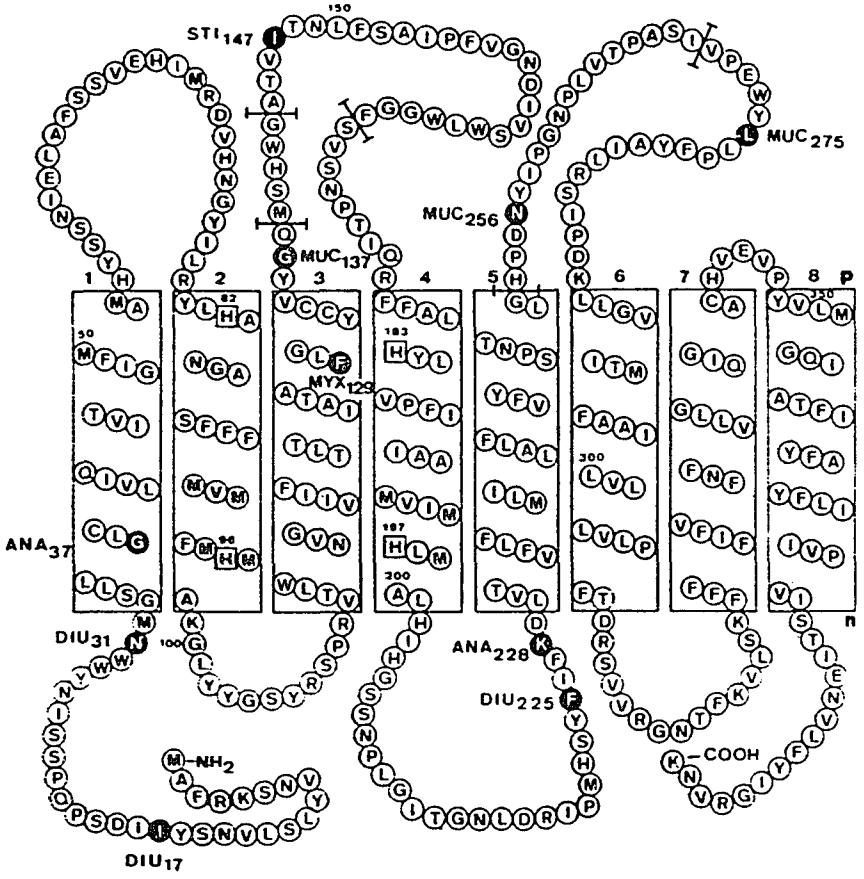

FIG. 4. **Cytochrome *b* folding model in the mitochondrial inner membrane.** This represents an eight-transmembrane helices model after the withdrawal of the fourth helix from the membrane. ——, indicates the positions of the corresponding exon limits of the split cytochrome *b* gene. Amino acids involved in the inhibitor resistance are shown in *white* surrounded by a *black circle* (*ANA*, antimycin; *DIU*, diuron; *MYX*, myxothiazol; *MUC*, mucidin; *STI*, stigmatellin). *Numbers* in *subscript* are the amino acid positions. *p* and *n* represent, respectively, positively and negatively charged sides of the mitochondrial inner membrane according to Cramer and co-workers (49).

Figure 15. Modified model of the predicted topography of yeast mitochondrial cytochrome b from hydrophobicity index and hydrophobic moments. Figure 4 of di Rago et. al. (1989) J. Biol. Chem. 264, 14543-14548. The predicted DCCD-binding aspartate residues 160 and 229 are indicated by asterisks.

localization of aspartate 160 which is in helix IV of the Widger model from a membrane spanning region to a region outside the membrane. The carboxyl amino acid which binds DCCD in other H^+-translocating enzymes were predicted to be localized in a membrane spanning region of a protein by hydropathic plots. Hence, if the model of di Rago and Colson is correct, then it seems less likely that asp 160 is the DCCD binding site in cytochrome b which results in the inhibition of H^+-translocation in the b-c_1 complex upon the binding of DCCD. Other carboxyl amino acids in cytochrome b are not dramatically affected by the modified model and therefore no new carboxy residues would be predicted to bind DCCD. Aspartate 229 would then be more likely to bind DCCD than aspartate 160, unless the DCCD binding site of cytochrome b is different from other H^+-translocating enzymes.

Another specific aim of the research was to investigate the effects of different types of inhibitors and Q-analogues of the b-c_1 complex on the binding of DCCD to cytochrome b.

In general, the results of the studies suggest that if the same carboxy amino acid binds DCCD regardless of which inhibitor or Q-analogue is present, then this carboxy amino acid residue can exist in different environments depending upon the conformation of cytochrome b. If the DCCD binding amino acid of cytochrome b serves as a proton carrier and is part of the mechanism of proton translocation in the b-c_1 complex, then this amino acid might be expected to exist in

different environments.

V. FOOTNOTES

1. Abbreviations:

ADP	adenosine diphosphate
AIA	allylisopropylacetamide
ALAS	õ-aminolevulinic acid synthase
ATP	adenosine triphosphate
BAL	2,3-dimercaptopropanol
CCCP	carbonyl cyanide m-chlorophenylhydrazone
DACM	N-(7-dimethylamino-4-methylcoumarinil) maleimide
DBH_2	2,3-dimethoxy-5-methyl-6-n-decyl-1,4 benzoquinone
DCCD	dicyclohexylcarbodiimide
DFP	diisopropylfluorophosphate
DM	dodecylmaltoside
DMSO	dimethylsulfoxide
DNFB	2,4-dinitrofluorobenzene
DQH_2	reduced duroquinone
DTNB	5,5'-dithiobis 2-nitrobenzoic acid
EDAC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EFA	ethoxyformic anhydride
EDTA	ethylenediaminetetraacetic acid
FMN	flavin mononucleotide
HPLC	high pressure liquid chromatography
HQNO	heptylhydroxyquinoline N-oxide
K_3H_2	menadiol
nonyl-HNQ	3-nonyl 2-hydroxy 1,4 napthoquinone
UHNQ	oxidized form of undecylhydroxynaphthoquinone
PMSF	phenylmethylsulfonylfluoride
Q_0H_2 and Q_6H_2	reduced forms of ubiquinone having a sidechains of 2 and 6 isoprene units respectivively
SDH	succinate dehydrogenase
SDS	sodium dodecylsulfate
SDS PAGE	SDS polyacrylamide gel electrophoresis
SMP	submitochondrial particles
SUDS gel	stacking urea-dodecyl sulfate polyacrylamide gel
TFA	trifluoroacetic acid

VI. APPENDIX

The Effects of Insulin and Glucose on the Intracellular Distribution and Translocation of õ-Aminolevulinic Acid Synthase

Prior to studying DCCD binding to cytochrome b, I investigated the effects of insulin and glucose on the intracellular translocation of õ-aminolevulinic acid synthase (EC 2.3.1.37). This research was discontinued for reasons explained later.

Hepatic õ-aminolevulinic acid synthase (ALAS) catalyzes the first and rate-limiting step of the heme biosynthetic pathway (Granick and Beale, 1978) and is thought to play a major role in the regulation of heme synthesis. ALAS is localized in the mitochondrial matrix (Zuyderboudt et. al., 1969; Scotto et. al., 1983) and catalyzes the oxidative decarboxylative condensation of glycine with succinyl-CoA to form õ-aminolevulinic acid.

The acitivity of ALAS is increased several fold by drugs such as allylisopropylacetamide (AIA), which causes experimental porphyria (Marver et. al., 1966; Narisawa et. al., 1966; and Granick and Sassa, 1971).

The enzyme is synthesized on cytoplasmic ribosomes as a higher molecular weight precursor form and translocated into mitochondria in a subsequent step concomitant with proces-

sing to the mature form of the enzyme (Yamauchi et. al., 1980; Kikuchi and Hayashi, 1981).

The translocation of ALAS from the cytoplasm to the mitochrondial matrix has been shown to be inhibited by heme (Hayashi et. al., 1972; Yamauchi et. al., 1980). When hemin was administered to rats, the activity of mitochondrial ALAS decreased and the ALAS activity in the cytosol increased relative to control animals, while the total activity of ALAS did not change significantly over time. This result suggested that heme may inhibit the intracellular translocation of ALAS from the cytosol into the mitochondrial matrix. Another observation by Yamauchi et. al. (1980) was that when AIA-treated rats were administered hemin followed by an injection of [^{3}H]-leucine, the amount of labeled immunoreactive ALAS increased in cytosol and decreased in the mitochondria, when compared to the amount of labeled ALAS in each fraction of control animals not treated with hemin. These results also provided evidence that heme inhibits the intracellular translocation of ALAS.

Further evidence of the inhibition by hemin on the translocation of ALAS came from turnover studies on ALAS activity Hagashi et.al.,(1980). For these experiments, AIA was administered to rats and four hours later either cycloheximide alone (control) or cycloheximide and hemin were injected into animals. The rats were then sacrificed and the ALAS activity was measured in both the cytosolic and

mitochondrial fractions. Rats were treated with cycloheximide to inhibit cytoplasmic protein synthesis, so that during hemin treatment, no new synthesis of ALAS would occur to complicate the measurement of turnover rates. The rationale behind these experiments was that if hemin inhibits the translocation of ALAS from the cytosol into the mitochondria, then the observed turnover rate of ALAS activity should increase in the mitochondria and decrease in the cytosol relative to the turnover rates of the control animals (cycloheximide treated). In other words, the half-life of ALAS activity in the mitochondria would decrease and the half-life in the cytosol would increase. In fact, the half-life of the activity of cytosolic ALAS increased from 20 minutes to almost no degradation after one hour of hemin treatment. Conversely, the half-life of the mitochondrial activity decreased from 60 min to 35 min upon hemin administration. The loss of ALAS activity in the mitochondria was shown to result in an actual decrease in the quantity in immunoreactive ALAS protein. The evidence presented clearly suggested that hemin inhibits the intracellular translocation of ALAS.

Similar experiments were done to study the effects of insulin and glucose on both the intracellular distribution and the translocation of ALAS from the cytosol into the mitochondrial matrix, for details see attached paper: DeLoskey and Beattie (1984).

The first set of experiments performed investigated the effects of insulin and glucose have on the intracellular distribution of ALAS activity. The administration of insulin or glucose to rats induced with AIA resulted in a significant increase in the percentage of total cellular ALAS activity in the cytosol, with a concomitant decrease in the amount in the mitochondria. This result suggested that these compounds may inhibit the translocation of ALAS from the cytosol into the mitochondrial matrix. Further evidence of the apparent inhibition of the intracellular translocation of ALAS by glucose and insulin was provided by studies on the effects of these compounds on the turnover of ALAS activity in the cytosol and in the mitochondria.

The effects of insulin and glucose on the turnover of ALAS activity was initially investigated using starved rats injected with AIA to induce ALAS activity 16 h prior to administering insulin, glucose, and cycloheximide. This time period was chosen to obtain maximum induction of ALAS and to ensure that no changes in ALAS activity would occur due to AIA during the administration of the other agents. The half-life of ALAS activity in the mitochondria from the livers of AIA-induced rats did not significantly change with insulin treatment. In contrast, the half-life of the cytosolic ALAS activity significantly increased from 21 minutes to 213 minutes when insulin was injected simultaneously with cycloheximide or 395 minutes if insulin was

administered two hours after cylcoheximide. Similarly glucose administration to AIA-induced rats resulted in no significant change in the half-life of the mitochondrial activity and a significant increase in the half-life in cytosol from 20.8 minutes to 102 minutes.

Another set of turnover studies was performed using fed rats, in which ALAS activity was induced with AIA in the same manner as the fasted animals. The half-lives of the control mitochondrial and cytosolic ALAS activities were 83.2 and 14.6 min, respectively, while the half-lives for the enzyme in the insulin-treated animals were 59.6 and 38.7 min. Again, the cytosolic turnover rate decreased significantly after insulin treatment but, like the starved animals, the turnover rate of mitochondrial ALAS did not change significantly.

Lastly, experiments with the purpose of investigating the insulin effect on ALAS activity using noninduced rats were performed. The half-life of ALAS decreased significantly in the mitochondria from 92.8 to 42.4 min, while the half-life increased significantly in the cytosol from 13.3 to 40.5 min after treatment with insulin.

The conclusion from the studies of the turnover and the intracellular distribution of ALAS suggested that insulin and glucose may inhibit the translocation of ALAS from the cytosol to the mitochondria matrix. Another possible explanation of the results is that insulin and glucose do

not inhibit the intracellular translocation of ALAS, but alter the rate of degradation of ALAS and/or alter the enzymatic activity. Therefore, I concluded that experiments quantitating the amount of actual ALAS protein in each fraction after glucose or insulin administration needed to be performed. The quantitation of ALAS was to be determined by the amount of immunoprecipitable ALAS.

In order to immunoprecipitate ALAS, a polyclonal antibody for the enzyme had to be obtained using purified ALAS as an antigen. A procedure to purify ALAS from rat liver was reported by Srivastava et. al. (1982) based on the purification of chick embryo liver mitochondrial ALAS (Borthwick et. al., 1983). The purification of the rat liver enzyme involved isolating mitoplasts and three column chromatography steps: molecular seive chromatography (Sephacryl S-200), chromatofocusing, and affinity chromatography on CoA agrose. The same purification scheme was repeatedly attempted in our laboratory, but I could not reproduce the results of Srivastara et. al. (1982). Many other purification attempts were performed with variations of published procedures, but with no success. We also tried to obtain the antibody to rat liver ALAS from Dr. G. Kukuchi's and Dr. A. Elliot's laboratories but they would not supply the antibody. As a last resort, we tried to obtain an antibody to chick embryo liver ALAS from Dr. I. Ades. The polyclonal antibody to chick embryo liver enzymes was reported to

cross-react with rat liver ALAS (Borthwick et. al., 1983). Unfortunately, Dr. Edith Gallob tested the cross reactivity of antibody to the chick embryo ALAS to rat liver enzyme and found no positive reaction (personal communication). We could not personally perform this crossreaction experiment since Dr. Ades declined to supply us with antibody.

Since no antibody which reacted with rat liver ALAS could be either made or obtained, further progress on this project was impossible. Therefore, the research on the effects of insulin and glucose on the intracellular translocation of ALAS was terminated.

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The Effects of Insulin and Glucose on the Induction and Intracellular Translocation of δ-Aminolevulinic Acid Synthase[1]

RICHARD J. DELOSKEY[2] AND DIANA S. BEATTIE[3]

Department of Biochemistry, Mount Sinai School of Medicine of the City University of New York, 1 Gustave L. Levy Place, New York, New York 10029

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The administration of insulin and glucose to young Sprague-Dawley rats (125-150 g) resulted in changes in the intracellular distribution and in the turnover rates of δ-aminolevulinic acid synthase (ALAS) activity in the mitochondria and the cytosol. When starved, allylisopropylacetamide (AIA)-induced rats were injected with either insulin or glucose, the percentage of the total ALAS activity found in the cytosol increased from 27% in control animals to 33-40% in insulin-treated and 50% in glucose-treated rats. Similar increases of the ALAS activity in the cytosol were observed after insulin treatment of noninduced, starved animals. Glucose administration also repressed 25-40% of the AIA induction of ALAS as previously reported; however, this effect apparently was not a result of elevated insulin levels, since there was no observed repression of AIA induction after insulin administration. The effects of insulin and glucose on the turnover rates of ALAS activity in the mitochondria and in the cytosol were investigated by observing changes in the half-lives of ALAS activity in the two intracellular compartments. Administration of both insulin and glucose resulted in an increased half-life of ALAS activity in the cytosol from 20.8 to over 100 min, while the mitochondrial half-life was not significantly changed. When insulin was given to either fed, AIA-induced or to starved, noninduced rats, the half-life of the cytosolic ALAS increased from about 14 to 40 min. In contrast to the starved, induced animals, the mitochondrial ALAS half-life in starved, noninduced animals decreased 50%. These results suggest that insulin and glucose treatment may inhibit the translocation of ALAS from the cytosol into the mitochondrial matrix.

Hepatic δ-aminolevulinic acid synthase (ALAS)[4] catalyzes the first and rate-limiting step of the heme biosynthetic pathway (1). The activity of ALAS, a mitochondrial matrix enzyme (2), is increased several-fold by drugs such as allylisopropylacetamide (AIA), which also cause an experimental porphyria (3-5). Administration of AIA not only increases the activity of mitochondrial ALAS but also results in a significant accumulation of ALAS in the cytosol (6, 7). The cytosolic form of hepatic ALAS in porphyric rats has been suggested to be a precursor form of the enzyme synthesized on cytosolic ribosomes and translocated into the mitochondria in a subsequent step concomitant with processing to the mature form of the enzyme (8, 9).

Mitochondrial ALAS has a very short half-life (or a rapid turnover rate) when compared to other mitochondrial proteins. Initially, the half-life of mitochondrial

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[3] To whom all correspondence should be addressed.

[4] Abbreviations used: ALAS, δ-aminolevulinic acid synthase; AIA, allylisopropylacetamide; TSE buffer, 10 mM Tris-HCl, 0.25 M sucrose, 1 mM EDTA, pH 7.6.

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ALAS was reported to be 60–70 min (3, 6, 10), but a more accurate method involving the hemin inhibition of ALAS translocation revealed that the apparent half-life of the mitochondrial enzyme is 35 min (11). This half-life is much less than that of the bulk of mitochondrial proteins, which have a half-life of approximately 8 days (12). The cytosolic ALAS had an apparent half-life of about 20 min when there was no known inhibition of ALAS translocation (11).

It has been reported that glucose inhibits the induction of ALAS by AIA (13, 14), the so called "glucose effect." An additional effect of glucose administration to AIA-treated rats was a change in the intracellular distribution of ALAS between the mitochondria and the cytosol (7). When compared to control animals, the ALAS activity in glucose-treated rats decreased in the mitochondria, while the cytosolic activity increased. In the current study, the observed effects of glucose and insulin on the AIA induction, the intracellular distribution, and the turnover rates of ALAS have suggested that these agents may act by inhibiting the translocation of ALAS from the cytosol into the mitochondria.

MATERIALS AND METHODS

Reagents and chemicals. δ-Aminolevulinic acid-HCl, coenzyme A, glycine, GTP, insulin, Lubrol WX, pyridoxal 5'-phosphate, and succinic acid were obtained from Sigma Chemical Company (St. Louis, Mo.). Succinyl-CoA synthase and dithiothreitol were purchased from Boehringer-Mannheim (Indianapolis, Ind.). [2,3-^{14}C]Succinic acid (100 mCi/mmol) was obtained from Amersham (Chicago, Ill.) and diluted with cold succinic acid to a specific activity of 80 mCi/mmol. [3,5 ^{3}H(N)]δ-aminolevulinic acid-HCl (2.3 Ci/mmol) and [4-^{14}C]aminolevulinic acid-HCl (53.2 mCi/mmol) were obtained from New England Nuclear (Boston, Mass.). Dowex AG-50-X8 (200–400 mesh) was supplied by Bio-Rad Laboratories (Rockville Center, N. Y.). AIA was a generous gift from Dr. William Scott from Hoffman La Roche Inc. (Nutley, N. J.). Other chemicals were of the highest purity obtainable from commercial sources.

Treatment of animals. Male Sprague-Dawley rats (125–150 g) were obtained from Perfection Breeders. Where indicated, rats were starved 24 h prior to injections. Rats treated with AIA were injected subcutaneously with a 20 mg/ml in 154 mM NaCl solution at a dose of 30 mg/100 g body wt. Animals were administered cycloheximide, insulin, and glucose by intraperitoneal injections using 5 mg/ml, 0.1 I.U./ml, and 0.5 g/ml in 154 mM NaCl solutions at doses of 5 mg, 0.2 I.U., and 1 g/100 g body wt, respectively, for the times indicated in the text or in the figure legends.

Preparation of mitochondrial and cytosolic fractions. Rats were killed and mitochondria were prepared according to Scotto *et al.* (15) with the following modifications. The second 600*g* supernatant was centrifuged at 8500*g* for 10 min to obtain the crude mitochondrial pellet. This pellet was then washed once with TSE buffer (0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA), and then centrifuged at 7500*g* for 10 min at 4°C to obtain the mitochondrial pellet. This pellet was resuspended to one-half the volume of the original liver weight with TSE buffer, resulting in a final protein concentration of about 20 mg/ml.

The cytosolic fraction was prepared by centrifuging the 8500*g* supernatant at 100,000*g* for 1 h at 4°C to remove the microsomes.

Both fractions were then stored at −20°C and assayed within a week; the ALAS activity was found to be stable to this treatment. Thawed mitochondria were treated with 50 μl Lubrol WX (20 mg/ml), a modification of Scotto *et al.* (15), to ensure that the mitochondria were permeable to succinyl-CoA during the enzymatic assay.

Protein concentration was determined by the modified Lowry method (16) according to Dully and Grieve (17).

Enzyme assay. The activity of ALAS in the Lubrol WX-treated mitochondrial fraction and the cytosolic fraction were assayed as described by Scotto *et al.* (2) with several modifications. One μCi of [^{14}C]succinate (50 μM) and 60 mU of succinyl-CoA synthase were used instead of 0.25 μCi and 6 mU, respectively. The reaction mixture was incubated with shaking at 37°C for 10 min in a New Brunswick gyratory water bath, and terminated by the addition of 1 ml of 10% sodium dodecyl sulfate.

RESULTS

Effects of insulin and glucose on induction of δ-aminolevulinic acid synthase activity by AIA and the intracellular distribution. Treatment of rats with AIA 4 h prior to killing resulted in an 8-fold increase in total ALAS activity, relative to saline-treated animals (Fig. 1). When insulin was injected 2 h after AIA, an even greater increase in ALAS activity was observed, to nearly 10-fold above the control; how-

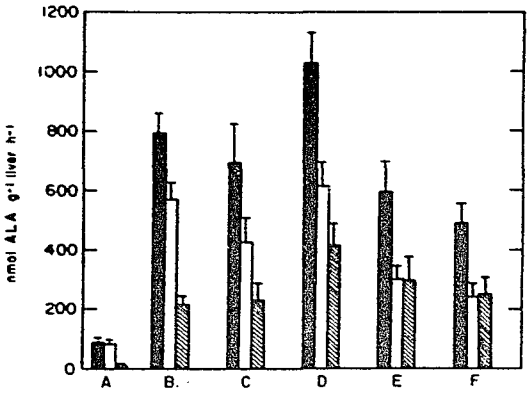

FIG. 1. The intracellular distribution of ALAS activity per gram liver in AIA-treated rats. The mitochondrial and cytosolic fractions were isolated and assayed as described under Materials and Methods. The total ALAS activity was obtained by adding the cytosolic and the mitochondrial activities, using the value of 60 mg mitochondrial protein/g liver as estimated by Scotto *et al.* (15). The solid bar graph is the total ALAS activity, the open bar graph is the mitochondrial activity, and the hashed bar graph is the cytosolic activity. Rats were injected with the drugs indicated and were sacrificed 4 h later. (A) saline-treated (control); (B) AIA-treated; (C) simultaneous AIA and insulin; (D) AIA-treated and then insulin-treated 2 h later; (E) simultaneous AIA and glucose treatment; and (F) AIA-treated and then glucose-treated 2 h later. Each bar graph represents the mean ± SE of 3-10 animals.

ever, when insulin was injected simultaneously with AIA, there was no effect on total ALAS activity (Fig. 1). By contrast, when glucose was injected 2 h after AIA, the total ALAS activity decreased 40% ($P < 0.08$) when compared to AIA-induced animals (Fig. 1). However, the simultaneous administration of glucose and AIA did not result in a significant change in total ALAS activity.

The effects of AIA, insulin, and glucose on the intracellular distribution of ALAS activity in the mitochondria and in the cytosol were also examined. When rats were treated with AIA only, about 27% of the total activity was present in the cytosol, whereas in saline-treated animals 7% of the activity was in the cytosol. Simultaneous administration of insulin with AIA resulted in no significant change in the total ALAS in the cytosol compared to AIA-induced controls; however, when the animals received insulin 2 h after AIA, 40% of the total ALAS activity was in the cytosol (significant to $P < 0.05$). Administration of insulin to control fasted rats also resulted in a time-dependent change in the intracellular distribution of ALAS (Table I). After 60 min a significant decrease in the percentage of ALAS in the mitochondria was observed, while a significant increase of ALAS in the cytosol was also observed at this time. Glucose administration to AIA-induced animals caused similar effects as insulin. When glucose was injected simultaneously with or 2 h after AIA, 50% of the total ALAS activity was observed in the cytosolic fraction (significant to $P < 0.05$). These effects of glucose and insulin on the intracellular distribution of ALAS suggest that these agents may inhibit the translocation of ALAS from the cytosol into the mitochondrial matrix, as previously suggested by Kim and Kikuchi (7).

Effects of insulin and glucose on turnover of ALAS activity. The suggested inhibition of ALAS translocation by insulin or glucose was tested by investigating the turnover rate or half-life of ALAS activity in the same manner as Hayashi *et al.* (11) used to show that heme inhibited the translocation of ALAS into the mitochondrial matrix. In our experiments, the rats were treated with AIA for 16 h prior to injections of insulin or glucose and cycloheximide. This time period was chosen to obtain maximum induction and to ensure that no changes in ALAS activity would occur due to AIA during the administration of the other agents. Rats were treated with cycloheximide to inhibit cytoplasmic protein synthesis, so that, during insulin or glucose treatment, no new synthesis of ALAS would occur to complicate the measurement of turnover rates. Previous studies (6) had indicated that the administration of cycloheximide completely blocked the induction of ALAS as well as total protein synthesis. The rationale behind the experiments was the following. If indeed insulin and glucose act to inhibit the translocation

TABLE I

THE INTRACELLULAR DISTRIBUTION OF ALAS ACTIVITY AFTER INSULIN TREATMENT OF STARVED, NONINDUCED RATS

Time (min)	Mitochondria		Cytosol	
	Specific activity	Percentage total activity/g liver	Specific activity	Percentage total activity/g liver
0	1.11 ± 0.20	82.4 ± 3.0	0.18 ± 0.02	17.6 ± 3.0
30	1.10 ± 0.09	81.9 ± 3.7	0.20 ± 0.03	18.1 ± 3.7
60	0.67 ± 0.07*	71.9 ± 1.4*	0.21 ± 0.01*	28.1 ± 1.4*
120	0.74 ± 0.06*	69.7 ± 2.0*	0.26 ± 0.01*	30.3 ± 2.0*

Note. Rats were injected with insulin and sacrificed at the indicated times. The mitochondrial and cytosolic fractions were isolated as described under Materials and Methods. The total ALAS activity was obtained by adding the cytosolic and the mitochondrial activities, using the value of 60 mg mitochondrial protein/g liver as estimated by Scotto *et al.* (15). The corresponding percentages were then calculated. The percentages were statistically analyzed by transforming the $(\text{percentages}/100)^{1/2}$ into the arcsine, and then these transformed values were analyzed by analysis of variance as discussed by Zar (26). The specific activities, expressed as nmol $mg \cdot hr^{-1}$, were also analyzed by analysis of variance. The values listed are the means ± SE. Five rats were used for the 0- and 30-min values, and six animals were used for the other times.

* $P < 0.05$. The probability that the value is not significantly greater than or less than the zero time value.

of ALAS from the cytosol into the mitochondria, then the observed turnover rate of ALAS activity should increase in the mitochondria and decrease in the cytosol relative to the turnover rates of the control animals (cycloheximide treated). Consequently, a decreased half-life of mitochondrial ALAS should be observed after treatment with either insulin or glucose, while an increased half-life of cytosolic ALAS should be observed. As seen in Table II and Figs. 2A and 3A, when starved, AIA-induced rats were treated with cycloheximide only, the half-life of mitochondrial ALAS activity was 45.8 min and that of the cytosolic enzyme was 20.8 min. Administration of insulin to the AIA-induced animals, simultaneously with cycloheximide, resulted in a large, statistically significant ($P < 0.05$), increase in the half-life of cytosolic ALAS to 213 min, with no observed difference in the half-life of the mitochondrial enzyme. When insulin was injected 2 h before cycloheximide, the observed half-lives were similar to the animals injected with insulin and cycloheximide at the same time (Figs. 2B and 3B). A possible explanation for the unchanged turnover rate of mitochondrial ALAS after insulin treatment may be that the 10-fold greater ALAS activity in the mitochondria relative to the cytosol may mask the actual inhibition of translocation. The turnover rates of fasted, induced animals treated with glucose were also investigated and were found to be similar to animals that were administered insulin (Table II).

Another set of turnover experiments was performed using fed rats, in which ALAS activity has been induced with AIA in the same manner as the fasted animals discussed above. AIA treatment of fed rats resulted in only a two- to fourfold increase of ALAS activity, or less than half the induction observed in the starved animals. The half-lives of the control mitochondrial and cytosolic ALAS activities were 83.2 and 14.6 min, respectively, while the half-lives for the enzyme in the insulin-treated animals were 59.6 and 38.7 min (Table II). Again, the cytosolic turnover rate decreased significantly ($P < 0.05$) after insulin treatment but, like the starved animals, the turnover rate of mitochondrial ALAS did not change significantly.

Lastly, experiments with the purpose of

TABLE II

THE HALF-LIFE OF MITOCHONDRIAL AND CYTOSOLIC ALAS ACTIVITY

	Half-life of ALAS activity (min)	
	Mitochondria	Cytosol
Induced	45.8 (39.4-54.7)	20.8 (16.7-27.3)
+ Insulin (simultaneous)	43.8 (35.5-57.2)	213 (74.9-$^{-}$253)**
+ Insulin (2 h prior)	48.6 (39.0-64.1)	395 (56.4-$^{-}$79.0)**
Glucose	41.7 (34.1-53.5)	102 (56.8-509)**
Fed-Induced	83.2 (57.0-154)	14.6 (12.7-17.2)
+ Insulin	59.6 (46.1-84.1)	38.7 (27.7-64.2)**
Noninduced	92.8 (67.5-148)	13.3 (8.32-33.1)
+ Insulin	42.4 (36.2-51.1)*	40.5 (34.4-49.2)*

Note. Rats were injected with AIA 16 h prior to the administration of the other agents. Glucose or insulin was given simultaneously with cycloheximide, unless indicated. The animals were then sacrificed at specific times after drug treatment, as in Figs. 3 and 4, and the cellular fractions were obtained as described under Materials and Methods. The half-life of ALAS activity in the mitochondria and the cytosol was calculated by using the slope and the y intercept of the log of activity as drawn by the method of least squares. The values in parentheses are the range of the half-lives calculated from the slope ± its SE.

* $P < 0.05$.

** $P < 0.05$.

The symbols above indicate the probability of the slope of the insulin or glucose treated animals not being significantly greater than or less than the slope of the corresponding control. The statistical methods used for the comparison of slopes were Student's t test, when comparing two slopes, and the analysis of covariance, for comparing more than two slopes as described in Zar (26).

investigating the insulin effect on ALAS activity using noninduced rats were performed. As shown in Table II, the half-life of ALAS decreased significantly ($P < 0.1$) in the mitochondria (corresponding to an increase in turnover rate) from 92.8 to 42.4

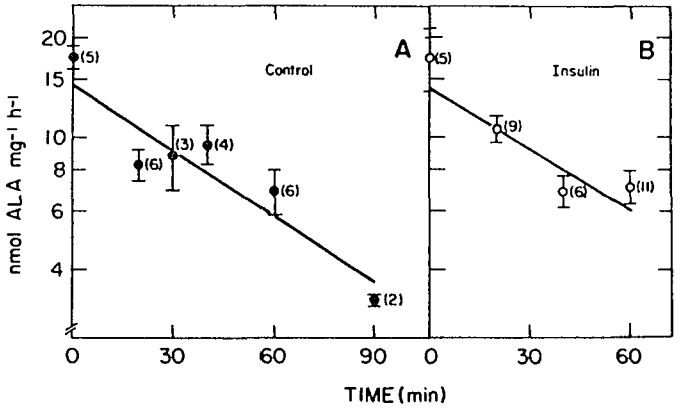

FIG. 2. Turnover of mitochondrial ALAS activity in control and insulin-treated rats. Rats were injected with AIA 16 h prior to the administration of cycloheximide or insulin. Insulin was injected 2 h before the administration of cycloheximide. The animals were sacrificed at the indicated times after cycloheximide injection. Each point represents the mean ± SE of mitochondrial ALAS specific activity (nmol ALA mg^{-1} h^{-1}). The number of rats for each time point is in parentheses. A (●) Cycloheximide treated (control animals); B (○) insulin and cycloheximide treated.

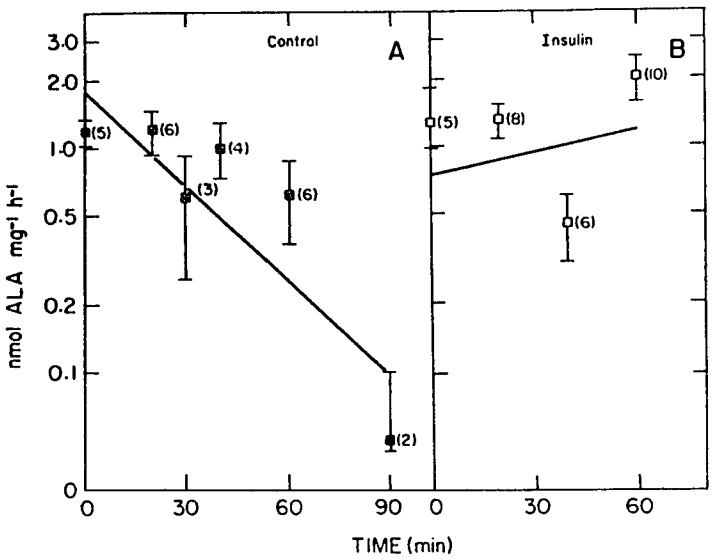

FIG. 3. Turnover of cytosolic ALAS activity in control and insulin-treated rats. Rats were treated as in Fig. 2. Each point represents the mean ± SE of ALAS specific activity in the cytosolic fraction. The number of rats for each time point is in parentheses. A (■) Cycloheximide treated (control animals); B (□) insulin and cycloheximide treated.

min, while the half-life increased significantly ($P < 0.1$) in the cytosol from 13.3 to 40.5 min after treatment with insulin.

DISCUSSION

The current study provides further evidence that glucose and insulin may inhibit the translocation of hepatic ALAS from the cytosol into the mitochondrial matrix, as previously suggested by Kikuchi and Hayashi (9). Administration of either agent to AIA-treated rats resulted in a significant increase in the percentage of the total ALAS present in the cytosolic fraction, with a concomitant decrease of the amount in the mitochondria. Furthermore, the turnover rate of the cytosolic ALAS was decreased after either glucose or insulin administration such that almost no loss in activity was observed for 1 h after cycloheximide treatment. It is possible that insulin may act by blocking degradation of ALAS in the cytosol rather than translocation of the enzyme from the cytosol into the mitochondria; however, the decreased percentage of total ALAS in the mitochondria after insulin or glucose treatment plus the increased turnover rate in the mitochondrial fraction observed after insulin treatment of noninduced animals makes this explanation seem less likely. Furthermore, no ALAS-specific protease appears to be present in the cytosolic fraction, as incubation of this fraction in the presence of pyridoxal 5'-phosphate at 37°C for 1 h did not result in any loss in ALAS activity. To establish unequivocally that an inhibition of translocation of ALAS into mitochondria occurs after insulin treatment will require the development of an *in vitro* system to measure translocation directly under physiological conditions.

These effects of glucose and insulin on the turnover of hepatic ALAS are similar to the results previously reported by Hayashi *et al.* (11), from which they concluded that hemin inhibits the translocation of ALAS from the cytosol to the mitochondria. One hour after hemin administration, a decrease in the half-life of mitochondrial ALAS from 60 to 35 min and an increase in the half-life of the cytosolic enzyme from

20 min to almost no degradation was observed. The loss of ALAS activity in the mitochondria was shown to result from an actual decrease in the quantity of immunoreactive ALAS protein, and provided strong evidence that heme acts to inhibit the translocation of ALAS from the cytosol to the mitochondria.

Recently, Yamamoto *et al.* (18) reported that the effects of insulin on the intracellular distribution of ALAS might be attributed to an increase in the heme levels resulting from insulin treatment. The intracellular heme levels were determined by examining the degree of heme saturation of tryptophan pyrrolase (1.13.11.11), an enzyme used as a sensitive marker for determining hepatic heme levels (19, 20). The degree of heme saturation was estimated by comparing the tryptophan pyrrolase activity in the absence and in the presence of added hemin. This group reported that the heme saturation of tryptophan pyrrolase increased from 45.6% in the controls to 77.6% in less than 1 h after animals were treated with insulin. This time course has been confirmed in our laboratory, and is consistent with the observed changes in the intracellular distribution (Table I). The apparent control of ALAS translocation by insulin via elevated heme levels fits in with the proposed "regulatory heme" pool concept (1, 21). The idea behind this concept is that a free-heme pool, in equilibrium with bound heme, is responsible for the regulation of ALAS levels found in the mitochondrial matrix. The free-heme level in chick embryo liver cells has been estimated to be 10^{-8} to 10^{-7} M (1, 22). The dissociation constant for heme of the purified rat liver tryptophan pyrrolase has been reported to be 1.3×10^{-8} by Sudo *et al.* (23), which is within the range of the estimated physiological heme levels. Hence, the apparent inhibition of ALAS translocation by insulin via an elevated heme content in the liver cell may occur physiologically. Evidence has been obtained indicating that heme also inhibits ALAS mRNA transcription (24) and ALAS translation (25) at heme levels close to the estimated physiological free-heme content. Interestingly, it has been reported that the induction of ALAS by porphyrinogens is impaired in diabetic rats (13).

The mechanism by which insulin might increase the content of the free-heme pool is unknown. Similarly, whether glucose affects hepatic ALAS activity in the whole animals either directly or by effecting changes in insulin levels is also unclear. Currently, we are investigating the effects of insulin, glucose, and hemin in isolated rat liver hepatocytes and perfused rat livers in an attempt to answer these questions.

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Pyridoxal Phosphate Protects against an Irreversible Temperature-Dependent Inactivation of Hepatic δ-Aminolevulinic Acid Synthase[1]

DIANA S. BEATTIE,[2] ANTHONY W. SCOTTO,[3] UMA REDDY, RICHARD DELOSKEY, AND CHANDRA G. BOSCH

Department of Biochemistry, Mount Sinai School of Medicine of the City University of New York, 1 Gustave L. Levy Place, New York, New York 10029

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The stability of hepatic δ-aminolevulinic acid synthase (ALAS), the first and rate-limiting enzyme of the heme biosynthetic pathway, was investigated. Incubation of the mitochondrial matrix fraction obtained from either control or allylisopropylacetamide-induced rats at 37°C in Tris-Cl, pH 7.4, EDTA, and dithiothreitol resulted in a rapid decrease in ALAS activity such that 50–70% of the activity was lost after 30 min. Similar decreases in ALAS activity were observed when a cytosolic fraction from the induced animals was incubated at 37°C. Addition of 0.1 mM pyridoxal-P, the cofactor of ALAS, to the preincubation medium completely prevented the observed loss of activity; however, dialysis of the inactive matrix fraction against several changes of buffer containing pyridoxal-P did not restore activity, suggesting that the inactivation was irreversible. These decreases in ALAS activity in the absence of pyridoxal-P were temperature dependent, as a 55% loss of ALAS activity was observed after a 60-min incubation at 30°C, while the enzyme was completely stable when preincubated at 22°C for 60 min. This inactivation of ALAS does not appear to involve proteolytic digestion, as addition of a wide spectrum of protease inhibitors to the preincubation medium in the absence of pyridoxal-P did not protect against the inactivation. The suggestion is made that the cofactor, pyridoxal-P, may dissociate from the enzyme during the preincubation and, consequently, the apoenzyme may be irreversibly inactivated at temperatures above 22°C.

Hepatic δ-aminolevulinic acid synthase (ALAS)[4] catalyzes the first and rate-limiting step of the heme biosynthetic pathway (1). The activity of ALAS, a mitochondrial matrix enzyme (2), is increased several fold by drugs such as allylisopropylacetamide, which also cause an experimental porphyria (3–5), as well as by ethanol (6) and heavy metals (7). Administration of allylisopropylacetamide not only increases the activity of mitochondrial ALAS but also results in a significant accumulation of ALAS in the cytosol (8, 9). The cytosolic form of hepatic ALAS in porphyric rats has been suggested to be a precursor form of the enzyme synthesized on cytosolic ribosomes and translocated into the mitochondria in a subsequent step concomitant with processing to the mature form of the enzyme (10, 11).

ALAS also has been reported to have a very rapid turnover *in vivo* of less than 1 h (11). The mechanism of this rapid

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[2] To whom correspondence should be addressed.

[3] Current address: Department of Medicine, Cornell University Medical College, 1300 York Avenue, New York, N. Y. 10021.

[4] Abbreviations: ALAS, δ-aminolevulinic acid synthase; AIA, allylisopropylacetamide; DTT, dithiothreitol; TSE buffer, 10 mM Tris-HCl, 0.25 M sucrose, 1 mM EDTA, pH 7.6.

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breakdown is not clear, although ALAS derived from bone marrow was reported to be sensitive to a specific protease (12). This protease was similar to, but not identical with, the group-specific protease(s) described previously for pyridoxal-P- and pyridoxamine-P-dependent enzymes (13). In addition, purified and active forms of ALAS have been shown to be sensitive to thiol proteases (14, 15).

In the current study, the stability of ALAS has been investigated in the mitochondrial matrix of control and AIA-induced rats and in the cytosol from the AIA-induced animals. ALAS in both the matrix and the cytosol was subject to a rapid temperature-dependent loss of activity in the absence of exogenous pyridoxal-P. The observed loss of activity at either 30 or 37°C was completely prevented by addition of pyridoxal-P, and partially prevented by addition of pyridoxamine-P. This inactivation does not appear to result from proteolysis, as addition of a variety of protease inhibitors to the incubation mixture did not prevent the loss of activity. The possibility of a two-step inactivation of hepatic ALAS is considered.

MATERIALS AND METHODS

Reagents and chemicals. Pyridoxal 5'-phosphate, GTP, coenzyme A, and aminooxyacetate were obtained from Sigma Chemical Company. Dowex AG-50-X8 (200–400 mesh) was supplied by Bio-Rad Laboratories. [^{14}C]Succinic acid (102.4 mCi/mmol) was obtained from Amersham, and was diluted with cold succinate to a specific activity of 80 mCi/mmol. δ-Amino[4-^{14}C]levulinic acid (53.2 mCi/mmole) was obtained from New England Nuclear. Allylisopropylacetamide (AIA) was a gift of Hoffmann-LaRoche. Succinyl-CoA synthase was purchased from Boehringer-Mannheim. Other chemicals were of the highest purity from commercial sources.

Treatment of animals. Male Sprague-Dawley rats (150–175 g) were obtained from Perfection Breeders. All animals were fasted 24 h prior to injection. Where indicated, AIA was administered by subcutaneous injection (20 mg/ml in 0.9% saline) at a dose of 40 mg/100 g body wt, and the animals were sacrificed after 1 h. Control animals received equivalent volumes of saline. No differences in enzymatic activity were observed between saline-treated and uninjected animals.

Preparation of liver mitochondria and the mitochondrial matrix fraction. Mitochondria were isolated in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.6, 1 mM EDTA (TSE buffer) according to the modifications (16) of Paterniti and Beattie (17). Subsequently, mitoplasts were prepared from the isolated mitochondria by treatment with digitonin by the method of Schnaitman and Greenawalt (18), and were solubilized with Lubrol to yield the matrix fraction (2). The cytosolic fraction consisted of the 100,000*g* supernatant of the homogenate (19).

Enzymatic assays. The activity of ALAS in the soluble matrix fraction or in Lubrol-solubilized mitochondria (19) was assayed in an incubation mixture containing 50 mM Tris-HCl, pH 7.4, 100 mM glycine, 10 mM EDTA, 20 mM $MgCl_2$, 10 mM coenzyme A, 1 mM GTP, 1 μC [^{14}C]succinate (50 μM), 1 mU succinyl-CoA synthase, and 50–200 g protein in a final volume of 250 μl. Pyridoxal-P was added as described in the text. The reaction was incubated with shaking at 37°C for 10 min in a New Brunswick gyratory water bath, and was terminated by the addition of 1 ml 10% sodium dodecyl sulfate. A known quantity of δ-amino-[^{3}H]levulinic acid was added to all samples to correct for losses during recovery, and δ-amino[^{14}C]levulinic acid was added to control tubes to correct for possible isotopic exchange. Carrier succinate (1 μmol) and ALA (100 mmol) were added to each tube. All samples were assayed in triplicate. Isolation of ALA was performed by chromatography on Dowex-50 as described previously (2).

Alanine aminotransferase activity was determined according to Segal *et al.* (20). Protein concentration was estimated by the method of Bradford (21).

RESULTS

The inactivation of ALAS in the absence of pyridoxal-P. Incubation of the soluble mitochondrial matrix fraction at 37°C in 10 mM Tris-HCl containing 1 mM EDTA and 0.1 mM DTT (buffer A) prior to assay resulted in a rapid decrease in the activity of ALAS (Fig. 1A). No loss of ALAS activity was observed, however, when the incubation medium was supplemented with 0.1 mM pyridoxal-P, suggesting that the cofactor protects against the inactivation of ALAS. Attempts to reverse the inactivation process by overnight dialysis of the matrix fractions at 4°C previously incubated for 30 min at 37°C in the absence of pyridoxal-P against buffer A containing pyridoxal-P were unsuccessful, suggesting that the observed loss of activity was irreversible.

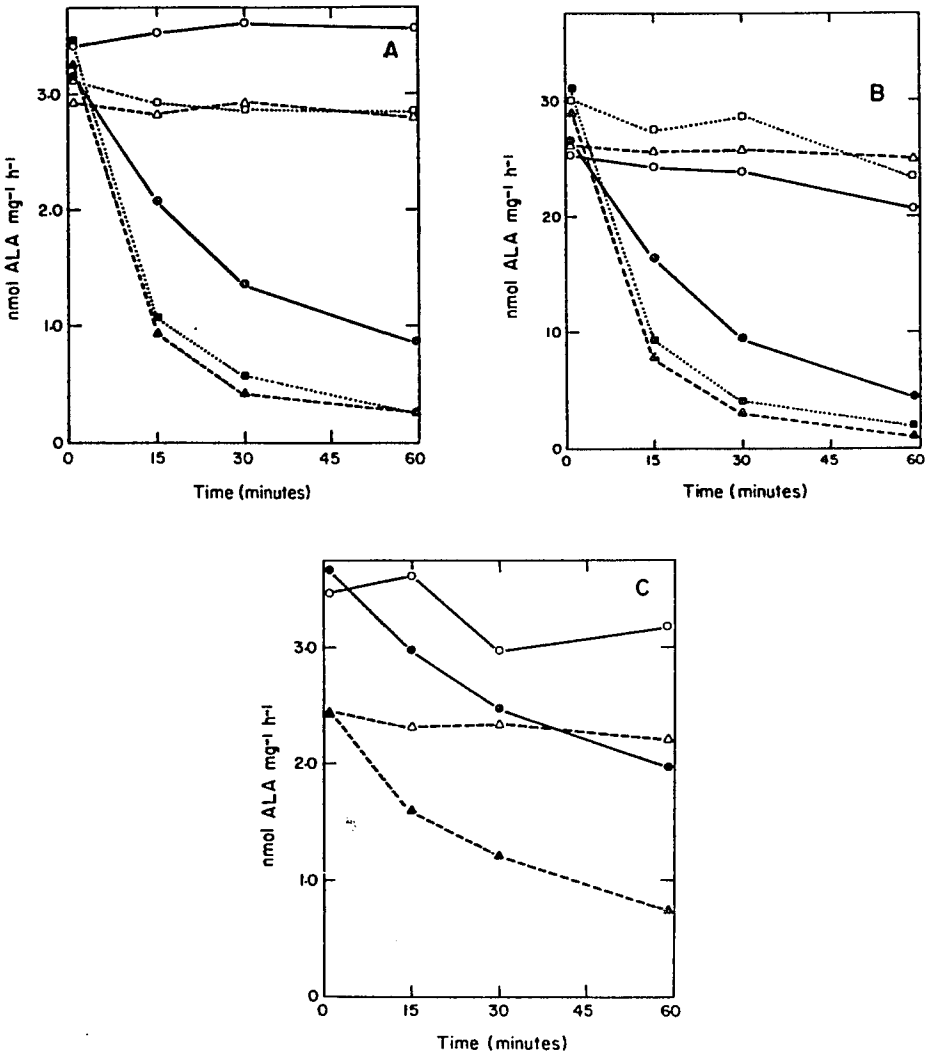

FIG. 1. The inactivation of ALAS in the absence of excess pyridoxal-P. Fractions containing ALAS were preincubated at 37°C for the times indicated in the buffer conditions specified, and were subsequently assayed for enzymatic activity as described under Materials and Methods with 0.1 mM pyridoxal-P. (A) Mitochondrial matrix fraction from normal rats; (B) mitochondrial matrix fraction from AIA-induced rats; (C) cytosolic fraction from AIA induced rats. A and B: (●) Buffer A (10 mM Tris-HCl, 1 mM EDTA, 0.1 mM DTT, pH 7.4); (○) buffer A supplemented with 0.1 mM pyridoxal-P; (▲) buffer A with 10 mM DTT; (△) buffer A with 10 mM DTT supplemented with 0.1 mM pyridoxal-P; (■) buffer A with 50 mM Tris-HCl and 10 mM DTT, pH 7.4; (□) buffer A with high Tris and DTT supplemented with 0.1 mM pyridoxal-P. C: (●) TSE buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.6, 1 mM EDTA); (○) TSE supplemented with 0.1 mM pyridoxal-P; (▲) TSE with 50 mM Tris-HCl, 10 mM DTT; (△) TSE with high Tris and DTT supplemented with 0.1 mM pyridoxal-P.

During preliminary purification attempts, we had noted that the stability of ALAS at 4°C was markedly improved by increasing the concentration of DTT in the medium to 10 mM and that of Tris to 50 mM. However, the addition of 10 mM DTT to the preincubation (buffer A) medium with or without increasing the Tris concentration did not protect against the loss of ALAS activity observed in the

absence of pyridoxal-P, but instead resulted in a doubling of the rate of ALAS inactivation at 37°C under these conditions (Fig. 1A).

A similar rate of inactivation of ALAS was observed when mitochondrial matrix fractions obtained from rats treated with AIA, the well-characterized inducer of ALAS, were incubated at 37°C in the absence of pyridoxal-P (Fig. 1B). Similarly, increasing the concentration of DTT to 10 mM increased the rate of inactivation at 37°C.

It was also of some interest to learn whether the ALAS, which accumulates in the cytosolic fraction of animals treated with the inducer, AIA, is subject to inactivation when incubated at 37°C in the absence of pyridoxal-P. The cytosolic fractions, obtained by centrifugation of the homogenate prepared in TSE buffer, were incubated at 37°C in this same medium. As seen in Fig. 1C, the ALAS present in the cytosol was also inactivated when incubated under these conditions in the absence of pyridoxal-P. Increasing the DTT concentration caused a significant inhibition of the observed activity; however, the rate of inactivation in the absence of pyridoxal-P was unchanged.

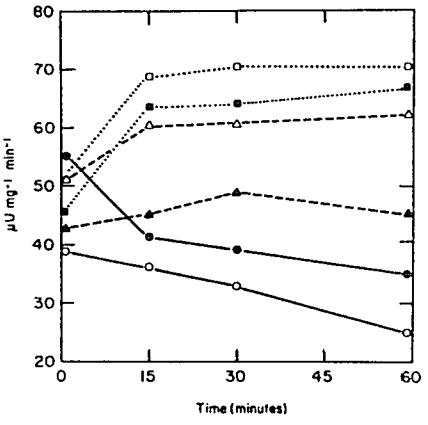

FIG. 2. The stability of alanine aminotransferase to preincubation at 37°C. Mitochondrial matrix fraction from normal rats were isolated in the specified buffers and preincubated at 37°C prior to assay alanine aminotransferase activity (21). (●) Buffer A; (○) buffer A supplemented with 0.1 mM pyridoxal-P; (▲) buffer A with 10 mM DTT; (△) buffer A with 10 mM DTT supplemented with 0.1 mM pyridoxal-P; (■) buffer A modified to contain 50 mM Tris-HCl and 10 mM DTT; (□) buffer A modified and supplemented with 0.1 mM pyridoxal-P.

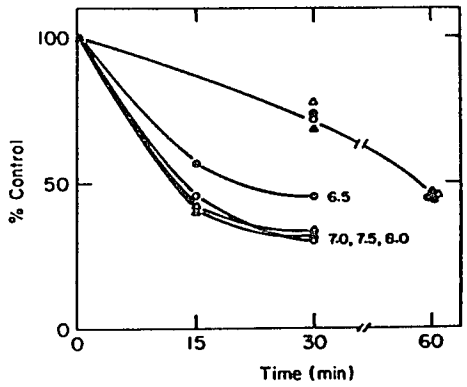

FIG. 3. Time course of inactivation of ALAS as a function of pH and temperature. Mitochondrial matrix fraction from AIA-treated rats was preincubated at 30 and 37°C in buffer A adjusted to the indicated pH values for the times indicated prior to assay, as described in Fig. 1.

In order to assess if this inactivation at 37°C is characteristic of pyridoxal-P-dependent enzymes in the mitochondrial matrix in general, the rate of inactivation of alanine aminotransferase was also investigated (Fig. 2). A 27% loss of alanine aminotransferase activity was observed when the mitochondrial matrix fraction was incubated for 1 h at 37° in the absence of pyridoxal-P; however, a similar decline in activity was observed in the presence of pyridoxal-P. Increasing the concentration of DTT in the buffer to 10 mM, with or without increasing the ionic strength to 50 mM Tris, resulted in an initial increase in transaminase activity during the first 15 min of incubation, and protected against any further loss in activity during a 60-min incubation.

Effect of pH, temperature, and buffers on the inactivation of ALAS. ALAS activity was stable at 4°C in the pH range of 5.8 to 8.4 in the presence or absence of pyridoxal-P for periods of greater than 24 h. Preincubation of the matrix fraction at 37°C in buffer A at pH 7.0 to 8.5 resulted in a similar time-dependent loss in ALAS activity (Fig. 3). At pH 6.5, however, a slower rate of inactivation was

observed whether the source of ALAS was the matrix fraction from control or AIA-induced rats.

Figure 3 also indicates that the loss of ALAS activity is temperature dependent. After preincubation at 30°C for 30 and 60 min, a progressive loss in ALAS activity was observed to 25 and 55% of the control, respectively. At the lower temperature, however, the loss of activity was identical at all pH values from 6.5 to 8.0. Furthermore, no loss of activity was observed in the absence of pyridoxal-P when the matrix fraction was incubated at 22°C.

The time- and temperature-dependent inactivation of ALAS observed in the matrix fraction was also investigated in Lubrol-solubilized mitochondria (19). The results of Fig. 4 indicate that a similar temperature-sensitive loss of activity occurred in the absence of pyridoxal-P in the solubilized mitochondria.

The buffer used in the experiments described above was Tris-Cl; however, substitution of a number of other buffers for Tris during the preincubation did not significantly change the extent of inactivation of ALAS at 37°C (Table I), suggesting that the inactivation of ALAS did not result because of the Tris in the medium. The possibility that DTT, which was present in all the preincubation mixtures, contributed to the inactivation of ALAS was also investigated. When DTT was omitted from the incubation mixture, similar rates of ALAS inactivation were observed as were observed in the presence of 0.1 mM DTT. In an additional experiment, we observed that the addition of optimal amounts of the substrate glycine (50 mM) to the preincubation mixture did not protect against the loss of ALAS activity in the absence of pyridoxal-P.

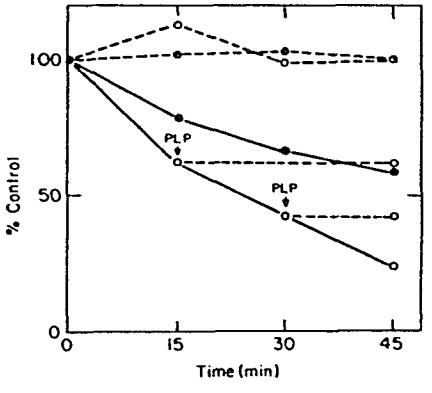

FIG. 4. Time course of inactivation of ALAS in mitochondria. Intact mitochondria from normal rats were resuspended in buffer A, pH 8.0, with (- - -) or without (——) pyridoxal-P, and were preincubated at 30°C (●) or at 37°C (○). At the times indicated the mitochondria were solubilized with Lubrol and assayed for ALAS as described previously (19).

TABLE I

EFFECT OF DIFFERENT BUFFERS ON THE INACTIVATION OF ALAS

	Buffer	Percentage of Loss ALAS
Experiment 1	Tris	63.4
	Hepes	61.3
	Mops	57.9
	Tricine	67.5
Experiment 2	Tris	59.2
	Bicine	66.3
	Tes	44.3
	Triethanolamine	37.4

Note. The mitochondrial matrix fraction was preincubated for 30 min at 37°C in the absence of pyridoxal-P in 10 mM buffer as indicated, pH 8.0, containing 1 mM EDTA and 0.1 mM DTT prior to assay, as described under Materials and Methods.

Effect of pyridoxine derivatives in protecting ALAS against inactivation. To investigate the degree of protection afforded by different pyridoxine derivatives, the mitochondrial matrix fraction was isolated without pyridoxal-P in buffer containing 10 mM DTT and supplemented with different concentrations of either pyridoxal-P or pyridoxamine-P. These fractions containing the various pyridoxine derivatives were either assayed directly or were preincubated for 15 min at 37°C prior to assay. Addition of 10^{-5} M pyridoxal-P provided significant protection against the observed loss of ALAS activity (Table II), while lower concentrations of pyridoxal-P had little protective effect. Similarly, addition of 10^{-5} M pyridoxal-P

to the assay mixture also resulted in maximum activity of ALAS in the matrix fraction without preincubation. Addition of 2×10^{-4} M pyridoxamine-P afforded a partial protection against the inactivation of ALAS (Table II). These concentrations of pyridoxamine-P also caused an increase in the ALAS activity of the control which was not preincubated at 37°C.

These effects of exogenous pyridoxal-P prompted us to investigate whether the coenzyme might work by activation of ALAS rather than by preventing its inactivation during the preincubation. As seen in Fig. 4, addition of pyridoxal-P to the preincubation mixture at the times indicated immediately prevented any further loss of ALAS activity. As an additional control, the solubilized mitochondrial fraction incubated for 60 min at 37°C without pyridoxal-P was added to a control fraction without preincubation and the ALAS activity was determined. The ALAS activity of the two fractions added together was identical to the mean of the activities of the two fractions assayed separately, suggesting that no inhibitory compound was generated in the absence of pyridoxal-P, and that the loss of activity is due to changes in the enzyme ALAS.

TABLE II

STABILIZATION OF ALAS BY PYRIDOXINE DERIVATIVES

Concentration (M)	No Preincubation (nmol mg^{-1} h^{-1})	Preincubation at 37°C for 15 min (nmol mg^{-1} h^{-1})
Pyridoxal-P		
10^{-2}	3.26 ± 0.21	3.09 ± 0.11
10^{-3}	3.25 ± 0.26	2.85 ± 0.15
10^{-4}	3.52 ± 0.17	3.07 ± 0.16
10^{-5}	3.63 ± 0.17	2.56 ± 0.08
10^{-6}	2.82 ± 0.04	0.67 ± 0.02
10^{-7}	2.20 ± 0.09	0.20 ± 0.01
0	2.01 ± 0.03	0.17 ± 0.02
Pyridoxamine-P		
10^{-3}	2.06 ± 0.45	0.44 ± 0.01
5×10^{-4}	2.08 ± 0.06	0.30 ± 0.01
2×10^{-4}	2.54 ± 0.09	1.13 ± 0.17
1×10^{-4}	2.46 ± 0.10	0.75 ± 0.03
5×10^{-5}	2.35 ± 0.06	0.42 ± 0.01
1×10^{-5}	2.27 ± 0.05	0.29 ± 0.01

Note. The mitochondrial matrix fraction, isolated in buffer A containing 10 mM DTT, was supplemented with pyridoxine derivatives prior to the preincubation at 37°C. ALAS activity was assayed as described under Materials and Methods without pyridoxal-P. Specific activities reported are the means ± standard deviations of triplicate determinations.

All of the data obtained indicate that ALAS may be rapidly inactivated in the absence of the cofactor at temperature above 22°C. It was, thus, of some interest to confirm how much of the enzyme present in the mitochondria is saturated with pyridoxal-P. Sufficient pyridoxal-P appears to be present in the mitochondria, as no loss of activity was observed if mitochondria or a Lubrol-solubilized matrix fraction were prepared with buffers without pyridoxal-P. Similarly, no loss of activity was observed when the matrix fraction was subjected to chromatography at 4°C on a molecular sieve in the absence of pyridoxal-P. In an additional approach, a detailed time course of the ALAS reaction was determined in the presence and absence of pyridoxal-P. The rate of ALAS activity was linear for 10 min at 37°C in the presence of pyridoxal-P (Fig. 5); however, the enzyme was active for only 5 min when pyridoxal-P was omitted. Addition of the cofactor at this time restored ALAS activity but at a lower rate than that of the control (Figs. 1–4). Similarly, at 30°C, ALAS was active for almost 7.5 min without pyridoxal-P, at which time no further activity was observed. These results suggest that, during these initial times, the cofactor dissociates from the enzyme and, subsequently, the irreversible inactivation of the enzyme occurs.

Effect of pyridoxal-P inhibitors on ALAS activity. Aminooxyacetate, a potent inhibitor of many pyridoxal-P-containing enzymes (22), also caused an inhibition of ALAS activity (Fig. 6). Inhibition was complete after a 10-min preincubation at 4°C of the mitochondrial matrix fraction with less than 1.0 mM aminooxyacetate. Spectral studies indicated that pyridoxal-

P was actually removed from the enzyme. The inhibition of ALAS activity in the soluble matrix fraction by aminooxyacetate was completely reversed by dialysis first against modified buffer without pyridoxal-P and subsequently against two changes of buffer containing pyridoxal-P.

The interaction of the inhibitor aminooxyacetate with pyridoxal-P has been reported to result in formation of a stable analogue (23). It was thus of some interest to investigate whether the complex formed with the inhibitor and the pyridoxal-P coenzyme of ALAS would protect the enzyme against the observed loss of activity at 37°C. After pretreatment of the mitochondrial matrix fraction with aminooxyacetate for 2 h, no protection against the loss of ALAS activity was observed during a subsequent incubation at 37°C in buffer A without pyridoxal-P. Subsequent dialysis as described above resulted in observed activities of ALAS comparable to those of fractions not pretreated with aminooxyacetate, suggesting that the complex formed between the inhibitor and pyridoxal-P of ALAS is probably unstable.

Gabaculine, 5-amino-1,3-cyclohexadienylcarboxylate, an enzyme-activated inhibitor of many pyridoxal-P-dependent enzymes (24), did not inhibit ALAS even when added in millimolar quantities.

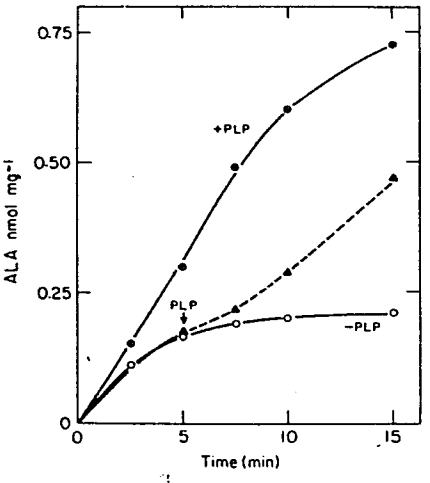

FIG. 5. Time course of ALAS activity in the presence or absence of pyridoxal-P. The mitochondrial matrix was incubated at 37°C with (●) or without (○) pyridoxal-P. After 5 min, 0.1 mM pyridoxal-P was added and the incubation was continued (▲).

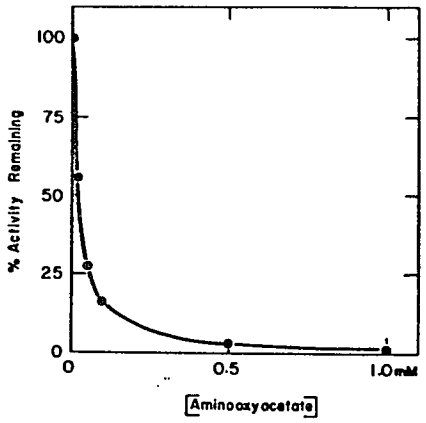

FIG. 6. Inhibition of ALAS by aminooxyacetate. Mitochondrial matrix fraction containing ALAS isolated in buffer A supplemented with 0.1 mM pyridoxal-P was treated with aminooxyacetate for 10 min at 4°C prior to assay, as described in Fig. 1.

Effect of protease inhibitors on the inactivation of ALAS. Earlier reports (12–15) indicating that ALAS was sensitive to proteases prompted us to investigate whether the inactivation observed during preincubation at 37°C might reflect proteolysis. None of the specific or general protease inhibitors tested arrested the inactivation observed at 37°C (Table III). Even certain inhibitors which had been reported by others to prevent the degradation of ALAS (14, 15) were ineffective, suggesting that the primary event in the thermal inactivation reported here is probably not a result of proteolysis.

DISCUSSION

The loss of ALAS activity in the absence of exogenous pyridoxal-P apparently results from a temperature-dependent inactivation of the enzyme. The protection afforded by both the cofactor, pyridoxal-P, as well as the analog, pyridoxamine-P, suggests that the irreversible inactivation at 30 or 37°C may occur after removal of the coenzyme from the holoenzyme. The time course of ALAS activity in the ab-

TABLE III

EFFECT OF PROTEASE INHIBITORS ON THE INACTIVATION OF ALAS AT 37°C

Inhibitor	Concentration	Preincubation at 4°C (nmol mg^{-1} h^{-1})	Preincubation at 37°C (nmol mg^{-1} h^{-1})
Pepstatin	10 μM	36.6	6.5
	100 μM	37.5	3.3
PMSF	1 mM	29.5	6.8
Soybean	100 μg/ml	36.3	11.1
TLCK	500 μM	38.9	6.1
TPCK	500 μM	30.1	3.6
Leupeptin	100 μM	33.8	10.1
	1000 μM	35.5	9.4
Antipain	100 μM	37.0	9.8
	1000 μM	33.7	8.8
Chymostatin	1.25 mg/ml	31.9	6.2
	12.5 mg/ml	36.4	5.4
1,10-Phenanthroline	1 mM	38.1	4.6
4-Chloromercuriphenol sulfonic acid	1 μM	43.3	10.1
	100 μM	40.6	10.7
	1000 μM	27.7	7.5
α_2-Macroglobulin	1 mg/ml	33.3	11.0
Elastinal	1 mg/ml	32.7	10.2
Diisopropylfluorophosphate	10^{-4} M	32.2	6.9
	10^{-6} M	32.8	9.9
Control H_2O		36.0	10.5
Control DMSO		35.1	6.6

Note. The mitochondrial matrix fraction obtained from treated rats was in buffer A containing 10 mM DTT. Protease inhibitors were dissolved in H_2O or DMSO, and were added to both experimental and control samples. All samples were treated with inhibitor for 15 min at 4°C before preincubation of the experimental samples at 37°C for 15 min. Samples were chilled to 4°C after preincubation, and all samples were assayed as described in Fig. 1.

sence of pyridoxal-P suggests that the cofactor is dissociated from the enzyme within 5 to 7 min, depending on the temperature. The subsequent inactivation of the apoenzyme, however, requires that the temperature be maintained above 22°C, as at lower temperatures ALAS appears to be stable even after removal of the cofactor. For example, prolonged dialysis of the matrix fractions in the absence of exogenous pyridoxal-P or extensive chromatography on molecular sieves, such as Ultrogel ACA-34 or Sephacryl-200, at 4°C did not result in the loss of enzymatic activity. Furthermore, treatment of the enzyme with the carbonyl reagent, aminooxyacetate, at 4°C results in the removal of the pyridoxal-P from the apoenzyme as determined spectrally; however, no permanent loss of ALAS activity was observed after treatment with the inhibitor. Subsequent dialysis of the aminooxyacetate-treated matrix fraction against pyridoxal-P resulted in the complete recovery of enzymatic activity, provided that the temperature was maintained at 4°C during dialysis. These results suggest that, after removal of the coenzyme from ALAS, the apoenzyme is stable at low temperatures and can be readily reconstituted by addition of coenzyme. This behavior of ALAS contrasts with that of many other pyridoxal-P-containing enzymes which become unstable after re-

moval of the pyridoxal-P (25). For example, the ALAS isolated from *Euglena gracilis* loses activity rapidly and irreversibly in the absence of pyridoxal-P, even at 4°C (26).

Such a temperature-mediated loss of activity in the absence of exogenous pyridoxal-P does not appear to be a common feature of pyridoxal-P-containing enzymes of the mitochondrial matrix. For example, the alanine aminotransferase present in the matrix fraction lost some activity when incubated at 37°C; however, addition of pyridoxal-P did not protect against the loss. This observation may reflect the previously reported tight binding of the coenzyme to this protein (25).

One explanation of the observed rapid inactivation of ALAS is that this process is a specific event which forms an important part of the intracellular regulation of this enzyme. The levels of ALAS within the cell are subject to regulation both by increases in synthesis after treatment of animals with specific inducers as well as by its very rapid turnover within the mitochondria (11). It would appear that the rapidly degraded ALAS of rat liver mitochondria [$T_{1/2}$ of 40 min (27)] must be subject to certain rigid controls such as loss of the cofactor, pyridoxal-P, influencing its sensitivity to proteolytic enzymes in the matrix.

Previous reports in the literature (12–15) had indicated that ALAS was sensitive to various proteases; however, the lack of protection of the observed inactivation at 37°C in the absence of exogenous pyridoxal-P by addition of a wide spectrum of protease inhibitors suggests that the primary event in the loss of activity does not involve proteolysis. The initial "tagging" of the apoprotein which occurs at 37°C appears to prevent the reassociation with pyridoxal-P, thus preventing catalysis. Consequently, the apoenzyme may then become subject to subsequent proteolytic digestion. This type of two-step processing for many enzymes was recently reported by Stadtman and co-workers (28). Glutamine synthetase of *Escherichia coli* is inactivated by several mixed-function oxidation systems by modification of a single histidine residue and, as a result, the enzyme is more susceptible to subsequent proteolytic degradation by a number of proteases in the cell. It is tempting to speculate that the inactivation of an enzyme, either by a mixed-function oxidase as described by Stadtman's group (29) or by some other mechanism as described in this paper for ALAS, may mark the protein for proteolytic digestion and thus play a key role in the regulation of protein turnover. Further studies on the initial inactivation step of ALAS and its relationship to the rapid turnover of this enzyme *in vivo* are currently in progress.

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