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**PURIFICATION, CHARACTERIZATION AND BIOGENESIS OF COMPLEX
III FROM YEAST MITOCHONDRIA**

City University of New York

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PURIFICATION, CHARACTERIZATION AND BIOGENESIS
OF COMPLEX III FROM YEAST MITOCHONDRIA

by

Anita Sidhu

A dissertation submitted to the Graduate Faculty in Biochemistry
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at the City University of New York.

1982

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

Purification, Characterization and Biogenesis
of Complex III from Yeast Mitochondria.

By

Anita Sidhu

Advisor: Professor Diana S. Beattie

Complex III was isolated and purified from bakers' yeast by ammonium sulfate fractionation and column chromatography. The purified complex contained 7.03 and 4.24 nmol/mg protein of cytochromes b and c₁, respectively. The specific activity of the complex was 17.1 μ mol/min/mg protein. Electrophoresis of the complex revealed the presence of seven polypeptides with molecular weights from 15,500 to 50,000. The isoelectric points of these subunits and their stoichiometry were determined.

The two core proteins and the iron-sulfur protein were purified, and antibodies against these subunits and complex III were raised. Electrophoretic analysis of the immunoprecipitates also confirm the presence of seven polypeptides in complex III with molecular weights from 15,000 to 47,000.

The topology of the subunits was studied by proteolytic digestion and immunoinhibition studies. The two core proteins appear to be embedded within the complex, while cytochromes b and c₁, the iron-sulfur protein and the 17,500-dalton subunit are substantially exposed to the surface.

Subunit interactions of complex III were studied by dissociation of mitochondria in low concentrations of SDS. Cytochromes b, c₁ and core protein I appear to be tightly associated and lie close together. The iron-sulfur protein and the 17,500-dalton subunit are loosely

attached to the complex.

Studies on the biogenesis of complex III indicates that only cytochrome b is synthesized on mitochondrial ribosomes. Pulse-labeling of (³H)leucine labeled cells with (³⁵S)methionine, followed by calculations of the half-life of the subunits suggests that core protein I and the iron-sulfur protein have large pools and low turn-over rates. Cytochromes b and c₁ appear to have small precursor pools and high turn-over rates.

Studies on diuron-resistant mutants of yeast show that these mutants are resistant to diuron in vitro and are not cross-resistant to antimycin or UHDBT. The mutants contained similar quantities of cytochromes a, b, and c₁ as the wild-type. No "extra reduction" of cytochrome b was observed in these mutants.

In completing one discovery we never fail to get an imperfect knowledge of others of which we could have no idea before,
so that we cannot solve one doubt without creating several new ones.

-Joseph Priestley, 1733-1804.

TO MY FAMILY

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CHAPTER I: INTRODUCTION

1. Mitochondrial Electron Transport Chain
2. Purification and Polypeptide Characterization of Complex III
3. Localization of the Subunits of Complex III
4. Biogenesis of Complex III
5. Biochemical Analysis of Diuron-Resistant Mutants of Yeast

1. MITOCHONDRIAL ELECTRON TRANSPORT CHAIN

The mitochondrion is a unique energy-transducing organelle which provides energy in the useful chemical form, ATP (adenosine triphosphate), by passing electrons through the respiratory chain. The respiratory chain has been dissected into four distinct lipid-protein complexes: complexes I, II, III and IV, each complex possessing a catalytic role in the oxidation-reduction reactions of the electron transfer process (Green and Hatefi, 1961). The sequence of electron transfer in the respiratory chain is determined by the redox midpoint potentials of the electron carriers in each complex (Chance, 1956). Complex V, the fifth complex in the mitochondrial inner membrane, is responsible for coupling the electron transport chain with ATP synthesis (Stiggall et al, 1978 & 1979). It is generally accepted that the electron transfer complexes convert the free energy of oxidation into an electrochemical proton gradient $\Delta\mu\text{H}^+$, which is used by complex V for the synthesis of ATP (Boyer et al, 1977).

Complex I, NADH-Coenzyme Q reductase, catalyzes the reduction of ubiquinones and ferricyanide by NADH. Reduction of the former compound is inhibited by both rotenone and piericidin. Complex I, the first site of oxidative phosphorylation in the respiratory chain, contains noncovalently bound FMN, a minimum of four non-heme iron centers and acid labile sulfide (Hatefi

and Stempel, 1969). Different purified preparations of the isolated complex I were found to have molecular weights ranging between 670,000 and 890,000 and consisted of six to fifteen polypeptide subunits (Ragan, 1976).

Complex II, succinate-coenzyme Q reductase, catalyzes the reduction of coenzyme Q by succinate. It contains succinate dehydrogenase, a low potential cytochrome b, b_{557.5} (Davis et al, 1973) and other proteins. Succinate dehydrogenase contains covalently bound FAD and two non-heme iron centers and has a molecular weight of about 97,000. The enzyme is composed of two subunits : a large subunit of 70,000 and a small subunit of 27,000 daltons (Hatefi et al, 1975). Recently, Hatefi and Galante (1980) isolated from complex II of beef heart mitochondria a cytochrome b₅₆₀ which contains in equimolar amounts two polypeptides of molecular weights about 15,500 and 13,500. This preparation of cytochrome b can be reconstituted with succinate dehydrogenase.

Complex III, coenzyme QH₂-cytochrome c reductase, catalyzes the reduction of cytochrome c by coenzyme QH₂ and constitutes the second site of oxidative phosphorylation. This complex has been isolated from several sources using different methods of purification. The complex isolated from either beef heart or Neurospora was present as a dimer in solution, as determined by ultracentrifugal studies (von Jagow et al, 1977; Weiss & Kolb, 1979). The molecular weight of the monomer were within the range

of 200,000 to 250,000 (Marres & Slater, 1977; Katan et al, 1976a, von Jagow et al, 1978). Only two groups had been able to isolate an enzymatically active complex III from yeast (Siedow et al, 1978; Beattie et al, 1980). The isolated complex from yeast was shown to contain 10 polypeptides consisting of : two "core" proteins (50,000 and 40,000), one cytochrome c₁ (31,000), one cytochrome b (30,000), one iron-sulfur protein (24,000) and five small polypeptides (17,000, 16,000, 12,000, 8,400, and 5,800). This isolated complex III from yeast was shown to catalyze the reduction of cytochrome c by the decyl analog of coenzyme Q, DBH₂, in a reaction which was both antimycin A and diuron sensitive.

Complex IV, cytochrome c oxidase, catalyzes the oxidation of reduced cytochrome c by molecular oxygen and forms the third site of oxidative phosphorylation. The catalytic reaction is inhibited by carbon monoxide, cyanide and azide. Complex IV contains two copper atoms and two moles of heme a, and a₃. Only heme a₃ can react with oxygen or carbon monoxide. The purified cytochrome oxidase from yeast contains seven polypeptide subunits: three large subunits (I, 40,000; II, 33,000; III, 22,000) are synthesized in the mitochondrion whereas the four small subunits (IV, 14,500; V, 13,700; VI, 12,500; VII, 4,600) are synthesized in the cytosol (Mason and Schatz, 1973; Rubin and Tzagoloff, 1973). Previous reports have suggested that either subunits I, II or IV

(Freedman et al, 1979) or subunit V (Tanaka et al, 1979) might be associated with heme in cytochrome oxidase from beef heart mitochondria.

Poyton and McKemie (1979) claimed that the four small subunits of cytochrome oxidase are synthesized in the cytoplasm as a single large precursor. Subsequently, this polypeptide would be transported into the mitochondrion and then processed after its association with the mitochondrial inner membrane. These observations were not confirmed by either Mihara and Blobel (1980) or Lewin et al (1980). Recently, Mihara and Blobel (1980) provided strong evidence that subunits IV, V and VI are synthesized as precursors larger by 1500 - 3000 daltons than their mature counterparts and that subunit VII is not synthesized as a larger precursor. These workers used subunit-specific antisera prepared against each of the four small subunits to precipitate polypeptides synthesized either in vitro or in vivo under conditions of pulse labeling, pulse-chase labeling and continuous labeling. The mitochondrially made subunit II is also synthesized as a precursor form which is processed post-translationally (Sevarino and Poyton, 1980).

Complex V, the oligomycin-sensitive ATPase, catalyzes the synthesis and hydrolysis of ATP in a process which is inhibited by oligomycin, rutamycin and DCCD. The purified complex from yeast contained ten polypeptides (Tzagoloff et al, 1973). Five of the six cytoplasmically made polypeptides are assembled in F_1 which faces the matrix and catalyzes ATP hydrolysis and

synthesis. The sixth subunit forms the stalk (oligomycin-sensitivity-conferring-protein) responsible for the binding of F_1 to the membrane. It was reported recently that non-membrane bound F_1 was capable of net synthesis of what may be an ATP synthesis and hydrolysis transition state analog (Bossard, Vik and Schuster, 1980). F_0 , or the membrane factor, is composed of at least three hydrophobic subunits (Tzagoloff et al, 1973) and functions as a proton conducting channel and contains the site for oligomycin and DCCD sensitivity (Enns and Criddle, 1977; Criddle et al, 1977). The small subunit with a molecular weight of 8,000 has been sequenced and shown to bind DCCD at the glutamic acid residue at position 65 (Sebald and Wachter, 1978) which is in the center of a hydrophobic sequence of amino acids. Oligomycin binds at a different but interacting site on the same subunit (Kiehl and Hatefi, 1980). Three of the F_1 -subunits are translated outside the mitochondria and imported into the organelles as individual precursors that are between 2,000 and 6,000 daltons larger than mature subunits, and then processed in the mitochondrion post-translationally (Macceccchini et al, 1979; Lewin et al, 1980)

2. PURIFICATION AND POLYPEPTIDE CHARACTERIZATION OF COMPLEX III

Complex III was first isolated from beef heart mitochondria in 1962 by Hatefi et al (1962). Since then, several methods of preparation of the complex from beef heart mitochondria have been reported from several laboratories (Yu et al, 1974; Gellerfors et al, 1976; Rieske, 1976; Capaldi et al, 1977). These isolation procedures are similar to the one originally developed by Hatefi, whereby the complex is first separated from the other respiratory chain components by dissolution with cholate (varying between 0.25 to 1.0 mg per mg of protein). The complex is then precipitated from the inner mitochondrial membrane with ammonium sulfate (varying between 35% to 50% saturation). A similar method has been recently used to isolate the enzyme from rat liver mitochondria (Gellerfors et al, 1981).

A newer method of isolation of complex III, differing in principle from those listed above, was developed by Riccio et al (1977) whereby beef heart mitochondria, in the presence of antimycin A, were solubilized in 5.5% Triton X-100. This was then followed by chromatography on a hydroxyapatite column. Antimycin was added to stabilize the complex, since it is known to prevent the dissociation of heme from cytochrome b apoprotein during chromatography in high ionic strength buffers (Rieske et al, 1967). The purified complex thus obtained, however, was inactive and moreover, it lacked the non-heme iron-sulfur protein.

Affinity chromatography was the method of choice for the isolation of complex III from Neurospora crassa (Weiss and Kolb, 1979). Cytochrome c-depleted submitochondrial particles were dissolved in 3% Triton X-100, followed by chromatography on an affinity column consisting of ferricytochrome c-Sepharose 4B. The conversion of ferricytochrome c to ferrocycytochrome c, upon addition of ascorbate, led to a concomitant release of the bound complex.

The isolation of complex III from yeast mitochondria required the development of new preparative procedures, since many of the established methods generally applied to the purification of beef heart complex III failed to work with yeast. In addition, it was very difficult to obtain a complete separation of complex III from complex II, and even mild dissociation procedures led to loss of heme from cytochrome b resulting in an inactive complex. Purification of complex III was first reported by Katan et al (1976), whereby submitochondrial particles, in the presence of antimycin, were solubilized by cholate followed by ammonium sulfate precipitation. This method yielded an inactive complex. Siedow et al (1978) then developed a method resulting in an enzymatically active complex, and although the basic method of preparation is similar to the original method of Hatefi et al (1962), there are nonetheless certain key modifications. Submitochondrial particles were dissolved in a low concentration of cholate (0.06 mg per mg of protein) and the complex was precipitated at a higher ammonium

sulfate saturation (60%). Contaminating complex II was removed by a series of ammonium sulfate fractionations and the complex was precipitated by ultracentrifugation. This same method, with modifications, has been used in this laboratory to obtain a pure complex III preparation, free of complex II (Beattie et al, 1980).

The polypeptide composition of complex III isolated from different sources using different methods of preparation varies considerably, as seen on SDS-polyacrylamide gels. There is a variation in the number, the molecular weights and in the relative amounts of the subunits, even when the complex is isolated from the same species. Table I summarizes the composition of complex III with regard to molecular weight, stoichiometry and heme content. The identity and function of the constituent polypeptides are far from certain, but it is generally agreed that there are two high molecular weight core proteins, of unknown function, present in the isolated complex. Core protein I has a molecular weight between 43,000 to 53,000 daltons, while core protein II has a molecular weight between 39,000 to 50,000. These core proteins contain no prosthetic groups (Sillman et al, 1967) but appear to be normal constituents of complex III, since their removal leads to loss of antimycin binding (Rieske et al, 1967), and since specific alkylation of core protein I with iodoacetamide leads to inactivation of enzyme activity (Gellerfors et al, 1976).

TABLE I

Comparison of the Composition of Complex III Isolated by Various Investigators

Subunit	Molecular weight and stoichiometry of the polypeptides of Complex III									
	Geller- fors <u>et al</u> (1976) ^a	Marres & Slater, (1976) ^a	Yu <u>et al</u> , (1974) ^a		Weiss & Kolb(1979) ^b		Katan <u>et al</u> , (1976a) ^c	Siedow <u>et al</u> , (1973) ^c	Beattie <u>et al</u> , (1980) ^c	
Core Protein I	50,000	43,000	1	53,000	2	52,000	1	44,000	47,000	50,000
Core Protein II	47,000	40,000	1	50,000	2	45,000	2	40,000	39,000	40,000
Cytochrome <u>b</u>	29,000	28,000	2	37,000	2	30,000	2	32,000	31,000	30,000
Cytochrome <u>c</u> ₁	29,000	29,000	1	30,000	3	31,000	1	32,000	31,000	29,000
ISP	26,000	24,000	1	28,000	2	25,000	1	-	23,000	24,000
	15,000	12,000	2	17,000	2	14,000	1	17,000	14,000	17,000
	13,000	8,000	2	15,000	5	12,000	1	14,000	13,000	16,000
	10,000	5,000	2			9,000	1	11,000	11,000	12,000
										8,400
										5,000
Cytochrome content in nmol/mg protein										
Cytochrome <u>b</u>		8.0				8.0		6.1	9.3	10.7
Cytochrome <u>c</u> ₁		4.2				4.0		3.0	4.6	4.6

Complex III isolated from: ^aBeef heart mitochondria, ^bNeurospora crassa, ^cYeast mitochondria.

Cytochrome b has a molecular weight between 23,000 to 37,000 and has been identified as an electron transferring, dimeric protein containing non-covalently bound protoheme IX. It is generally distinguished from other cytochromes by its characteristic maximum absorption spectra: α -band at 562 nm, β -band at 532 nm and γ or Soret band at 429 nm in the reduced state. It has been extensively studied in the past few years, (Weiss, 1976; von Jagow and Sebald, 1980), but the exact mechanism of its function is still unknown.

The other two electron-transferring polypeptides, cytochrome c₁ and the iron-sulfur protein (ISP), have been isolated and well characterized. The molecular weight of the monomeric cytochrome c₁ varies between 29,000 to 32,000 and contains covalently bound protoheme IX. It also has a characteristic absorption spectra with an α -band at 553 nm and a β -band at 523 nm. The monomeric ISP has a molecular weight of 23,000 to 26,000 daltons. It contains nonheme iron present in a single iron-sulfur cluster, and has been identified by its EPR spectra with a g value of 1.90 (Trumpower, 1981a). To date, very little is known about the function of the low molecular weight subunits; 3 to 5 of these subunits can be seen in various complex III preparations and their molecular weights vary from 5,000 to 17,000. By using 6M guanidine-HCl to subfractionate complex III from beef heart, Bell and Capaldi (1976) have suggested that subunits of molecular weight 14,000 and 12,500 are associated with cytochrome c₁ and cytochrome b, respectively; while Yu

and Yu (1980) have claimed that a polypeptide with molecular weight of 15,000 to 17,000 is a ubiquinone-binding protein. Earlier evidence indicated an 11,000 dalton species to be the antimycin A-binding subunit (DasGupta and Rieske, 1973).

The first goal in this dissertation was thus to use the methods of Siedow et al (1978) and Beattie et al (1980) for the isolation of a highly purified and enzymatically active complex. III. Next, a thorough characterization of complex III would be performed, with regard to its enzymatic activity as a function of pH and its sensitivity to inhibitors of the respiratory chain, namely antimycin A and UHDBT. In order to understand the function of the isolated complex, an indepth analysis of the subunits of complex III would be made, with an emphasis on the determination of their molecular weights, their stoichiometry, their electrophoretic behaviour on SDS-gels and SDS-8M urea gels, and their pI values using isoelectric focusing.

3. LOCALIZATION OF THE SUBUNITS OF COMPLEX III

Complex III not only catalyzes the transfer of electrons from reduced coenzyme Q to cytochrome c, it is also an energy-conserving site and during electron transport causes an electrogenic translocation of protons across the inner mitochondrial membrane (Leung and Hinckle, 1975; Alexandre et al, 1980). Thus, considerable interest in the localization of the various protein components of complex III in the inner mitochondrial membrane has been generated to explain the unidirectional proton translocation at this site of the respiratory chain. Complex III in solution appears to be a dimer with a molecular weight ranging from 440,000 to 550,000 (Engel et al, 1980; Weiss and Kolb, 1979). Crystalline preparations of complex III from Neurospora crassa have been studied by electron microscopy (Leonard et al, 1981) and computer-aided image analysis also suggests that the membrane-bound complex exists as a dimer, with a symmetry axis perpendicular to the membrane plane. Each monomeric unit is 7nm x 5nm, while the dimer is 7nm x 10nm. The complex appears to be S-shaped in projection protruding 70 Å from one side of the membrane and 30 Å from the opposite side. Thirty percent of the enzyme mass seems to be buried in the lipid bilayer.

The topographical distribution of individual subunits in isolated and membrane-bound complex has been studied biochemically

by surface labelling techniques that use both hydrophilic and hydrophobic reagents. Thus, labeling studies with the membrane-nonpermeant reagent, diazobenzene sulfanilic acid (DABS), in beef heart mitochondria and submitochondrial particles have indicated that cytochrome c₁ and the ISP are on the cytoplasmic side of the inner membrane, while cytochrome b appears to span the membrane (Bell et al, 1979; Beattie et al, 1981). Using a similar approach with lactoperoxidase-catalysed iodination of mitochondria from Neurospora crassa, Weiss and Eiganke (1976) concluded that the bulk of cytochrome b is localized in the interior of the membrane and hence almost inaccessible to the probe. Hydrophobic photo-labelling using photoreactive lipid analogs showed that cytochrome b is in contact with lipids (Gutweniger et al, 1981) and is buried within the lipid bilayer, based on its inaccessibility to digestion by trypsin (Beattie et al, 1981). Electron spin resonance studies in pigeon heart mitochondria indicated that the hemes of the dimeric b are localized on the cytoplasmic side of the membrane (Case and Leigh, 1976).

Cytochrome c₁ appears to be amphipathic with its hydrophilic domain exposed on the cytoplasmic side of the membrane since it is labeled by both hydrophilic and hydrophobic probes and it binds cytochrome c (Trumpower and Katki, 1975; Broger et al, 1980). Primary sequence analysis of cytochrome c₁ show a large hydrophilic domain with a single 21 hydrophobic amino acid stretch and it is presumed that this hydrophobic region near the

-COOH terminus may be responsible for its membrane location and integration into the bc₁ complex (Wakabayashi et al, 1980). The external localization of the ISP on the C-side of the mitochondrial membrane has also been largely confirmed by Onishi et al (1980), based on paramagnetic perturbation of its EPR signal at $g = 1.90$. Cross linking studies (Smith et al, 1978), a high mid-point potential of $\sim +280$ mV and the dependance of its oxidation-reduction status on cytochrome c₁ indicates that the ISP is located adjacent and peripheral to cytochrome c₁, and that it transfers electrons to cytochrome c₁ from ubiquinol (Trumpower, 1981b). Based on X-ray crystallography, however, Leonard et al (1981) suggested that the ISP is buried within the lipid bilayer. This seems unlikely, since the evidence for an external localization of the ISP is vast and overwhelming.

DABS labeling of the two core proteins (Mendel-Hartvig and Nelson, 1978; Beattie et al, 1981) in both the mitochondria and SMP suggests that these proteins span the bilayer of the inner mitochondrial membrane. The results of hydrophobic labeling with photoreactive lipids indicate that part of core protein I is exposed to lipids, while very little, if any, of core protein II is present at the lipid-protein boundary of the enzyme complex (Gutweniger et al, 1981). These results suggest that core protein II is buried within the complex through protein-protein type interactions. Smith et al (1980), through cross linking studies, found that the two core proteins lie next to each other. A

A totally different picture of the topology of these core proteins is presented in X-ray crystallographic analysis, whereby it is proposed that the bulk of the two core proteins are substantially exposed to the matrix side of the membrane (Leonard et al, 1981).

The topology of the smaller subunits is not clear due to the large variation seen in both the number and molecular weights of these subunits in isolated complex III preparations. However, Beattie et al (1981) have observed that a 17,000 dalton species is substantially labeled with DABS in mitochondria and not in submitochondrial particles, suggesting that this subunit is exposed to the cytoplasmic side of the membrane. Similar DABS labelling studies (Bell et al, 1979) along with chemical cross-linking data (Smith et al, 1978) indicate that two low molecular weight polypeptides, 17,000 and 15,000, are exposed to the cytoplasmic side of the membrane.

The second goal in this dissertation was thus to study the orientation of the subunits in both the isolated and purified complex III and in the membrane bound enzyme, using two different approaches. The first approach was to perform proteolytic digestion of the isolated complex III, using trypsin and chymotrypsin, in order to determine the accessibility of these subunits to proteolytic enzymes. Since the complex has been reported to undergo a conformational change upon reduction (Baum et al, 1967), the accessibility of these subunits to proteolytic enzymes was studied as a function

of the redox state of the complex by using oxidised, reduced and antimycin A treated complex III. The second approach for studying the topography of membrane-bound complex III was an immunological one. Complex III antibodies and subunit-specific antibodies, against purified core protein I, core protein II and the ISF were obtained and immunoinhibition studies were performed on mitoplasts, submitochondrial particles and cholate-solubilized mitoplasts. Such immunoinhibition studies would indicate if these subunits were at all implicated in the catalytic activity of the ubiquinol:cytochrome c reductase in membranes having different orientations.

4. BIOGENESIS OF COMPLEX III

Early studies on the biogenesis of complex III of the yeast mitochondrial respiratory chain have indicated that products of both mitochondrial and cytoplasmic protein synthesis are required for the proper functioning and assembly of this complex (Beattie et al, 1974; Lin et al, 1974; Brown and Beattie, 1978). The identification of mitochondrial and cytoplasmic translational products were greatly facilitated by using selective inhibitors of protein synthesis: chloramphenicol, erythromycin, acriflavin for the mitochondrial system, and cycloheximide for the cytoplasmic system. Lin et al (1974) in this laboratory have shown that both chloramphenicol and cycloheximide block the increase of the activities of coenzyme QH₂:cytochrome c reductase, cytochrome oxidase as well as oligomycin-sensitive ATPase in yeast undergoing glucose derepression, suggesting that the formation of complexes III, IV and V requires products of both mitochondrial and cytoplasmic protein synthesis. From similar experiments, it was demonstrated that the development of NADH-coenzyme Q reductase activity of complex I and succinate dehydrogenase activity of complex II during glucose derepression was not inhibited by chloramphenicol suggesting that these complexes did not contain products of mitochondrial protein synthesis. The formation of complex III

was also observed to require the coordinated synthesis of proteins in the two genetic systems by other groups (Weiss and Ziganke, 1974; Ferlman and Mahler, 1974).

Cytochrome b in complex III was shown in Neurospora to be labeled with radioactive leucine in the presence of cycloheximide, while chloramphenicol prevented the labeling of this protein (Weiss and Ziganke, 1974; Weiss, 1976). Lin et al (1978), and Chen and Beattie (1982), by using a specific antiserum raised against a highly purified cytochrome b, have demonstrated that the incorporation of (³H)leucine or (³⁵S)methionine into the apoprotein of cytochrome b isolated by immunoprecipitation followed by gel electrophoresis was insensitive to cycloheximide and sensitive to acriflavin, erythromycin and chloramphenicol. Recent studies have demonstrated that mitochondrial DNA of yeast codes for cytochrome b (Tzagoloff et al, 1976; Slonimski and Tzagoloff, 1976; Haid et al, 1979), thus confirming the mitochondrial origin of cytochrome b. The synthesis of cytochrome b-apoprotein was found to be heme-independent (Clejan et al, 1980).

All the other polypeptides of complex III, including cytochrome c₁, have been shown to be products of cytoplasmic translation in experiments with yeast (Katan et al, 1976b; Beattie et al, 1980), and Neurospora (Weiss and Ziganke, 1977). The possibility that other proteins of complex III may be

synthesized by mitochondria has not been ruled out, since most studies on the biogenesis of this complex were performed with enzymatically inactive complexes that may have lost key proteins (Katan et al, 1976b; Weiss and Eiganke, 1977). Cytochrome c₁ was shown to be synthesized in the cytoplasm (Ross and Schatz, 1976). It is synthesized in a precursor form as a polypeptide 6,000 daltons larger than the mature subunit (Nelson and Schatz, 1979; Neupert and Schatz, 1981). The identity of the precursor was established by: 1. in vitro translation of yeast mRNA, 2. labeling cells in the presence of an uncoupler, CCCP, to block import into the mitochondria and to thus accumulate a large precursor pool in the cytoplasm, 3. pulse-labeling cells for very short periods in the absence of any inhibitor (Ohashi et al, 1982). The precursor in all these experiments was immunoprecipitated using subunit-specific anti-cytochrome c₁ antiserum. The precursor was then shown to be imported into the mitochondria (Gasser et al, 1982) and cleaved into a membrane-bound intermediate form (4,000 daltons larger than the mature subunit) by an o-phenanthroline-sensitive matrix protease (Ohashi et al, 1982). Furthermore, these authors demonstrated that the intermediate form was converted to the mature cytochrome by the covalent linkage of heme to the polypeptide, accompanied by cleavage by another unknown protease. This step was shown to be heme-dependent and was blocked in heme-deficient mutants or in

wild-type cells treated with levulinic acid, an inhibitor of heme synthesis.

The iron-sulfur protein was also shown to be synthesized in the cytoplasm as a precursor 2,000 daltons larger than the mature form (Cote et al, 1979). The precursor was detected by in vitro translation of yeast mRNA and by pulse-labeling cells for short periods, followed by immunoprecipitation using subunit-specific antiserum directed against the ISF. The precursor is thought to be cleaved into the mature form either during or after its entry into the mitochondria.

To date, very little is known about the biogenesis of the two core proteins and the low molecular weight subunits. This may be due, in part, to a lack of antigenicity of subunit-specific antisera obtained from the two core proteins (Schatz, personal communication). However, Beattie et al (1980) showed that when cells were labeled in the presence of chloramphenicol, a large subunit with a molecular weight of 100,000 daltons accumulated in the cytoplasm. A subsequent chase with unlabeled amino acid showed a direct correlation between the disappearance of the label in the 100,000 subunit and an increase in the label incorporated into the two core proteins. It was postulated that the 100,000 dalton species may either be a precursor of the core proteins or a partially assembled complex of these two core proteins.

Thus, the third project in this dissertation was to:

1. Determine which of the subunits of complex III were synthesized in mitochondria and cytoplasm, 2. To investigate the relationship between the 100,000 dalton subunit and the two core proteins, using the subunit specific antisera raised against core protein I and core protein II.

5. BIOCHEMICAL ANALYSIS OF DIURON-RESISTANT MUTANTS OF YEAST

The mechanism of electron transfer through the cytochrome b-c₁ segment of the mitochondrial respiratory chain is not understood. An especially enigmatic aspect of this electron transfer process is the phenomenon known as "oxidant-induced reduction of cytochrome b" whereby addition of an oxidant to mitochondria, in which cytochrome c₁ is reduced and the b cytochromes are oxidized, results in transient reduction of cytochrome b coincident with cytochrome c₁ oxidation (Erecinska *et al*, 1972). The oxidant-induced reduction of cytochrome b requires a source of reducing equivalents (succinate, NADH or ubiquinol), depends on the presence of ubiquinone and involves both cytochromes b₅₆₂ and b₅₆₆ (Wikstrom and Berden, 1972). The oxidant-induced reduction of cytochrome b is most readily demonstrated in the presence of antimycin, with a concomitant shift of the absorption maximum of cytochrome b₅₆₂ towards the red ("red-shift"). This reduction behaviour of cytochrome b has also been observed at low temperatures, suggesting that this reaction is intrinsic to the mechanism of electron transfer in the cytochrome b-c₁ segment and not an aberrant response elicited by antimycin (Erecinska and Wilson, 1972).

The oxidant-induced reduction of cytochrome b is best explained by the Q cycle (Mitchell, 1975) or one of its modifications (Trumpower, 1981b) than by the classical electron

transport chain. A schematic diagram of the Q cycle is depicted below (Trumppower, 1981b).

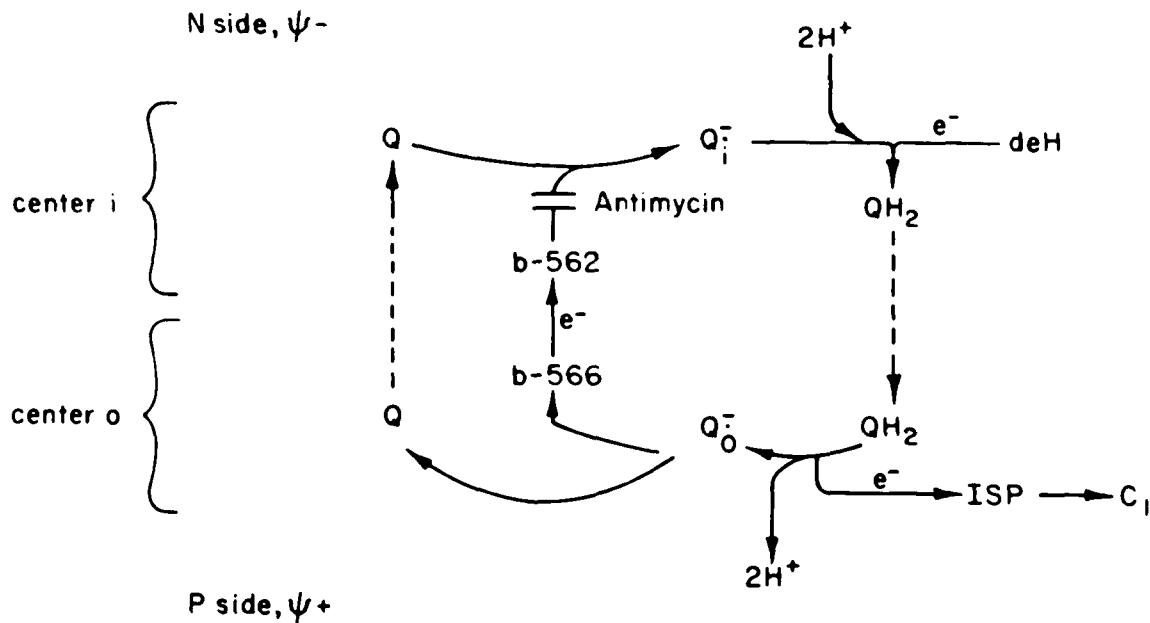


Fig. 1. Proton-motive Q cycle mechanism for electron transfer through the cytochrome b - c_1 complex. The subscripts i and o designate specific species of semiquinone anion, which react at the electronegative, N side (center i) or electropositive, P side (center o) of the inner mitochondrial membrane.

Thus according to the Q cycle hypothesis, ubiquinol is oxidized at the cytoplasmic side of the inner mitochondrial membrane by transfer of an electron to cytochrome c_1 (via the ISP) and the low potential ubisemiquinone ($Q_o^\bullet$), which then reduces cytochrome b_{566} , which in turn reduces cytochrome b_{562} . The electron on cytochrome b_{562} is used along with an electron delivered by the substrate to reduce ubiquinone to ubiquinol.

Antimycin inhibits reoxidation of cytochrome b₅₆₂ thus "trapping" electrons in the b cytochromes, while the c cytochromes continue being oxidized.

Recently, the gene for cytochrome b has been localized on the mitochondrial DNA of both yeast (Nobrega and Tzagoloff, 1980a) and mammals (Anderson et al, 1981) and sequenced (Nobrega and Tzagoloff, 1980b; Lazowska et al, 1980). Mutants, which are resistant to 3-(3,4-Dichlorophenyl)-1,1-dimethyl urea (diuron), have been isolated and shown to be allelic with various regions of the cytochrome b gene (Colson and Wouters, 1980). Diuron, a herbicide, inhibits the electron flow in yeast mitochondrial respiratory chain. Its inhibitory site is localized between cytochrome b and cytochrome c₁, since diuron blocks cytochrome b oxidation and cytochrome c₁ reduction (Briquet and Goffeau, 1981). Although antimycin also inhibits electron transport between cytochromes b and c₁, the mode of action of the two inhibitors is not strictly identical and the kinetics of inhibition by these inhibitors differ slightly. Indeed, Convent and Briquet (1978) in studying the response of normal yeast cells to these inhibitors, showed the the kinetics of inhibition of NADH oxidase are non-sigmoidal for diuron and are sigmoidal for antimycin. Diuron induces the "extra-reduction" of cytochrome b, in the same way as antimycin, but does not induce the "red-shift" of cytochrome b₅₆₂.

In our laboratory we felt that such diuron-resistant mutants

might be useful in investigating the structural features of the cytochrome b protein which contribute to its functional behaviour. Three diuron-resistant mutants were chosen in these studies, Diu^R-740, Diu^R-742 and Diu^R-752. Genetic analysis revealed that the mutant Diu^R-742 mapped in the diuron-II locus, which is allelic with Box 4 or the α -exon of the cytochrome b gene (Colson and Wouters, 1980). The other two mutants, Diu^R-740 and Diu^R-752, mapped in a new locus, diuron-III, which appears to be allelic to Box 3, the first intron of the mosaic cytochrome b gene (Lazowska et al, 1980).

Hence, the fourth goal of this dissertation was to study the biochemical response of these mutants to diuron, antimycin and UHDBT, inhibitors of the complex III region of the respiratory chain, with an emphasis on the kinetic and spectral behaviour of the mutants. UHDBT, the synthetic analog of ubiquinone, 5-n-undecyl-6-hydroxy-4,7-dioxobenzothiazole, is a highly potent and specific inhibitor of electron transfer in complex III in both yeast (Roberts et al, 1978) and beef heart mitochondria (Trumpower and Haggerty, 1980). Part of these studies are presented in a manuscript entitled "Studies on the function and biogenesis of cytochrome b in mutants of Saccharomyces cerevisiae resistant to 3-(3,4-dichlorophenyl)-1,1-dimethylurea". (Clejan, L., Sidhu, A., and Beattie, D.S. (1982), Biochemistry, In press).

CHAPTER II: EXPERIMENTAL PROCEDURES

1. Purification of Complex III
2. Polyacrylamide Gel Electrophoresis
3. Two Dimensional Electrophoresis
4. Effect of Proteolytic Digestion
5. Purification of Subunits of Complex III
6. Immunological Studies
7. Labelling of Yeast Cells
8. Growth of Yeast and Preparation of Membranes
9. Enzyme Assays
10. Analytical Methods
11. Materials

1. PURIFICATION OF COMPLEX III

Isolation of mitochondria by the freeze-fracture method:

Mitochondria were isolated from 25 pounds of commercial bakers yeast cells (Red Star) as described by Lin and Beattie (1978). The yeast cells (1.75 lb at a time) were crumbled in a gallon size, stainless steel Waring Blendor container and liquid nitrogen was added until the yeast cells were completely frozen. The frozen cells were homogenized for a total of 5 min in 4 equal bursts. The final product, as a fine powder, was transferred to 1.5 liters of buffer containing 0.25M sucrose, 0.01M K_2HPO_4 , pH 7.5, 1mM EDTA and 1mM IMSF (Buffer A). After thawing the powder at room temperature, the suspension was homogenized for 50 seconds in the Waring Blendor. The homogenate was centrifuged twice in a Sorvall GSA rotor for 10 min at 5,000 $\times g$ to remove unbroken cells. The supernatant was then centrifuged at 18,000 $\times g$ for 20 min in the same rotor to sediment the mitochondria. The mitochondrial paste was washed twice with a glass-Teflon homogenizer in Buffer A and the washed mitochondria were frozen at a concentration of 30-40 mg of protein per ml and stored at $-70^{\circ}C$. Twenty-five pounds of yeast gave an average yield of 42 g of mitochondrial protein.

Preparation of submitochondrial particles: The mitochondria prepared above were thawed and pooled together and the protein concentration adjusted to 17 mg/ml in Buffer A. The mitochondrial suspension was sonicated in 40 ml aliquots in a Branson Sonifier

at a power setting of 9.5 for 1.5 min in 2 equal bursts with constant cooling in an ice bath. The sonicated suspension was centrifuged 2 times for 10 min at 10,000 xg and again for 30 min at 100,000 xg. The submitochondrial particles were suspended in 0.25M sucrose, 0.01M K_2HPO_4 , pH 7.2, 1mM EDTA and 0.5mM PMSF, and the protein concentration was adjusted to 27 mg/ml. Starting with 42 g of mitochondrial protein, an average yield of 17 g of submitochondrial protein was obtained.

Ammonium sulfate fractionation; A crude pellet of complex III was obtained by ammonium sulfate fractionation according to the method of Biedow et al (1978). To the 27 mg/ml of submitochondria, a 20% solution of potassium cholate was added to a final concentration of 1.6%, and the volume of cholate added (V_{cho}) was noted. This mixture was brought to 20% saturation in ammonium sulfate (114g/l) and the pH was adjusted to 7.2-7.3 with 4N KOH. The mixture was stirred overnight and additional salt (91g/l of V_{cho}) added to raise the salt concentration to 35% saturation. After stirring for 30 min, the suspension was centrifuged at 18,500 xg for 40 min in a Sorvall GSA rotor. This same centrifugation was employed in all subsequent steps. The light brown pellet was discarded and more ammonium sulfate added to the cloudy reddish supernatant to 45% saturation (63g/l). After centrifugation, the large green pellet, consisting of cytochrome oxidase was discarded. The pH of the supernatant was raised to 8.1 and made 50% saturated with respect to ammonium sulfate (32g/l).

After centrifugation, the resultant supernatant was brought to 55% ammonium sulfate saturation (66g/l). The clear, light orange supernatant contained cytochrome c and was discarded. The large pink pellet, containing crude complex III, was dissolved in 0.1M potassium phosphate, pH 7.4, 1mM EDTA and 0.5mM PMSF, and the volume brought to 15% of V_{cho} . The volume of the pellet was also noted.

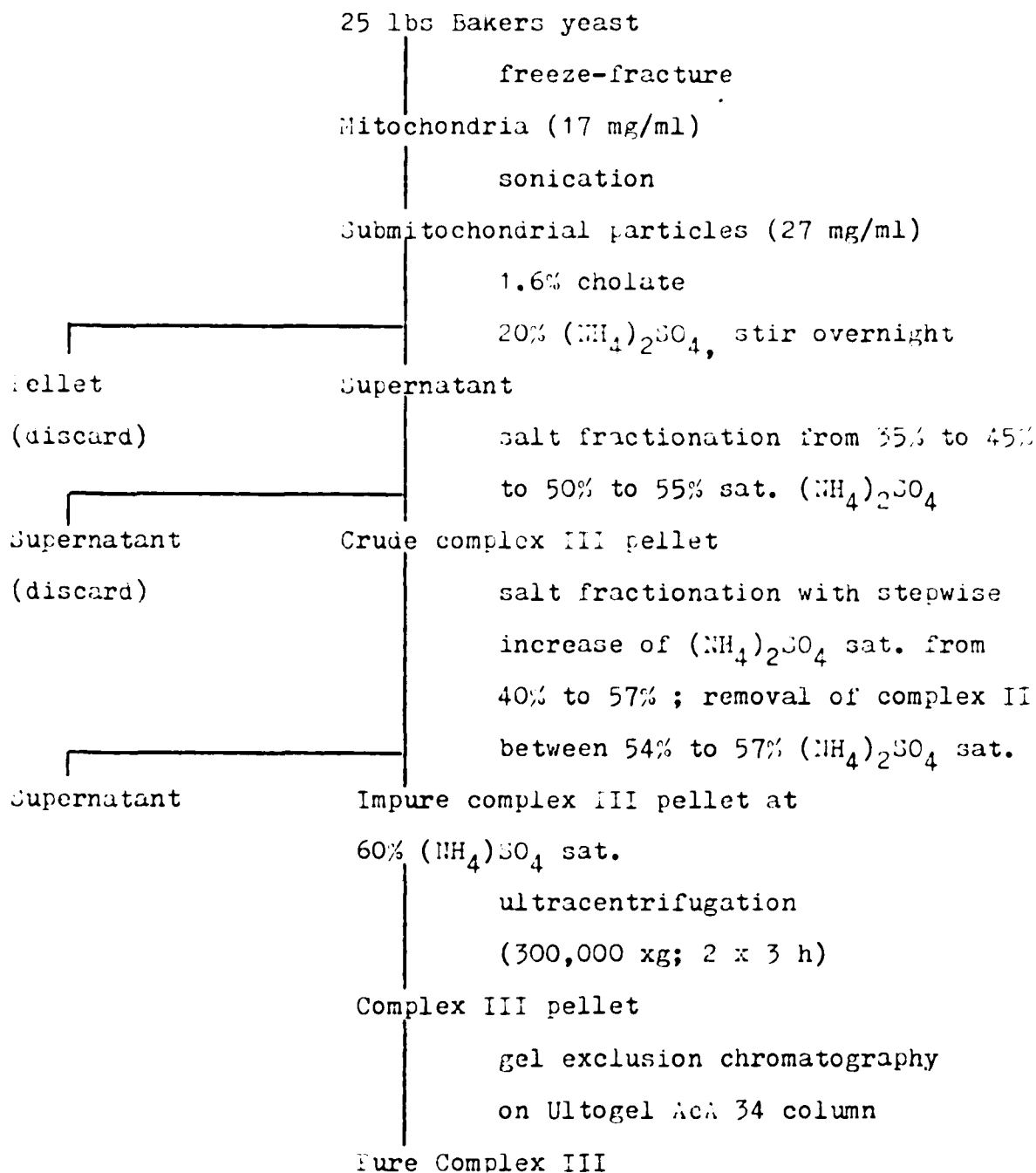
An impure complex III pellet was next obtained, concomitant with a loss of complex II, by repeating the salt fractionations. The removal of complex II was monitored by measuring the enzymic activity of succinate dehydrogenase. The crude complex III solution was brought to 40% ammonium sulfate saturation by addition of a 100% saturated ammonium sulfate solution, after calculating the salt concentration in the mixture at the start. The mixture was centrifuged and the pellet discarded. The ammonium sulfate concentration was then increased stepwise from 45 to ~54% saturation, with increments of 3%, accompanied by centrifugation prior to the addition of more salt. Then the salt concentration in the supernatant was raised from 54 to ~57% saturation and the increments were between 0.3% to 0.7%. It is at this step that most of the complex II precipitated out of solution, as noted by the absence of succinate dehydrogenase activity in the supernatant. The supernatant was then made 60% saturated in ammonium sulfate and upon centrifugation, impure complex III was obtained as a

pink pellet.

Ultracentrifugation of complex III: Complex III, free of ammonium sulfate, was obtained as described by Beattie et al (1980). The pink pellet of complex III obtained above was dissolved in 0.1M potassium phosphate, pH 7.4, and 0.5mM FMSE and centrifuged twice at 100,000 xg for 3 hr. The bright blood-red sticky pellet of complex III was dissolved in 40 mM Tris-acetate, pH 7.0, 5% sucrose, 0.05% Triton X-100 and 0.2mM FMSE (Buffer B), prior to further purification by column chromatography.

Gel exclusion chromatography of complex III: Complex III in Buffer B, was chromatographed twice on an Ultrogel ACA 34 column (1.2 x 50 cm), preequilibrated with Buffer B containing 0.1% sodium azide (Weiss and Kolb, 1979). The columns were run at 6ml/h at 4°C and 1ml fractions were collected. The elution profile was monitored by measuring the absorbance of the Soret bands at 420 nm. The fractions containing complex III were concentrated, using Diaflo XM 300 ultrafilters and frozen in small aliquots at -70°C at a concentration of 26mg/ml. The purification of complex III from yeast cells is shown in Fig.2.

Fig. 2: Purification scheme of complex III from yeast cells.



2. POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-polyacrylamide gel electrophoresis: was performed on analytical slab gels (14 cm long, 1.2 mm thick) using the discontinuous SDS-Tris buffer system of Laemmli (1970). The stacking gels were 5% acrylamide and the running gels were 12.5% acrylamide, except where indicated. A calibration kit (Pharmacia), which contains standard proteins of phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100) and α -lactalbumin (14,400) was used for molecular weight determination. Slab gels containing non-radioactive samples were stained for 2 h with 1.25% Coomassie brilliant blue in 45% methanol and 9.2% acetic acid, according to the method of Weber and Osborn (1979). The gels were then destained with gentle shaking, using the destaining solution containing 5% methanol and 7.5% acetic acid until a clear background was obtained (usually 2 to 3 days).

For fluorography of gels containing radioactive samples, a fluor, sodium salicylate, was used for enhanced detection of radioactivity, according to the method of Chamberlain (1979). The gels were first stained in Coomassie brilliant blue as described above, and then soaked in distilled water for 30 min in order to remove the acetic acid and thus prevent precipitation of sodium salicylate. The gels were soaked in a 1M solution

of sodium salicylate for 30 min. The slab gels were dried for autoradiography and the position of the standard proteins noted. The dried gels were exposed to Kodak MS-5T X-ray film at -70°C .

For a quantitative determination of radioactivity in slab gels, the lanes containing the samples were sliced 2 mm thick with a manual gel slicer made of a row of 100 razors, without prior staining of the gels. The gel slices were digested with 0.5 ml of hydrogen peroxide at 65°C for 36 h. After addition of Liquiscint, they were counted in a Beckman counter. The lanes containing the standard proteins were stained and destained as described above for non-radioactive samples. In order to eliminate variations due to gel elongation during staining and destaining, the length of the gel before and after staining was determined and the migration of the standard proteins corrected accordingly.

Ferguson plots: The R_f values of the subunits of complex III and the standard proteins were plotted against the acrylamide concentration, as previously described (Beattie et al, 1979). Running gels had varied acrylamide concentrations of 10, 11, 12.5 and 15%. The "free mobilities" of the proteins were obtained by extrapolating the respective lines to zero acrylamide concentration.

Sample preparations: Except where noted, all samples prior to electrophoresis were dissociated in a buffer containing 0.05M Tris-HCl, pH 6.8, 10% glycerol, 2mM EDTA, 5% SDS,

5% 2-mercaptoethanol, 1mM EMSF, 1mM TFCK and 1mM TLCK, and were stored overnight at room temperature. Radioactive immunoprecipitates, coupled to Sepharose-bound protein A, were boiled for 5 min at 95°C in the dissociation buffer listed above. This step dissociated the protein A from the IgG of the immunoprecipitate. After heating, the samples were centrifuged at 15,000 xg for 2 min to pellet the Sepharose-protein A and the supernatant was stored overnight prior to electrophoresis in the presence of the protease inhibitors.

SDS-8M urea gel electrophoresis: Gel electrophoresis in the presence of 8M urea was performed as described by Swank and Munkres (1971), using a running gel consisting of 12.5% acrylamide. In order to adapt the original tube method to a discontinuous vertical slab (14 cm long, 1.2 mm thick), it was essential to have a stacking gel consisting of 8M urea, 0.1% SDS, 5% acrylamide, 0.17% bisacrylamide, 0.034M H_3PO_4 , 0.07% ammonium persulfate and 0.075% N,N,N',N'-tetramethylethylenediamine, with solid Tris added to give a final pH of 6.8. Protein samples were dissociated in 1% SDS, 8M urea, 1% 2-mercaptoethanol and 0.01M H_3PO_4 adjusted to pH 6.8 with Tris, heated 10 min at 60°C and stored overnight at room temperature prior to electrophoresis.

3. TWO DIMENSIONAL ELECTROPHORESIS

Isoelectric focusing: in the first dimension was performed by adapting the method of Cabral and Schatz (1979) for a flat bed gel. 40 ml of the gel solution (containing 6% acrylamide, 8M urea, 3% Nonidet P-40, 0.017% TEMED, 0.08% ammonium persulfate and 2% ampholines, pH 3.5-10) were cast between two vertical glass plates to form a slab gel (12 cm x 13 cm x 1.2 mm). After polymerization was complete, one glass plate was removed and the other glass plate supporting the gel was placed in a Desaga Double Chamber apparatus. Paper wicks connected the flat gel to the reservoir buffers consisting of 0.4% ethylene diamine and 0.2% H_2SO_4 . Complex III (150 μ g) was dissociated in a buffer containing 8M urea, 0.025M Tris-HCl, pH 7.2, EDTA, 5% Triton X-100 and 1% 2-mercaptoethanol (Cabral and Schatz, 1979) to which 2 mg of SDS/mg of complex III were added (Stephenson et al, 1980), and it was applied either directly on top of the gel or onto a piece of filter paper (10 x 15 mm) pre-wetted with sample overlay solution (Cabral and Schatz, 1979). Isoelectric focusing was performed at a constant power of 6 watts for 5-6 h until a voltage of 1500 V was reached. To monitor the focusing visually, hemoglobin was applied to the gel. The pH gradient was determined by slicing a strip of a blank portion of the gel into 5 mm pieces, using a manual gel slicer, and eluting the ampholines in 0.1% NaCl.

The remainder of the gel was then soaked for 36 h in 50% methanol, 7% acetic acid and 0.005% Coomassie blue to remove the ampholines (O'Farrell, 1975). Following rehydration of the gel in 1% acetic acid and 0.005% Coomassie blue, the gel was stained and destained. Lanes from the gel were then cut and equilibrated in 0.05M Tris, 0.38M glycine, pH 8.3, and 2% SDS for 30 min.

For the second dimension, the equilibrated lane was placed on top of a slab gel (15 cm x 14 cm x 1.2 mm) containing a 5% acrylamide upper stacking gel and a 12.5% acrylamide lower gel. The contact of this lane with the gel was maintained by a flat piece of Teflon. After electrophoresis the gels were stained and destained, and the molecular weights of the subunits were calculated by comparison to standard molecular weight proteins.

4. EFFECT OF PROTEOLYTIC DIGESTION

Proteolytic digestion: of complex III was performed by suspending the purified complex at 5 mg/ml in 0.67M sucrose, 0.05M Tris-HCl and 1mM histidine, pH 8.0, in the presence of ferricyanide (oxidized form), sodium dithionite (reduced form), or antimycin A. The complex was then treated with 0.02 mg of trypsin/mg of complex III (Ball et al, 1977) or with varying amounts of chymotrypsin. After incubation at room temperature for 30, 60 and 90 min, aliquots were removed and the digestion was stopped by the addition of 1mM EMSF and 4 mg of trypsin inhibitor/mg of trypsin to the trypsin-treated complex, or by adding 2 mM EMSF to the chymotrypsin-treated complex. Each aliquot was either examined for DBH₂:cytochrome c reductase activity or dissociated in buffer overnight and analyzed on a 12.5% polyacrylamide gel, using the SDS-Tris glycine buffer system described above. Densitometric scans of the stained gels were obtained at 590 nm, using a Canalco microdensitometer. Areas under the peaks were calculated, and the percentage of each subunit remaining was obtained by comparison with a gel containing untreated complex III.

5. PURIFICATION OF SUBUNIT OF COMPLEX III

Preparative gel electrophoresis: Approximately 2 mg of the purified complex III were dissociated in 0.05M Tris-HCl, pH 6.8, 10% glycerol, 2mM EDTA, 5% SDS and 5% 2-mercaptoethanol and were boiled for 2 min at 100°C. Electrophoresis was then performed on a preparative slab gel (24 x 16 x 0.2 cm) containing 10% polyacrylamide for 16 h at 20 mA, using the Tris-glycine buffer system of Laemmli (1970). After electrophoresis, the gels were stained with Coomassie blue for 4 h and then destained for 2-3 days with several changes of destaining solution. The polypeptide bands corresponding to core protein I ($M_r=49,000$), core protein II ($M_r=39,000$), and the iron-sulfur protein ($M_r=24,000$) were excised from several gels and pooled. These gel slices were then cut into small pieces and equilibrated with 10-fold excess of buffer containing 0.05M Tris, 0.38M glycine, pH 8.3, and 2% SDS. An equilibration time of 30 min with 2 changes of buffer was sufficient to change the pH of the gel from acidic to basic. Protein was then electrophoretically eluted from the equilibrated gel pieces on a funnel-shaped gel (Chen and Beattie, 1982) using a 5% stacking gel. The elution was performed at 4°C for 16 h until the dye front had entered the dialysis tubing attached to the bottom of the funnel.

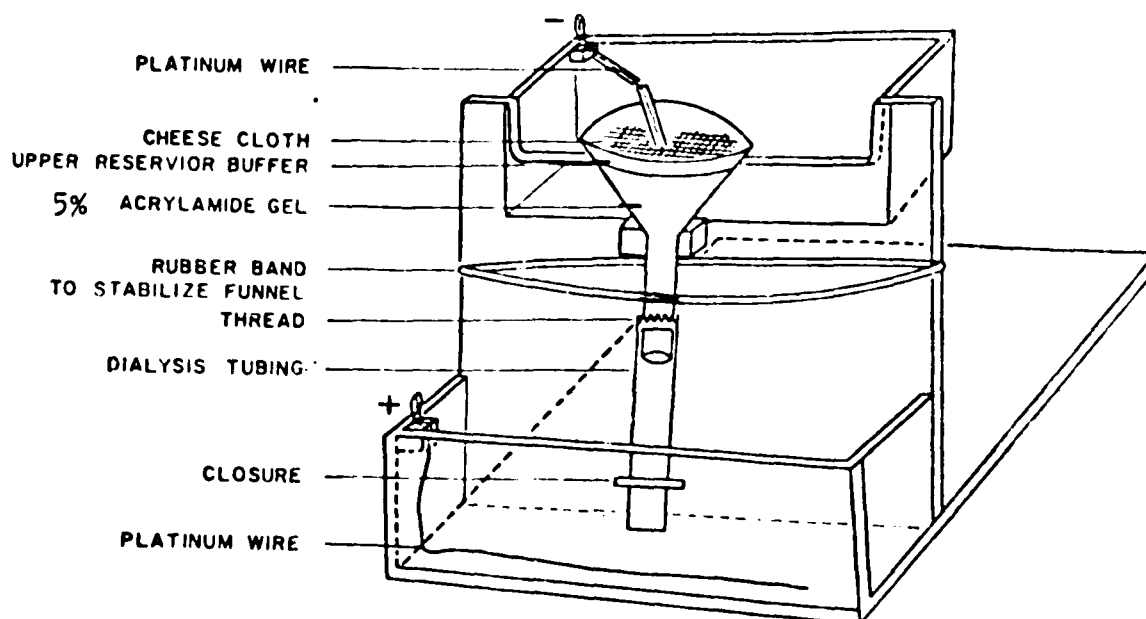


Fig. 3: Modified funnel-shaped acrylamide gel for electroelution of protein from Coomassie blue stained gel slices.

Acetone extraction: The electrophoretically eluted proteins were precipitated from solution by adding 90% acetone containing 0.1N HCl, at -20°C for 16 h. The acetone mixture was then centrifuged at 3,000 xg and the acid removed by washing the precipitates with 90% iced acetone (Chua and Blomberg, 1979). The purified polypeptides were dissolved in 10mM sodium phosphate, pH 7.0, containing 0.2% SDS, and were boiled at 100°C for 2 min. To improve the solubilization, the samples were sonicated for 10 s and the ~~non~~soluble material was removed by centrifugation at 15,000 xg for 2 min. The purity of the eluted protein was determined by analytical SDS- gel electrophoresis.

6. IMMUNOLOGICAL STUDIES

Preparation of antibodies: The purified subunits (200-250 μ g of either core protein I, core protein II, or ISF) were suspended in a small volume of 10mM sodium phosphate, pH 7.0, containing 0.2% SDS, and were mixed with an equal volume of Freund's complete adjuvant. Complex III in Buffer B containing 0.05% Triton X-100 was mixed directly with Freund's complete adjuvant. The proteins were then injected subcutaneously into rabbits at multiple sites. Booster injections containing 50-100 μ g of purified subunit and 200-300 μ g of complex III were administered to each rabbit every 2 weeks. Antiserum obtained from the immunized rabbits were centrifuged at 15,000 xg for 10 min and stored in small aliquots at -20°C.

In some experiments an IgG enriched fraction was prepared from antisera as described by Chan and Schatz (1979). Antisera were incubated for 90 min with 34% saturated ammonium sulfate at 4°C and centrifuged at 3,000 xg for 25 min. The precipitates were suspended in 10mM sodium phosphate, pH 7.2, at a concentration of 10 mg protein per ml, and fractionated twice with the same saturation of ammonium sulfate. The IgG fraction in phosphate buffer containing 0.1% sodium azide was dialyzed against the same buffer at 4°C for 24 h to remove the ammonium salt. The protein concentration of the dialyzed IgG fraction was determined and stored in small aliquots at -20°C.

Control serum was obtained from non-immunized rabbits and an IgG fraction was also prepared.

Double immunodiffusion: Immunodiffusion analysis of the antibodies was performed for 2 days in Petri dishes containing 1% agarose dissolved in 33mM Tris-HCl, pH 8.6, 150mM NaCl, and 1% Triton X-100 (Buffer C). The purified acetone-precipitated core proteins I and II and the ICI and purified complex III were dissolved in Buffer C containing 5% Triton X-100, prior to addition to the wells. The appropriate antiserum and control serum were placed directly into the wells without further treatment. After the immunodiffusion was complete, the agarose gels were gently dislodged from the Petri dishes and the non-precipitated material was rinsed for 3 days with several changes of Buffer C. The agarose gels were stained with Coomassie blue and destained.

Solubilization of proteins: Prior to immunoprecipitation, the membrane-bound proteins were solubilized in several ways. Labelled mitochondria were suspended at 1.5 mg protein/ml in 0.1M sodium phosphate, pH 8.0, and 1mM PMSF and then Triton X-100 was added from a 20% stock solution to give a final detergent concentration of 1% (Werner and Sebald, 1981). After incubation at 0-4°C for 1 h with occasional vortexing, the extracts were centrifuged at 100,000 xg for 45 min to remove any unsolubilized material. The clear supernatant was mixed with the appropriate antiserum and subjected to immunoprecipitation.

Alternatively, labeled mitochondria were suspended to 3mg/ml in 0.1M phosphate, pH 8.0, and lysed in 1 vol of boiling SDS solution containing 3% SDS, 1mM EDTA, 1mM EGTA, pH7.4, and boiled for 5 min at 95°C. The SDS-solubilized mitochondria were then diluted with immunoprecipitation buffer (0.1M sodium phosphate, pH 7.4, 150mM NaCl, 1mM EDTA, 1% Triton X-100 and 1mM DMSF) such that the final SDS concentration in the mixture was $\leq$ 0.2%. Following incubation at room temperature for 1 h, the solubilized material was centrifuged at 100,000 xg for 1 h.

In some experiments, whole cells were directly solubilized prior to immunoprecipitation (Chashi et al, 1982). The labeling of whole cells was stopped by the addition of 20% TCA, trichloroacetic acid, (w/v, final concentration). The TCA denatured cells were then broken by agitating them with an equal volume of glass beads on a vortex mixer for 1 min at maximal speed. The suspension was centrifuged at 3,000 rpm for 10 min in a Sorvall SS34 rotor and the supernatant was discarded. The pellet was resuspended in sample buffer (4% SDS, 0.15M Tris, pH 7.4, 5mM EDTA and 0.1% bromophenol blue). The solution was neutralized with 1M Tris base, heated for 5 min at 95°C and centrifuged at 3,000 rpm for 10 min. The supernatant was removed and the glass beads washed several times with TNET buffer (0.1M Tris, pH 7.4, 150mM NaCl, 5mM EDTA, 1% Triton X-100 and 1mM DMSF) and the washes were

pooled. The volume of the supernatant was adjusted with TNET buffer so that the SDS concentration in the supernatant was $\leq$ 0.2%. The mixture was centrifuged twice at 23,000 xg for 30 min and again at 100,000 xg for 45 min.

Immunoprecipitation: Proteins, solubilized by any of the above techniques, were mixed with an appropriate amount of an antiserum and incubated overnight at 4°C, with or without gentle shaking. The antigen-antibody complexes were then precipitated by the addition of a 10-20% solution of Sepharose-bound protein A. Following a one hour incubation period at 4°C, the immunoprecipitates were centrifuged at 15,000 xg for 2 min in an Eppendorf centrifuge or at 13,000 xg for 10 min in a Sorvall SS 34 rotor. The immunoprecipitates were then washed 3 times with buffer containing 10mM sodium phosphate, pH 7.4, 150 mM NaCl and 0.15% Triton X-100, and 2 times in the same buffer without the Triton. The washed immunoprecipitates were dissociated in dissociation buffer (5% SDS, 5% 2-mercaptoethanol, 50mM Tris-HCl, pH 6.8, 10% glycerol, and 1mM FMSF) and boiled for 5 min. The protein A-Sepharose was removed by centrifugation at 15,000 xg for 2 min and the dissociated immunoprecipitate was analyzed on polyacrylamide gels.

Immunotitration: Prior to any immunoprecipitation experiment, the optimal antiserum:antigen ratio was determined.

Thus, a fixed amount of labeled mitochondria (75 μ l; 1.5 mg/ml), solubilized in Triton X-100, were incubated with increasing amounts of various antisera. The immunoprecipitation was performed as described above and the washed and dissociated immunoprecipitates were counted directly in Liquiscint. The ratio of antiserum:antigen giving maximum precipitation of counts was used in subsequent immunoprecipitation experiments.

7. LABELING OF YEAST CELLS

Uniform labeling with (^3H)leucine: Labeling of total cellular protein with (^3H)leucine was performed by 2 different methods. In the first method, cells were grown overnight in semi-synthetic medium (Schatz and Novac, 1974) containing 2% galactose, 0.2% yeast extract and 5mCi of (^3H)leucine per liter of growth medium. The cells were harvested, washed and mitochondria prepared.

In the second method, a much higher specific radioactivity was obtained. Yeast cells were grown in the semi-synthetic medium described above, but in the absence of any label. The cells were harvested, washed twice with water and resuspended at 200 mg/ml in labeling medium. This medium consisted of the salts normally present in the semisynthetic medium (0.04% CaCl_2 , 0.05% NaCl , 0.07% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1% KH_2PO_4 , 0.12% $(\text{NH}_4)_2\text{SO}_4$ and 0.5mg% FeCl_3), and in addition it contained 1% ethanol, 0.1% glucose or 0.3% galactose, and 0.05% yeast extract. The cells were labeled with 400mCi/ml of (^3H)leucine for 1 h and then chased with 1mM unlabeled leucine for 5 min.

Double labeling of cells: Yeast cells were uniformly labeled with (^3H)leucine by growing the cells overnight to mid-log phase in semi-synthetic medium containing 5mCi/ml of the label. The cells were harvested, washed and resuspended at

200mg/ml in labeling medium, and preincubated at 30°C for 10 min. They were then pulsed with 200μCi/ml of (³⁵S)methionine and 3 min later, the label was chased by the addition of 10mM unlabeled methionine. At various time intervals after the addition of (³⁵S)methionine, an aliquot of cells was removed and broken immediately with glass beads. The broken cells were chilled to 0°C, centrifuged, and mitochondria isolated. The mitochondria were solubilized with 1% Triton X-100 and subjected to immunoprecipitation.

In vivo labeling of cells with (³⁵S)sulfate: Yeast cells were grown to early stationary phase in semi-synthetic medium. The cells were then diluted with 4 vol of fresh, sterile growth medium and regrown for 2-3 h. The cells were harvested, washed and resuspended at 20mg wet weight per ml of low sulfate medium (LSM), which contained salts, vitamins, rare elements, 0.5μg/ml sulfate and 0.3% glucose (Douglas et al, 1979). The cells were shaken for 1 h in LSM at 30°C in order to deplete the endogenous sulfate levels. The yeast cells were once again harvested, washed 1 time with LSM and resuspended at 200mg/ml of LSM. Approximately 200-400μCi of (³⁵S)sulfate per ml of medium was added and the cells were labeled for 2-4 h. At the end of the labeling period, the label was chased by addition of an equal vol of chase medium containing 1% casamino acids plus 2 mg/ml sodium sulfate, for 10 min. The cells were cooled to ice-temperature and washed twice with

ice-cold chase medium, and mitochondria prepared. The mitochondria were solubilized with either 1% Triton X-100 or with boiling 3% SDS solution. In some experiments, a known quantity of mitochondrial protein were spotted on filter disks and the (^{35}S)sulfate incorporated was determined as a function of trichloroacetic acid-precipitable protein (Hans and Novelli, 1961).

Labeling of cells in the presence of CAF and CHI: Selective labeling of yeast cells in the presence of chloramphenicol (CAF) or cycloheximide (CHI) was performed using (^{35}S)methionine. Cells were grown to mid-log phase and resuspended at 200mg/ml in labeling medium described above, containing 0.1% glucose. After a preincubation period of 5 min at 30°C, CHI (1mg/ml) or CAF (4mg/ml, in ethanol) was added and the incubation continued for 5 and 15 min, respectively. The cells were pulsed for 30 min with 200-300 $\mu\text{Ci/ml}$ of (^{35}S)methionine. The label was chased for 5 min by the addition of 10 mM unlabeled methionine and the cells were then chilled to 0-4°C and mitochondria prepared. Control cells, containing no inhibitor, were labeled and processed simultaneously.

For the pulse-chase experiments, yeast cells were pulsed for 15 min with (^{35}S)methionine in the presence of 4mg/ml CAF as described above. At the end of the pulse period, an aliquot of cells was removed and denatured in 20% TCA to stop further incorporation of the label. The remaining cells were chased for 5 min with 10mM unlabeled methionine and also denatured with 20% TCA. Control cells, containing no inhibitor, were treated in the same manner as the pulse-chased CAF-treated cells.

3. GROWTH OF YEAST AND PREPARATION OF MEMBRANES

The strain of yeast used in immunological studies and the biogenesis of complex III was the haploid KL14-4A. This strain was also used as the parent-type along with the three diuron-resistant mutants, Diu^{r} -740, Diu^{r} -742 and Diu^{r} -752, in the study of these mutants.

Cells were grown aerobically at 30°C with 2-3% galactose as carbon source, under the conditions described previously (Brown and Beattie, 1977). The semisynthetic growth medium (Schatz and Kovac, 1974) contained 0.2-0.3% yeast extract, 0.04% CaCl_2 , 0.05% NaCl , 0.07% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1% KH_2PO_4 , 0.12% $(\text{NH}_4)_2\text{SO}_4$ and 0.5mg% FeCl_3 .

Isolation of mitochondria by cell-breakage with glass beads: Yeast cells were harvested by centrifugation for 5 min at 5,000 xg, washed with distilled water, and suspended in STE buffer (0.25M sucrose, 0.02M Tris-HCl, 1mM EDTA and 1mM DMSF, pH 7.5). Cells were then broken by shaking with glass beads 2 times for 45 sec in a Bronwill shaker, as described by Kim and Beattie (1973). Small aliquots of cells were broken by placing them in Eppendorf tubes (1.5ml vol) containing glass beads and using an Eppendorf adaptor in the Bronwill shaker. The suspension was centrifuged 2 times for 5 min at 5,000 xg to remove unbroken cells and debris. After

sedimenting the membranes at 18,000 xg for 20 min, mitochondria were washed by suspension in a glass-glass homogenizer in STE buffer and centrifuged for 20 min at 18,000 xg.

Isolation of mitochondria from spheroplasts: Spheroplasts were prepared as described by Finzi et al(1981). Washed yeast cells were suspended in a buffer containing 20mM Tris-HCl, pH 9.0, 20mM EDTA, and 50mM 2-mercaptoethanol at a concentration of 1 g wet weight of cells/3 ml of buffer and incubated at 30°C for 15 min. The cells were harvested, washed three times with distilled water and suspended at a ratio of 1 g wet weight of cells/4 ml of buffer D (1M sorbitol, 20mM potassium phosphate, pH 7.4). Three mg of Osmolyase 5000 was added for each g of cells and the suspension incubated at 30°C for 60 min, by which time digestion was complete. The spheroplasts were harvested by centrifugation, washed once with Buffer D and carefully resuspended in ice-cold buffer E (0.6M mannitol, 1mM EDTA, 1mg/ml of bovine serum albumin, 10mM potassium phosphate, pH6.8) and lysed at low speed in a Waring Blendor for 25 s. The broken spheroplasts were then diluted 5 to 10 times with Buffer E and the cell debris removed by 3 centrifugations at 1,200 xg for 10 min. The supernatant was centrifuged for 10 min at 18,000 xg, washed once with a buffer and resuspended in the buffer.

Preparation of mitoplasts: Mitoplasts were prepared as described by Chen and Beattie (1981). Mitochondria,

isolated from spheroplasts as described above, were suspended in 0.6M mannitol, 10mM sodium phosphate, pH 7.0, at a protein concentration of 10mg/ml. A freshly prepared stock solution of 35mg/ml of recrystallized digitonin was added to the mitochondrial suspension to a final concentration of 3mg digitonin per 10mg mitochondrial protein. After vortexing the mixture gently for 1 min, the suspension was diluted with buffer and the mitoplasts were sedimented by a 10 min centrifugation at 12,000 xg. The mitoplasts were washed once and used directly for immunoinhibition studies, or were solubilized by adding 1% Lubrol WX and 2% cholate per 1.5mg of protein. The mixture was incubated at 4°C for 1 h and then centrifuged at 24,000xg for 30 min. The clear supernatant was used immediately for immunoinhibition studies.

Preparation of cytochrome c-depleted SMP: Mitochondria were suspended in 0.1M sodium phosphate, pH 7.5, 0.5mM EDTA and 1mM PMSF, and sonicated for 1.5 min in 2 equal bursts, at a power setting of 4.5. The suspension was centrifuged for 10 min at 10,000 xg and the supernatant was centrifuged at 100,000 xg in a Spinco type 40 rotor. The submitochondrial particles (SMP) were resuspended in the sodium phosphate buffer.

9. ENZYME ASSAYS

Succinate dehydrogenase activity: was measured by following the reduction of 2,6-dichlorophenolindophenol, DCPIP, at 600 nm by succinate and phenazine methosulfate (PMS) in a reaction mediated by the enzyme (King, 1976).

Ubiquinol :cytochrome c reductase activity: was performed as described by Brown and Beattie (1977), using either sodium succinate (10mM) or the coenzyme Q analog, 6-decyl,2,3-dimethoxy, 5-methyl,1,4-benzoquinone, QBH_2 , (25mg/ml), in 25mM sodium phosphate buffer, pH 7.6. The coenzyme Q analog was reduced by the method of Rieske (1976) and added as an ethanolic solution. For the inhibition studies, varying concentrations of antimycin A (in ethanol), UHDBT (also in ethanol) and diuron (methanolic solution) were preincubated with the enzyme prior to the assay.

The rate of cytochrome c reduction was monitored by following the increase in absorption at 550 nm. An extinction coefficient of $18.5 \text{ cm}^{-1} \text{ cm}^{-1}$ was used to calculate the rate of nmol of cytochrome c reduced per minute. In order to study the activity of purified complex III as a function of pH, the pH of the assay medium was varied from 6.0 to 8.0.

For studies involving the diuron-resistant mutants, intact mitochondria and cytochrome c -depleted SMP were used or solubilized in cholate (0.5mg per mg of protein). Values

of K_m and V_{max} were calculated from Eadie-Hofstee plots (Segel, 1975).

For the immunoinhibition studies, 15 μ g of either the intact mitoplasts, cholate-solubilized mitoplasts or intact submitochondrial particles were incubated with varying amounts of IgG, obtained from antisera against core protein I, core protein II, iron-sulfur protein and complex III. Control experiments contained IgG obtained from pre-immune serum. After incubation at room temperature for 45 min, DEH_2 or succinate:cytochrome c reductase activity was determined. In order to compensate for inhibition by the control IgG, enzyme activity was calculated as the activity obtained in the presence of immune IgG relative to the activity obtained in the presence of an identical amount of pre-immune IgG.

All the enzyme assays were performed at room temperature.

10. ANALYTICAL METHODS

Spectral determinations: A Perkin-Elmer double beam spectrophotometer (model 557) was used for spectral analysis at room temperature. A slit width of 2 nm was used and spectra were scanned at 120 nm/min. Difference spectra were obtained by scanning the dithionite-reduced sample against the ferricyanide-oxidized reference. Cytochrome content was determined using extinction coefficients of 12, 25.6 and 20.9 mM/cm for cytochromes a, b and c₁, respectively (Van Gelder, 1978).

The extra reduction of cytochrome b was studied in cytochrome c-depleted SMF of the diuron mutants and the wild-type, as described by Roberts et al (1980). SMF were suspended in 0.1M phosphate buffer, pH 7.5, at a protein concentration of 1.5-2 mg/ml and the following spectra were recorded: 1) Succinate-reducible: 15 min after the addition of 60 mM succinate the spectrum was recorded; 2) Succinate-reducible in the presence of either diuron or antimycin: 2 min after the addition of the inhibitor, 60mM succinate was added and the spectrum was recorded after 15 min; 3) Total dithionite reducible: the spectrum was recorded 2 min after the addition of a few grains of dithionite to the sample cuvette.

Protein determinations: were made by the method of Lowry (1951), using bovine serum albumin as the standard. To minimize interference by Triton X-100 that could lead to an overestimation of protein content, whenever proteins were dissolved in buffers

containing this detergent, all determinations were modified as described previously (Dulley and Grieve, 1975).

Essentially, a 10% SDS solution was added to all the test tubes containing the protein standard and the Triton-solubilized protein sample. The assay was then performed as described by Lowry (1951), except that just prior to determining the absorbance of the solutions, the solutions were centrifuged at 5,000 xg in order to remove the yellow precipitate caused by a combination of the SDS and Triton.

11. MATERIALS

Commercially grown bakers' yeast (Red Star) was obtained from Universal Corporation. Ultrogel ACA 34 was purchased from LKB. Sodium cholate and sodium deoxycholate were from Calbiochem. Antimycin A, Triton X-100, digitonin, Lubrol WX, sodium dodecyl sulfate, trypsin inhibitor, cycloheximide and chloramphenicol were obtained from Sigma. Trypsin and Nymolyase 5000 were from Miles Laboratories Inc. α -Chymotrypsin was a Worthington product. L-(^{35}S)methionine ($1000 \text{ Ci.mmol}^{-1}$), L-(4,5- ^3H)leucine (55 Ci.mmol^{-1}) and carrier free (^{35}S)sulfate ($1,000 \text{ Ci.mol}^{-1}$) were purchased from Amersham. Protein A-Sepharose was from Pharmacia. DBH_2 and UHDET were gifts from Dr. B. Trumpower (Dartmouth), while diuron was a gift from Dr. A. Goffeau (University of Louvain). All other reagents were of the highest purity commercially available.

The yeast strains used in this study, the wild-type K114-4A and the three Diu^{R} mutants, were a generous gift of Dr. A. Goffeau (University of Louvain).

CHAPTERS III AND IV: RESULTS AND DISCUSSION

1. Purification and Characterization of
Complex III
2. Immunological Characterization of
Complex III
3. Localization of the Subunits of
Complex III
4. Biogenesis of Complex III
5. Biochemical Analysis of Diuron-
Resistant Mutants of Yeast

1. PURIFICATION AND CHARACTERIZATION OF COMPLEX III

Purification of complex III: As seen in Fig. 2, the purification of complex III from bakers' yeast involved several steps, the most crucial of which was the ammonium sulfate precipitation of the crude complex III. This fraction contained mainly complex II, complex III and a small amount of complex IV. Complex IV precipitated readily out of solution between 45 to 40% ammonium sulfate saturation. However, the removal of complex II from complex III was very difficult. Complex II was extremely sensitive to ammonium sulfate in the saturation range of 54 to 57%, and increments of even 0.3% in salt saturation were sufficient to precipitate this complex. Unfortunately, complex III also started to precipitate out of solution at ammonium sulfate saturations of 55 to 57%. Hence, an intricate balance had to be maintained such that the ammonium sulfate added to the solution would precipitate all of the complex II and not complex III. To this end, it was essential to use a 100% saturated solution of ammonium sulfate instead of the solid sulfate suggested by Siedow et al (1978), in the fractionation of crude complex III. Improved results were also obtained when the volume of the crude complex III pellet (precipitated out of solution at 55% sulfate saturation) was calculated, and assumed to have a salt saturation of 55% prior to

refractionation. All salt fractionation steps were performed at 4 °C and this temperature was strictly maintained, since an increase in temperature of the solution by even a few degrees could cause a variation in the ammonium sulfate saturation by several percent.

Thus, the salt fractionation method of Siedow et al (1978), with modifications, resulted in a relatively pure form of the complex completely free of both succinate dehydrogenase activity and other cytochromes (King, 1967). The complex, however, could be further purified 1.5 times by gel filtration on an Ultrogel ACA 34 column, under nonreducing conditions, in the presence of Triton X-100. Fig. 4 illustrates a typical elution profile of complex III, monitored by measuring the absorbance at 420 nm due to the Soret bands of the cytochromes. These fractions also contained a DBH₂:cytochrome c reductase activity (data not shown). The fractions were concentrated using Diaflo XM 300 ultrafilters, which also removed excess Triton X-100 micelles. Complex III, frozen in small aliquots at -70 °C, was stable for over 6 months. The protein content of the purified and concentrated complex III was determined, using the modified Lowry (Dulley and Grieve, 1975). A typical yield of 150 to 250 mg of the purified complex was obtained from 25 lbs of yeast.

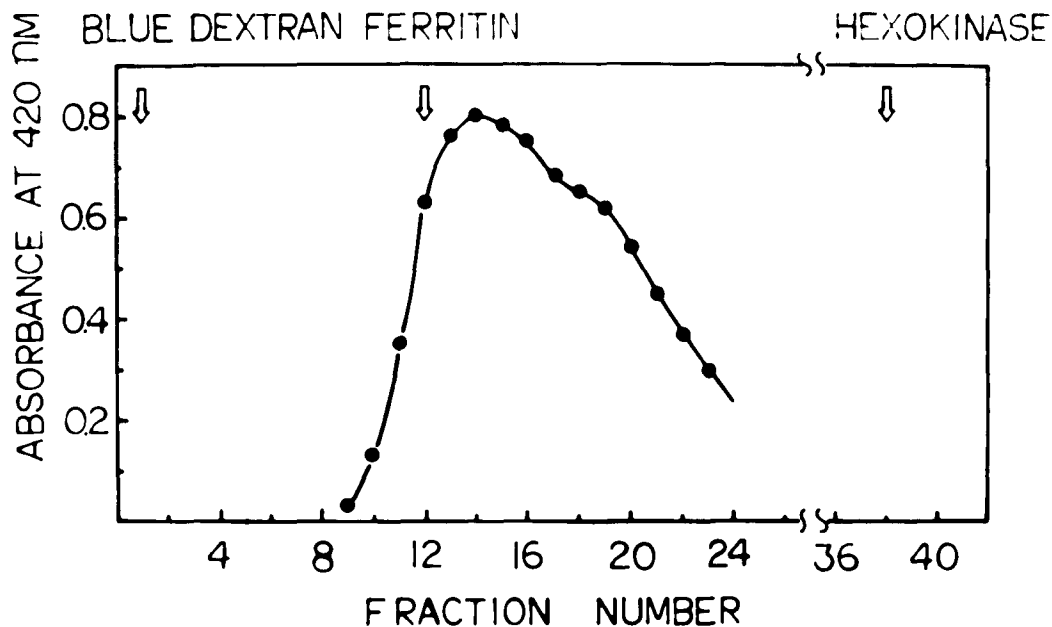


Fig. 4: Elution profile of complex III from Ultrogel AcA 34 column. Approximately 15 mg of complex III were dissolved in buffer B, as described under "Experimental Procedures", and loaded twice on an Ultrogel AcA 34 column (1.2 x 50 cm), pre-equilibrated with the same buffer. The columns were run at 6 ml/h at 4 °C. Fractions (1 ml) were collected and the elution profile was monitored by measuring the absorbance of the Soret bands at 420 nm. Marker proteins used were blue dextran (void volume), ferritin ($M_r = 450,000$), and hexokinase ($M_r = 102,000$).

Spectral and enzymic properties of complex III:

Difference spectra of the purified complex (Fig.5) showed the presence of only cytochrome b (α -band at 562 nm and β -band at 532 nm) and cytochrome c₁ (α -band at 554 nm and β -band at 523 nm). From this, the cytochrome content of the complex was determined and found to contain 7.03 nmol/mg of protein and 4.24 nmol/mg of protein of cytochromes b and c₁, respectively (Table II). The enzymic activity of complex III (diluted to 1 mg/ml) as a function of pH, showed a maximum value of 14.9 μ mol/min/mg of protein at pH 7.5 (Fig.6). At low pH and at pH = 8, the activity was decreased, probably due to the instability of the complex at these values of pH. Subsequent enzyme assays of the undiluted complex III (26mg/ml), conducted in an assay mixture having a pH of 7.4, resulted in a maximum enzyme activity of 17.1 μ mol/min/mg of protein. This suggested that some enzyme activity, probably due to a destabilization of the complex, was lost upon dilution. Several attempts were made to stabilize the complex during dilution, such as the inclusion of glycerol or bovine serum albumin in the dilution buffer, without success. Thus, calculations of enzyme activity and heme content indicated a 52- to 56-fold purification of complex III from mitochondria (Table II).

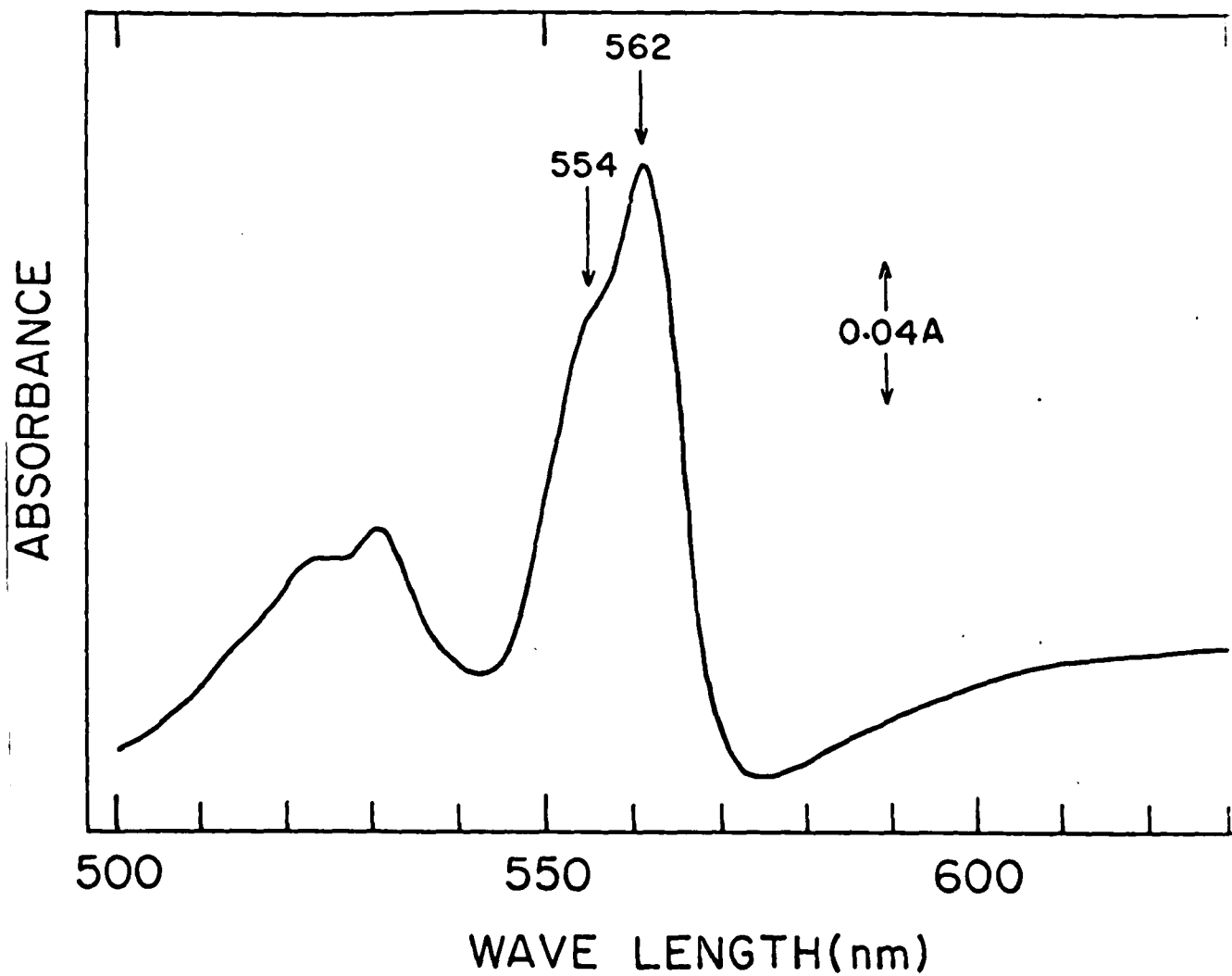


Fig. 5: Difference spectra of purified complex III. Complex III was diluted to 1 mg/ml in Tris-acetate buffer, pH 7.4, and the dithionite-reduced versus ferricyanide-oxidized spectrum was recorded at room temperature.

TABLE II

Purification of Complex III

The cytochrome content and DBH_2 :cytochrome c reductase activity of purified, twice chromatographed complex III was compared in mitochondria and submitochondrial particles, as described under "Experimental Procedures".

Fraction	Cyto- chrome <u>b</u> ^a	Cyto- chrome <u>c</u> ₁ ^a	Enzyme activity ^b	Fold purification of complex III	
				Cyto- chrome <u>b</u>	enzyme activity
Mitochondria	0.125		0.39		
Submitochon- drial parti- cles	0.289		0.357		
Purified com- plex III	7.03	4.24	17.1	56 X	52 X

^a Nanomoles/mg of protein.

^b Micromoles/min/mg of protein.

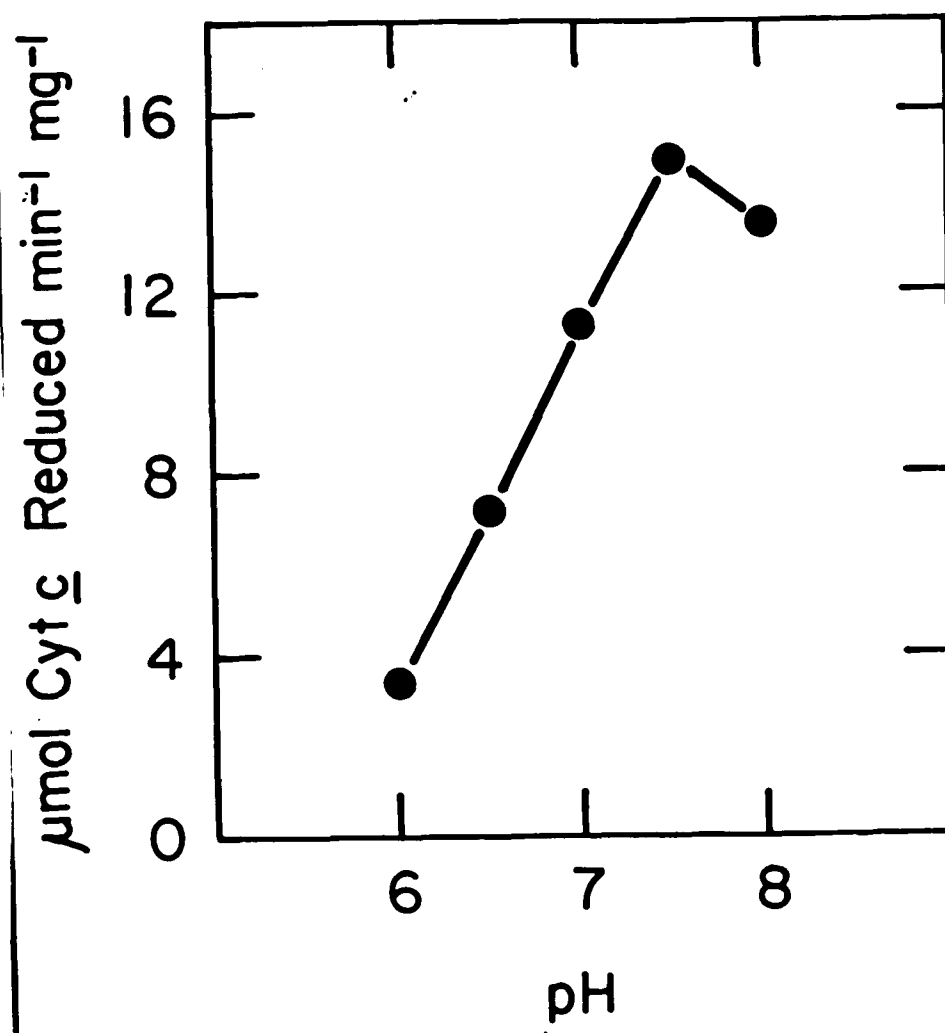


Fig.6 : Enzyme activity of complex III as a function of pH. Complex III was diluted to 1 mg/ml in Tris-acetate buffer, pH 7.4, and DBH_2 :cytochrome c reductase activity was determined as described under "Experimental Procedures", except that the pH of the assay medium was varied between 6.0 to 8.0.

The enzymic activity of complex III was sensitive to inhibitors of the cytochrome b-c₁ segment of the respiratory chain, antimycin A and UHDBT, which inhibited over 95% of the activity. A 50% inhibition value at 0.75 mol UHDBT per mol of cytochrome c₁ was obtained (Fig.7).

Subunit composition of complex III:The column chromatography step resulted in the removal of several polypeptides from complex III without loss of enzymic activity or heme content, suggesting that these proteins were contaminants. SDS-gel electrophoresis in 12.5% acrylamide gels revealed the presence of 10-15 bands in complex III prior to column chromatography (Fig.8, lane C). All the observed polypeptides with a molecular weight greater than 50,000 plus those polypeptides having molecular weights of 22,000, 18,000, and 15,000 were removed by column chromatography. The purified complex contained seven different polypeptides with molecular weights of 49,000 (core protein I), 39,000 (core protein II), 30,000 (cytochrome b apoprotein), 29,000 (cytochrome c₁), 24,500 (iron-sulfur protein), 17,500 and 15,500 (Fig.8, lane A and Table III). A few other proteins can also be seen in this gel, but none of these proteins were present in stoichiometric amounts. It was necessary to make these molecular weight determinations in gels of 12.5% acrylamide, since Ferguson plots of the complex in gels of varying acrylamide concentrations indicated that cytochrome b

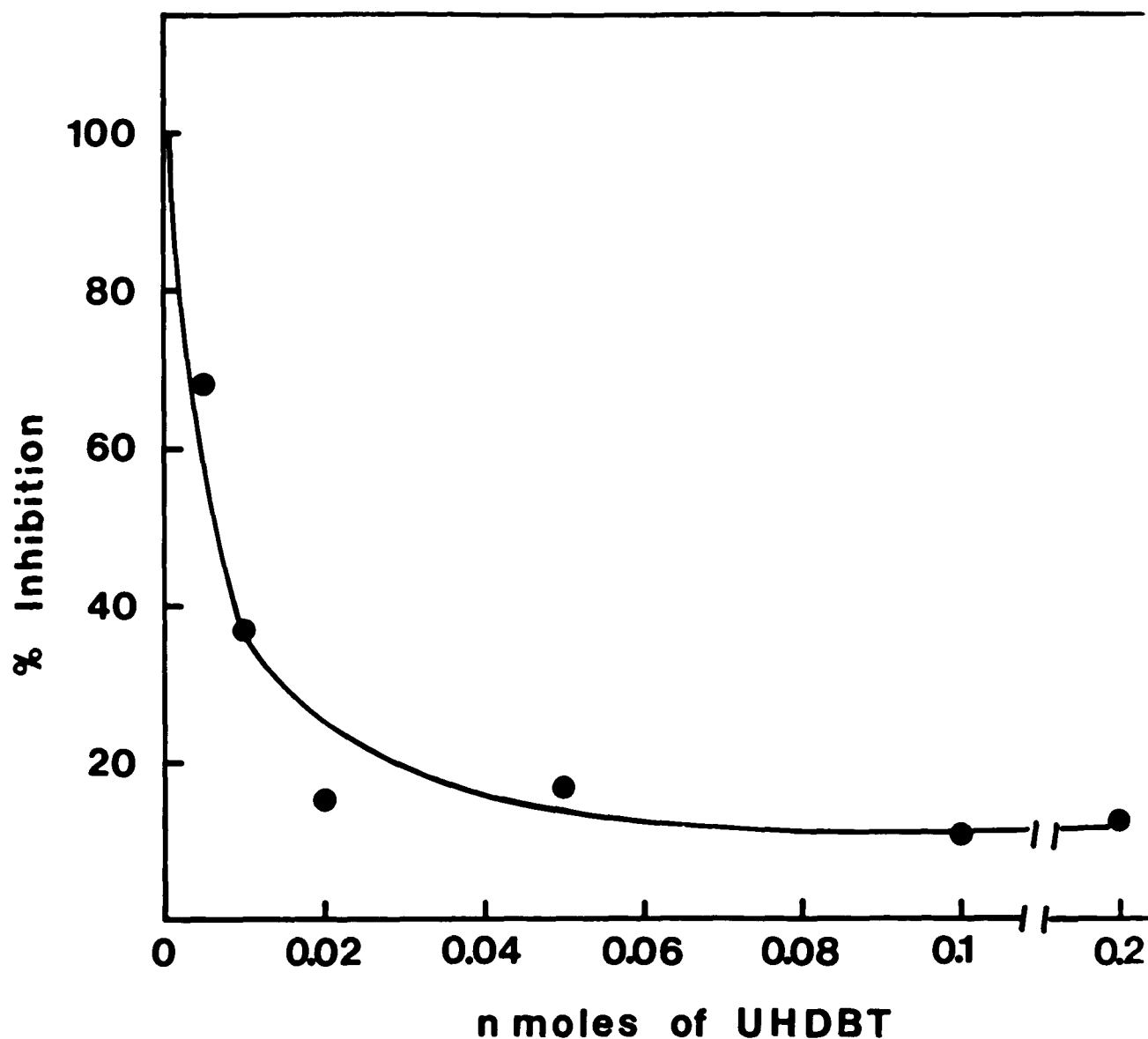


Fig.7: Inhibition of DBH₂:cytochrome c reductase activity by UHDBT in isolated complex III. Varying concentrations of UHDBT (ethanolic solution) were added to the assay mixture, and the DBH₂:cytochrome c reductase activity was assayed upon the addition of the enzyme to the assay mixture. The assay was performed as described under "Experimental Procedures".

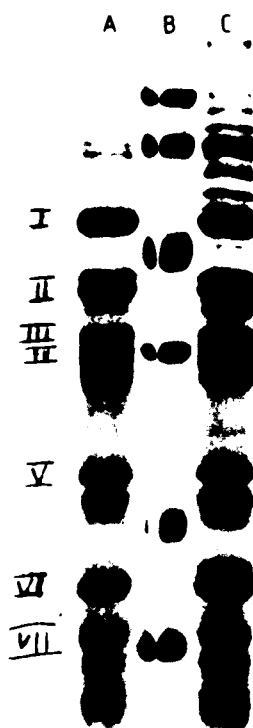


Fig. 8: SDS-PAGE of complex III before and after chromatography on Ultrogel AcA 34 columns. Samples of complex III were dissociated overnight in the presence of 1mM TPCK, 1mM PMSF and 1mM TLCK, as described under "Experimental Procedures", and electrophoresis was performed on a 12.5% gel. A, twice chromatographed complex III; B, molecular weight standard proteins; C, complex III prior to column chromatography. Subunits are numbered from largest to smallest molecular weights. The molecular weight standards used were phosphorylase b, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,100; and α -lactalbumin, 14,400.

TABLE III

Subunit Composition of Complex III

Electrophoresis of complex III on 12.5% acrylamide gels, using the SDS-Tris or SDS-8 M urea buffer systems was performed as described under "Experimental Procedures". The gels were stained, destained and scanned at 590 nm. From the areas under the peaks, the amount of each subunit present was calculated relative to one cytochrome c_1 . The pI values, obtained from isoelectric focusing, represent the average of seven such experiments.

Subunit Number	Identity of Subunit	<u>Molecular Weight</u>		Stoichio- metry	pI
		SDS-Tris	SDS-Urea		
I	Core Protein I	49,000	50,000	1.42 $\pm$ 0.32	6.53
II	Core Protein II	39,000	41,000	0.83 $\pm$ 0.31	6.37
III	Cytochrome b	30,000	30,000	0.68 $\pm$ 0.08	8.30
IV	Cytochrome c_1	29,000	30,000	1	6.45
V	ISP	24,500	24,250	0.67 $\pm$ 0.13	6.57
VI		17,500	17,700	0.94 $\pm$ 0.30	6.20
VII		15,500	15,500	0.83 $\pm$ 0.56	7.15

migrates anomalously (Fig.9). In gels having an acrylamide concentration $\leq 10\%$, the cytochrome b apoprotein migrated faster than that of cytochrome c₁, while at acrylamide concentrations $> 12.5\%$, the apoprotein of cytochrome b migrated more slowly than that of cytochrome c₁. At an acrylamide concentration of 11%, the migration of cytochrome b apoprotein was identical with that of cytochrome c₁ and the two polypeptides could not be resolved. None of the other subunits of complex III (core protein I, core protein II, cytochrome c₁ and the iron-sulfur protein) showed a similar anomalous migration pattern.

Similar molecular weight determinations were calculated for the polypeptides of complex III after electrophoresis in a 12.5% acrylamide, 8M urea gel (Fig.10 and Table III). On the SDS-urea gel, the molecular weights of the subunits increased slightly or remained the same. The SDS-urea gel, however, did not resolve the cytochrome b apoprotein from cytochrome c₁, and instead, a single band was observed with an apparent molecular weight of 30,000.

In recent years, several laboratories have reported the presence of polypeptides in complex III having molecular weights lower than 15,000 (Hatefi, 1978; Bell et al, 1979; Beattie et al, 1980; Engel et al, 1980; Gellerfors et al, 1981). Prior to column chromatography, our preparation of complex III also contained low molecular weight proteins

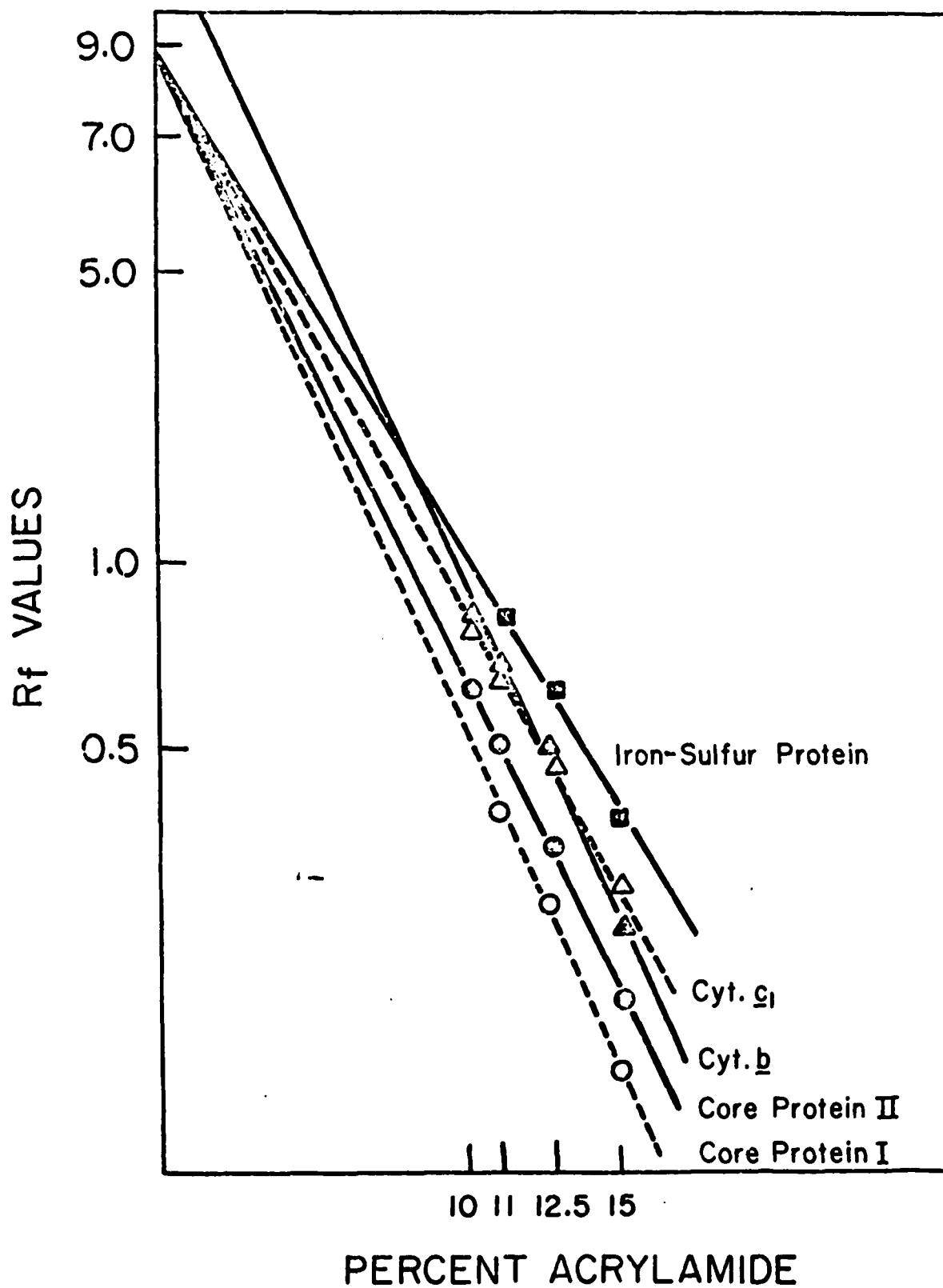


Fig. 9: Ferguson plot of the subunits of complex III. Core protein I (○---○), core protein II (●—●), cytochrome b (▲—▲), cytochrome c_1 (△---△), iron-sulfur protein (■—■).

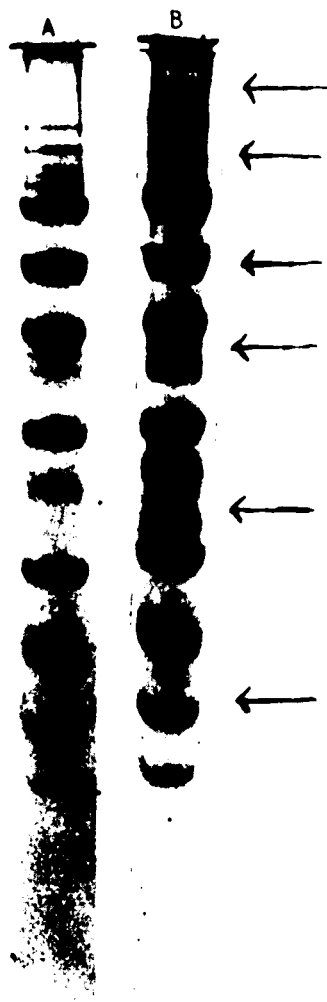


Fig. 10: SDS-8M urea gel electrophoresis of complex III before and after chromatography on Ultrogel Aca 34 columns. Samples of complex III were dissociated overnight in the presence of protease inhibitors, and electrophoresis was performed on a 12.5% polyacrylamide gel, using the SDS-8M urea system described under "Experimental Procedures". A, twice chromatographed complex III; B, complex III prior to column chromatography. Arrows denote molecular weight standards having molecular weights identical to that in Fig.8.

plus a polypeptide with a molecular weight of 22,000 (Fig. 8, lane C). Analysis of the complex on SDS-8M urea gels failed to indicate the presence of stoichiometric amounts of polypeptides with molecular weights less than 15,000 (Fig. 10, lane 1). In addition, low molecular weight polypeptides were sometimes detected in some gels of purified complex III and were absent in other gels. This suggested that some of the proposed low molecular weight constituents of complex III arise as a result of proteolysis during dissociation of the complex prior to electrophoresis.

To test this suggestion, the most purified preparation of complex III was dissociated overnight in the presence or absence of protease inhibitors, PMSF, TPCK and TLCK, and analyzed on 12.5% SDS-polyacrylamide gels. The gel was stained, destained and scanned in a Canalco microdensitometer at 590 nm. As seen in the scan of the gels in Fig. 11, the relative amounts of the polypeptides of molecular weights 22,000, 14,000, and 12,000 (indicated by arrows) increased dramatically when the dissociation was performed in the absence of the protease inhibitors. As a result of this finding, all subsequent purifications of complex III and its subunits were performed in the presence of 0.2 to 1mM PMSF, even when these procedures called for the explicit omission of the protease inhibitor (Siedow et al, 1978). Additionally, the dissociation of all samples prior to

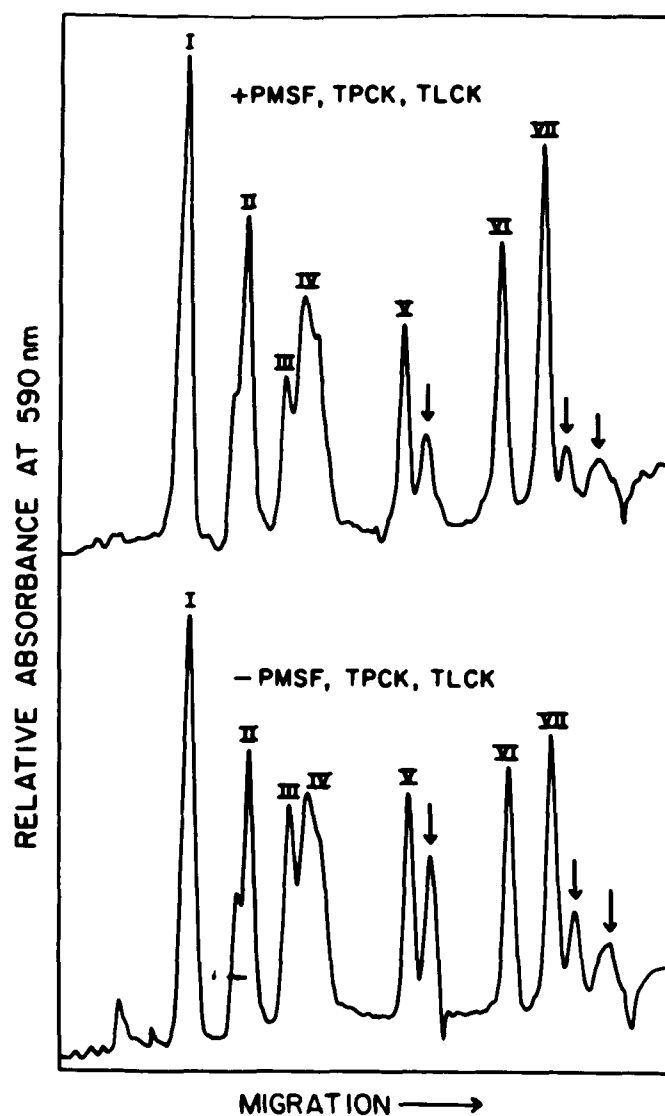


Fig. 11: Dissociation of complex III in the presence and absence of proteolytic inhibitors. Complex III (50 μ g) was dissociated overnight in the presence and absence of 1mM PMSF, 1mM TPCK, and 1mM TLCK, and electrophoresis was performed on 12.5% gels, as described under "Experimental Procedures." The gels were stained, destained, and scanned at 590 nm. Top, complex III dissociated in the presence of proteolytic inhibitors; bottom, complex III dissociated in the absence of proteolytic inhibitors. Arrows indicate contaminating polypeptides of $M_r = 22,000$; $14,000$, and $12,000$.

electrophoresis was performed by including 1mM PMSF, 1mM TPCK and 1mM TLCK in the dissociation buffer.

Stoichiometry and molecular weight of complex III:The stoichiometry of the individual subunits of complex III was calculated from a Coomassie blue-stained gel. Different amounts of complex III (30 to 100 μ g) were loaded onto a 12.5% acrylamide gel to ensure sufficient staining. The stained and destained gels were subsequently scanned in a Canalco microdensitometer. The relative amounts of each subunit were determined by measuring the area under the curve for each subunit and calculating concentrations relative to 1 mol of cytochrome c_1 /mol of complex III monomer. The results indicated that complex III of yeast mitochondria contains 2 mol of subunit I (core protein I) and 1 mol of each of the remaining six subunits (Table III). Using this stoichiometry plus the molecular weight of each subunit for the purpose of calculation, the molecular weight of the complex III monomer was determined to be 253,000. Similarly a minimum molecular weight of 238,000 can be calculated for complex III from the heme content of cytochrome c_1 . The complex, however, may exist as a dimer during purification, since it eluted from the Ultrogel column with an apparent molecular weight of 450,000.

Isoelectric focusing:The isoelectric points of the various subunits of complex III were determined by two-

dimensional electrophoresis. Isoelectric focusing in the presence of 8M urea in the first dimension was followed by SDS-polyacrylamide gel electrophoresis in the second dimension to identify the subunits by their determined molecular weights (Fig.12). To prevent aggregation, it was also necessary to add 2 mg of SDS/mg of complex III in the dissociation buffer prior to the isoelectric focusing (Stephenson et al,1980).The pI values for the individual subunits range from 6.2 to 8.3 (Table III). It should be noted that subunits VI and VII could not always be clearly resolved in the second dimension. The observed pI values for cytochrome b and core protein I agree well with those previously reported (Stephenson et al, 1980; Mendel-Hartvig and Nelson, 1981); however,our value for core protein II is considerably more acidic. This difference may have occurred because this other group (Mendel-Hartvig and Nelson, 1981) used the isolated protein rather than intact complex III for determination of the pI.

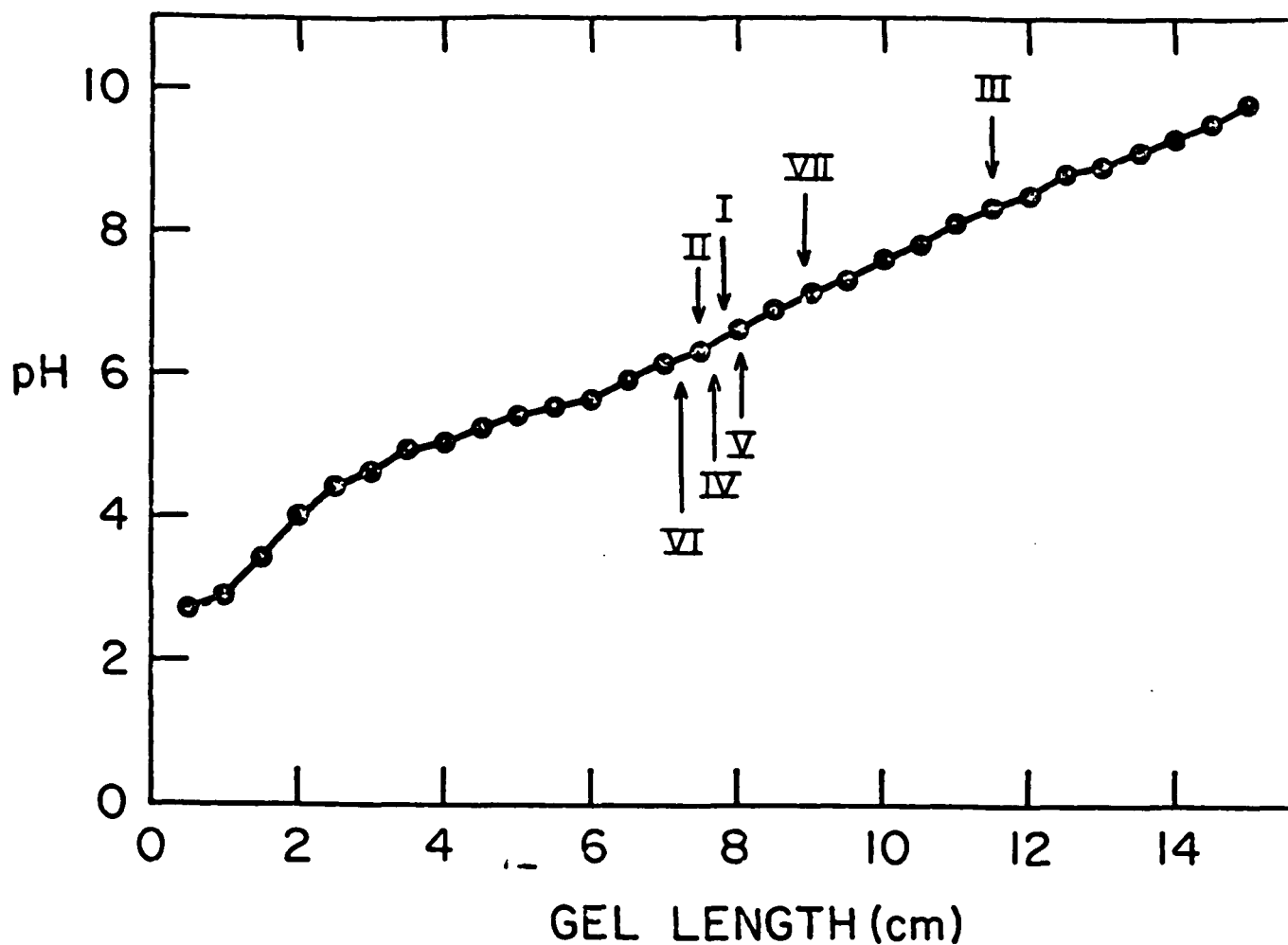


Fig. 12: Isoelectric focusing of complex III. Isoelectric focusing in the first dimension was performed as described under "Experimental Procedures", using 2% ampholines, pH 3.5 to 10. The pH gradient of the gel was determined and the distance migrated by the subunits was noted. The subunits were identified by SDS-gel electrophoresis in the second dimension, and are numbered according to the legend in Fig. 8.

1. DISCUSSION

In the current study, the subunit composition of an enzymatically active complex III from yeast mitochondria has been determined. Contrary to recently published studies of complex III from beef heart (Marres and Slater, 1977; Hatefi, 1978; Bell et al, 1979; Engel et al, 1980), rat liver (Gellerfors et al, 1981) and N. crassa (Weiss and Kolb, 1979), where the complex was resolved into eight or nine polypeptides, complex III from yeast contained only seven subunits. Whereas other reports indicate the presence of several low molecular weight species (M_r values of 15,000 to 4,000) associated with complex III, our purified preparation of the enzyme showed a complete absence of polypeptides of molecular weight lower than 15,00. Electrophoresis on SDS-8M urea gels (Swank and Munkres, 1971) also failed to reveal any low molecular weight species. While some low molecular weight components were present in our impure preparation for yeast, the ease at which these proteins were removed upon gel filtration suggested that they were present as contaminants. Moreover, when protease inhibitors were not included in the dissociation buffer, there was a dramatic increase in the amounts of these same low molecular weight subunits. It should be noted that whenever low molecular weight polypeptides have been detected in various preparations by

different investigators, such protease inhibitors were not added to dissociation buffers. We have thus concluded that low molecular weight protein species are not an integral part of complex III in yeast mitochondria and that they arise as a result of proteolysis either during isolation of the complex or during dissociation of the complex prior to electrophoresis.

The molecular weight of the seven subunits of yeast complex III are in good agreement with the subunit molecular weights previously published (Hatefi, 1978; Engel et al, 1980; Beattie et al, 1980; Siedow et al, 1978; Gellerfors et al, 1981). Our assignment of subunit III ($M_r = 30,000$) as the apoprotein of cytochrome b is based on the observation that only subunit III of complex III showed an anomalous migration pattern on SDS-polyacrylamide gels (Marres and Slater, 1977; Chen and Beattie, 1982), an observation previously attributed to the hydrophobic nature of this polypeptide (Von Jagow and Sebald, 1980). In addition, when tall preparative gels of 10% acrylamide were used in the purification of the subunits, the red color due to cytochrome c₁ (subunit IV, $M_r = 29,000$) could be seen to migrate more slowly than subunit III (data not shown).

Cytochrome b, cytochrome c₁, and the iron-sulfur protein, have been extensively characterized (Lin and Beattie, 1978; Chen and Beattie, 1982; Von Jagow and Sebald, 1980). The

function of the two core proteins, the 17,500 and the 15,500 dalton subunits are unknown. The low molecular weight subunits have been alternately characterized as being associated with cytochromes b-c₁, as the ubiquinone-binding protein or the antimycin A-binding protein (Bell and Capaldi, 1976; Yu and Yu, 1980; DasGupta and Rieske, 1973).

The molar ratios of the subunits of complex III indicate the presence of 2 mol of core protein I and 1 mol each of the remaining subunits/mol of complex III, which is contrary to recently published reports (Marres and Slater, 1977; Hatefi, 1978; Engel et al, 1980; DasGupta and Rieske, 1973), where the molar ratios for these subunits range from 1 to 5. It is generally accepted that there are 2 mol of cytochrome b apoprotein/mol of complex III, based on heme content relative to that of cytochrome c₁. While we see only 1 mol of subunit III, this observation could have occurred because the apo-protein stained faintly with Coomassie blue. Furthermore, sometimes two species of cytochrome b are observed, one with an apparent molecular weight of 30,000 and the other with an apparent molecular weight of 28,500 (Figs. 8 and 11). The isolation of two forms of cytochrome b with these molecular weights has been recently reported (Chen and Beattie, 1982). Using the calculated stoichiometry and the molecular weight of the subunits, as well as the heme content of cytochrome c₁, minimum molecular weights of 253,000 and 238,000 for the complex III monomer were obtained, respectively.

In addition, yeast complex III eluted from the Ultogel column as a species of molecular weight of 450,000, indicating the existence of a dimer of the complex in solution. Similar hydrodynamic calculations from beef (Engel et al, 1980) and L. crassa (Weiss and Kolb, 1979) have indicated molecular weights of 440,000 and 550,000, respectively, for the isolated complex. In addition, x-ray crystallographic analysis suggests that complex III from Neurospora has a molecular weight of approximately 550,000 (Hovmoller et al, 1981).

2 . IMMUNOLOGICAL CHARACTERIZATION OF COMPLEX III

Purification of the subunits of complex III: Core protein I ($M_r = 49,000$), core protein II ($M_r = 39,000$), and the iron-sulfur protein ($M_r = 24,500$) were isolated and purified by electroelution of the proteins from polyacrylamide gels, followed by acetone-precipitation, as described under "Experimental Procedures". Analysis of the purified subunits on SDS gels revealed a single band corresponding to the purified polypeptides (Fig. 13A and 13B). Moreover, the molecular weight of all three purified polypeptides were identical with the values obtained for the complex III-associated polypeptides. While the purified iron-sulfur protein was highly soluble in 10mM sodium phosphate, pH 7, containing 0.2% SDS, the acetone-precipitated core proteins did not dissolve to any appreciable extent in the same buffer. Sonication of the resuspended core proteins for 10 s greatly improved their solubility.

Double immunodiffusion: The antisera raised against complex III and its purified subunits were tested for antigenicity by double immunodiffusion on agar gels, as described under "Experimental Procedures". Prior to staining the gels with Coomassie blue, it was essential to rinse these gels with for at least 3 days in order to remove any non-precipitated protein. Ochterlony double



Fig. 13: Electrophoretic analysis of the purified subunits of complex III. A, core protein I (lane 3), core protein II (lane 1), and B, the iron-sulfur protein (lane 2) were purified from complex III, as described under "Experimental Procedures." Approximately 4 μ g of each purified subunit were loaded on the gel. The molecular weight standard proteins are in lane 2 of A and in lane 1 of B.

diffusion tests revealed that the antiserum against the purified subunits reacted with its corresponding antigen, producing a single sharp line (Fig.14,A-C). The antiserum against each purified subunit did not cross-react with any other subunit. In the reaction between complex III and its antiserum, however, two precipitin lines can be seen (Fig.14D), suggesting the presence of two antigenic sites in the complex. The antisera against the purified subunits all cross-reacted with complex III, producing a single precipitin line, indicating that the antisera recognize the corresponding antigen present in complex III. In addition, this result suggests that the purified SDS-treated subunits retained the (an) antigenic site, present in complex III, in their native state. SDS treatment followed by boiling and sonication apparently did not affect the antigenic sites of the purified subunits. Control experiments using preimmune serum and purified proteins failed to give any precipitin lines under similar conditions.

Immunotitration of antisera: Prior to any immunoprecipitation experiment, the optimal antigen:antiserum ratio was determined. Yeast cells were grown overnight in the presence of (^3H)leucine and mitochondria were prepared. Mitochondria (10mg/ml), were then solubilized by several techniques: 1) 1% Lubrol WX and 1% SDS at 4 °C for 1 h (Lin et al,1978b), 2) 1M KCl and 1% Triton X-100 at 37 °C

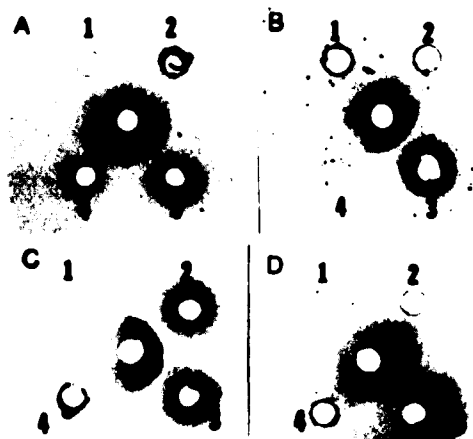


Fig. 14: Immunodiffusion of complex III and the purified subunits of complex III with various antisera. Antisera (10 μ l) to complex III and its purified subunits were placed in the central wells. A, anti-iron-sulfur protein; B, anti-core protein II; C, anti-core protein I; and D, anti-complex III. The antigens, dissolved in Buffer C containing 5% Triton X-100, were loaded in the peripheral wells. 1, 10 μ g of core protein II; 2, 15 μ g of core protein I; 3, 25 μ g of complex III; and 4, 10 μ g of iron-sulfur proteins. The plates were allowed to develop at room temperature for 48 h and then washed in several changes of Buffer C for 3 days prior to staining and destaining.

for 2 h (Bell et al, 1979), 3) 5% Triton X-100 and 0.1% SDS at 37 °C for 2 h (Nelson and Mendel-Hartvig, 1977). The solubilized extracts were then centrifuged at 24,000 xg for 1 h and the clear supernatant was used for immunoprecipitation. Unfortunately, none of these methods gave reproducible results. Even though complex III was detected spectrally in the solubilized extracts, the antiserum failed to recognize its antigenic determinant(s). Results were not improved when protein A-Sepharose was included to precipitate any immunocomplex.

Thus after trial and error, the best results were obtained when a much more dilute mitochondrial suspension (1.5mg/ml) was solubilized with 1% Triton X-100 (Werner and Sebald, 1981) and then centrifuged at higher speeds, 100,000 xg for 45 min. A constant aliquot of the solubilized material was incubated with 0-200 µl of the various antisera and the immunocomplex precipitated by addition of protein A-Sepharose. As seen in Fig.15, the complex III antiserum precipitated the highest number of counts while the antiserum against the two core proteins precipitated slightly more counts than did the control serum.

SDS-gel analysis of immunoprecipitates: An inexpensive way to label cells is by using (³⁵S)sulfate. Thus, the method of Douglas et al (1979) was used whereby a yeast culture (in mid-log phase) was labeled in LSM for 1 h with 200 µCi of (³⁵S)sulfate per ml. This method, however, gave a very low incorporation of the label, 200-400 cpm/min/µg of

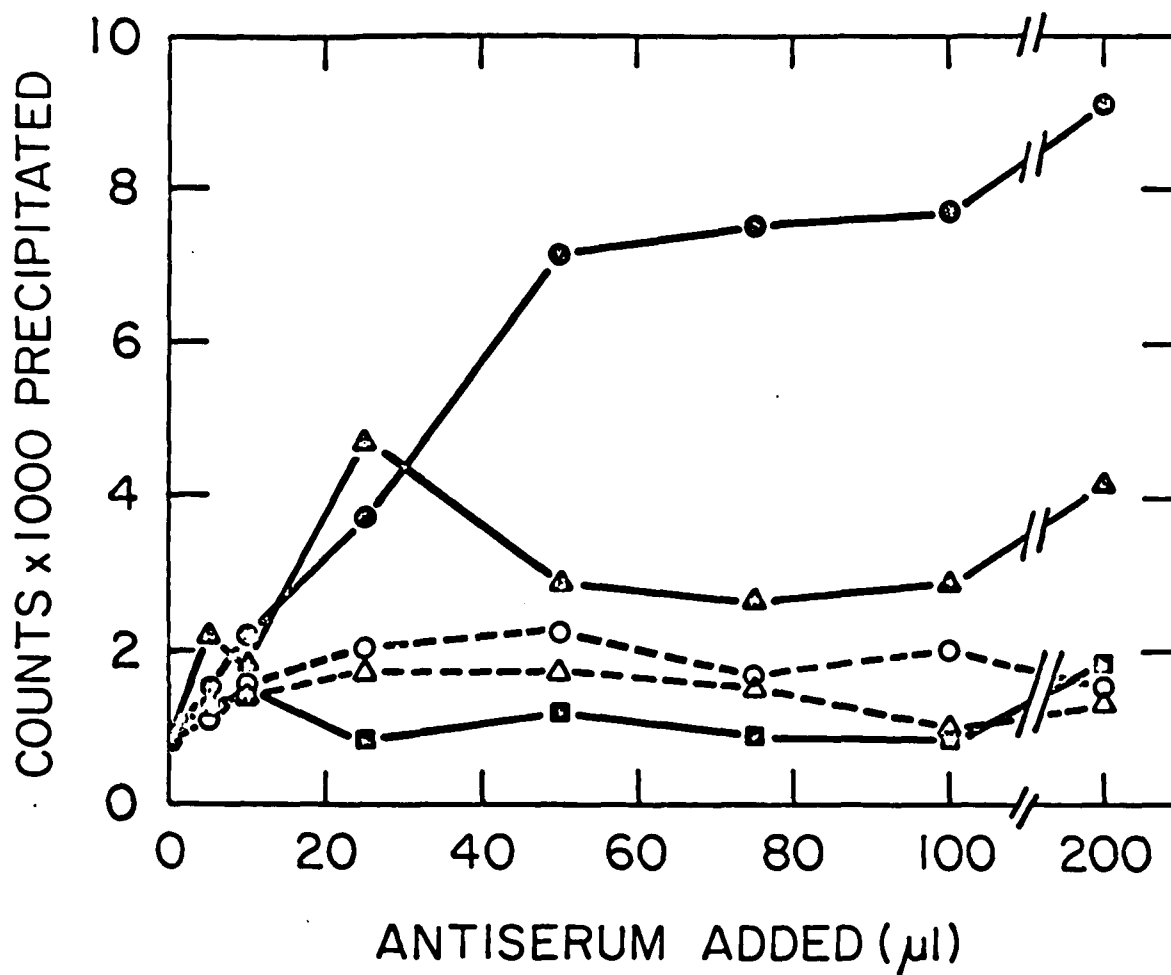


Fig. 15 : Immunotitration of the various antisera. Mitochondria were isolated from yeast cells grown overnight in the presence of 5 mCi/l of (³H)leucine, and solubilized with 1% Triton X-100, as described under "Experimental Procedures". The solubilized mitochondrial protein (112 μg) was incubated with 0 to 200 μl of either antisera against core protein I (●—●), core protein II (△---△), iron-sulfur protein (▲---▲), complex III (●—●), or with control sera (■—■).

mitochondrial protein, as determined by TCA-precipitable counts (Mans and Novelli, 1961). It was then necessary to modify this method by first preincubating the cells for 1 h in the low sulfate medium (LSM) prior to labeling. This step greatly increased the incorporation of (^{35}S)sulfate in mitochondrial protein by 5-8 fold. This result suggested that the preincubation step depleted the endogenous sulfate levels in the cells. In order to test the optimal time required for labeling, cells were labeled for 4 h and at various time intervals, aliquots were removed and the label incorporated was determined (Fig. 16). The incorporation of the label closely parallels the growth curve of yeast cells (doubling time = 1.5 h), indicating that the cells were labeled under growing conditions. Although a maximum incorporation of the label was obtained after 4 h, for the sake of convenience, a labeling time of 2 h was subsequently used, whereby 6000-7000 cpm/min were incorporated per μg of mitochondrial protein.

Thus, yeast cells were labeled with (^{35}S)sulfate, mitochondria prepared and solubilized in 1% Triton, and the solubilized extracts were subjected to immunoprecipitation using an optimal amount of all four antisera. The immunoprecipitates were analyzed on 12.5% polyacrylamide gels by fluorography (Fig. 17). The polypeptide composition of the immunoprecipitate obtained by using complex III

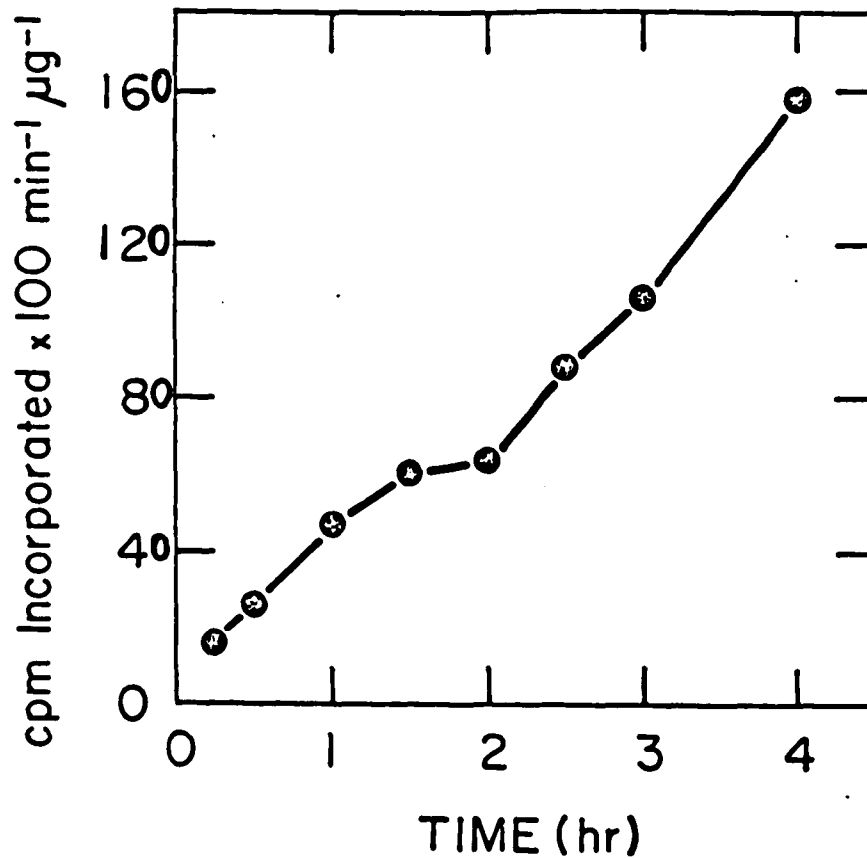


Fig. 16: Incorporation of (³⁵S)sulfate into mitochondria. Yeast cells were labelled in vivo, in low sulfate medium, using 200 µCi/ml of (³⁵S)sulfate and mitochondria were prepared, as described under "Experimental Procedures". A constant aliquot of mitochondrial protein was placed on paper filter disks, and the TCA-precipitable counts were determined, according to the method of Mans and Novelli(1961).



Fig. 17: Autoradiogram of immunoprecipitates from yeast cells labeled in vivo with (^{35}S)sulfate. Yeast cells were labeled for 2 h with 200-400 μCi of the label per ml of low sulfate medium. Mitochondria were isolated, solubilized in 1% Triton and subjected to immunoprecipitation using antisera against complex III (lane 3), core protein II (lane 4), core protein I. (lane 5) and iron-sulfur protein (lane 6). Lanes 1 and 2 contained the molecular weight standard proteins and immunoprecipitate obtained by using control serum, respectively. The immunoprecipitates were analyzed on a 12.5% polyacrylamide gel, followed by fluorography.

antiserum (Fig. 17, lane 3), is almost identical to that of the purified complex III (Fig. 8, lane A). Thus the immunoprecipitate contained several polypeptides with molecular weights of 47,000, 29,000, 24,000, 17,000 and 15,000. These molecular weights correspond very well to those of core protein I (49,000), cytochromes $\underline{b}-\underline{c}_1$ (30,000 and 29,000), iron-sulfur protein (24,500), the 17,500 and the 15,500 subunits, respectively. In this autoradiogram, cytochrome $\underline{c}_1$ and the apoprotein of cytochrome $\underline{b}$ were not clearly resolved, and instead a single broad band at 29,000 is seen. There are also a few contaminating proteins seen in the gel with molecular weights of 20,000, 25,000 and $> 52,000$. Subunits with molecular weights $< 15,000$ were not detected on the gel, again suggesting the possible absence of these proteins in complex III. Surprisingly, core protein II could not be detected on the gel, and even the antiserum against core protein II (lane 4) failed to reveal the presence of this polypeptide. Core protein II was also absent in the immunoprecipitates obtained from anti-core protein I antiserum (lane 5) and anti-iron-sulfur protein (lane 6). Core protein I antiserum immunoprecipitated 2 major bands: at 47,000 (core protein I) and at 29,000 (cytochromes $\underline{b}-\underline{c}_1$), along with several minor bands corresponding to the various subunits of complex III. ISP antiserum precipitated cytochromes $\underline{b}-\underline{c}_1$, a minor band at

25,000 and a major band at 24,000.

Immunoprecipitation of (^3H) labeled cells using core II antiserum: The absence of core protein II in the immunoprecipitates obtained in Fig. 17 was surprising, especially in view of the fact that antisera against core protein II was shown to be antigenic to core protein II and complex III, by double diffusion tests (Fig. 15B). This at once suggested several possibilities: 1. A non-optimal ratio of antigen:antibody was used, 2. Solubilization of the mitochondria was such that the antibody did not recognize the antigenic site(s), 3. The antiserum was not antigenic and that the results obtained by the Ouchterlony tests were an artifact, 4. Core protein II was not an integral part of the complex and that it cofractionated with the complex during purification as a contaminant. Several different experiments were then performed where antigen:antibody ratio and the solubilization of the mitochondria were varied. All these experiments, however, again failed to reveal the presence of core protein. Indeed, it seemed as if suggestions 3. and 4. provided a good explanation for the absence of core protein II, especially as it had been suggested by Jeffrey et al (1979) that core proteins were not part of the complex and that they were present only as an artifact during isolation of the enzyme.

Finally, at a much later date, during the studies involving the biogenesis of complex III whereby yeast cells

were double-labeled with (^3H)leucine and (^{35}S)methionine (Fig.30), it was seen that complex III did indeed immunoprecipitate core protein II as part of the complex, but only when cells were labeled with (^3H)leucine could the core protein II be seen. When cells were labeled with (^{35}S)-methionine, this label did not get incorporated into the 39,000 dalton polypeptide and consequently core protein II was not seen. The possibility that core protein II did not contain methionine or cysteine residues was not seriously considered, since amino acid analyses from several laboratories had previously shown that mixtures of core proteins I and II from beef heart mitochondria (Silman et al, 1967; Bell and Capaldi, 1976) contained between 1-1.8 mol of methionine per 100 mol of amino acid residues. Similar studies from yeast mitochondria using core protein II (Katan et al, 1976a) showed this protein to contain 1.5 mol methionine/100 mol of amino acids. In addition, several groups (Katan et al, 1976b; Beattie et al, 1980) had shown that this protein does get labeled with (^{35}S)methionine. Moreover, it seemed unlikely that a polypeptide with a molecular weight of 39,000 daltons would lack methionine or cysteine residues.

Yeast cells were then subsequently pulsed with 400 μCi of (^3H)leucine/ml for 1 h and the cells were denatured with

20% TCA. The denatured cellular proteins were boiled for 5 min in 4% SDS buffer, and a solubilized extract obtained as described under "Experimental Procedures". The immunoprecipitate obtained using core protein II antiserum was analyzed on a 12.5% gel. Soaking the gel in 1M sodium salicylate prior to flourography, enhanced the radioactivity, making it possible to detect the weak β -rays of the ^3H label on a X-ray film. Thus, Fig.18 shows a single subunit with a molecular weight of 39,000 corresponding to core protein. None of the other subunits of complex III were seen on the gel, partly because the protein-protein associations were disrupted by the 4% SDS solution prior to immunoprecipitation, and partly because the antibody was subunit-specific recognizing only core protein II.

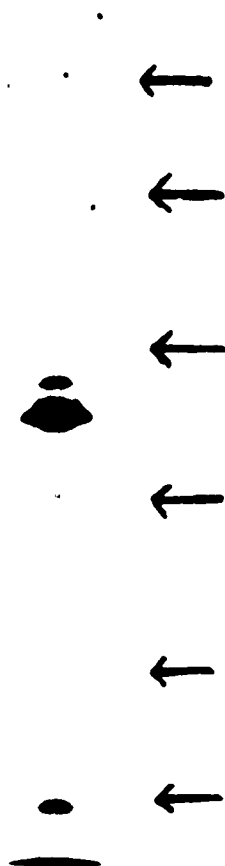


Fig. 18: Autoradiogram of immunoprecipitate obtained using antisera against core protein II. Yeast cells were labeled with 400 $\mu\text{Ci/ml}$ of (^3H)leucine for 1 h and the label was chased for 10 min by the addition of 1mM unlabeled leucine. The cells were denatured with 20% TCA (w/v), and solubilized as described under "Experimental Procedures". The solubilized extracts were subjected to immunoprecipitation with anti-core protein II antisera, and the immunoprecipitate was analyzed on a 12.5% gel. The gel was soaked in 1M salicylate prior to drying and fluorography. Arrows denote position of molecular weight standards.

2. DISCUSSION

The core proteins of complex III were first identified in 1967 and were found to make up 40-50% of the total protein of the complex (Silman et al, 1967). Since core proteins contain no prosthetic groups, it has not been possible to establish their function within the complex. A major problem in studying these core proteins has been the lack of specific probes. To learn more about their function and location in the yeast mitochondria, we turned to immunological techniques. In the present study, the core proteins, along with the ISI, were purified and antibodies raised against the purified polypeptides. These antibodies were shown to be strongly antigenic and subunit-specific by double immunodiffusion (Ouchterlony, 1953). The lack of cross-reactivity between the subunit-specific antisera and the other subunits indicates that the two core proteins and the ISI do not share common antigenic determinants. The presence of a complex reaction in complex III, as revealed by double immunodiffusion tests Fig. 14D, is not surprising in view of the size of the complex, since it is well known that the number of antigenic determinants usually increases with the size of the antigen (Kabat and Mayer, 1961). Complex III antiserum did not cross-react with core proteins I and II, and the ISF. This observation contradicts earlier finding (Nelson and Mendel-Hartvig, 1977), where the antiserum to complex III was

reported to cross-react with these proteins. The purity of the isolated subunits by these authors, however, was not established.

SDS-gel analysis of (^{35}S)sulfate labeled immunoprecipitate, obtained by using complex III antiserum, revealed the presence of six of the seven subunits of complex III. The molecular weights of these subunits are in good agreement with those of the purified complex III. Our assignment of the ISI as the 24,000 dalton subunit rather than the 25,000 dalton subunit, is based on the observation that only the 24,000 dalton species was sensitive to cycloheximide inhibition (Fig. 28, lane 4), indicating the cytoplasmic origin of this polypeptide (Beattie *et al*, 1980; Katan *et al*, 1976a and 1976b). Furthermore, the 25,000 dalton protein was found to be also insensitive to chloramphenicol, and was sometimes absent from the gels, suggesting that it is a contaminant of unknown origin.

The polypeptide band at 29,000 daltons was attributed to cytochrome b apoprotein and cytochrome c₁. It was not possible to separate these two polypeptides, even though a 12.5% acrylamide gel was used, and instead a broad diffused band was seen. The nature of this band maybe due to the use of salicylate as a fluor, since it had been reported earlier that sodium salicylate, while being an excellent fluor, caused a diffusion of radioactive bands (Chamberlain, 1979).

The presence of cytochrome b apoprotein in this band has been demonstrated in Fig. 28, lane 4, as the cycloheximide insensitive polypeptide (Beattie et al, 1980; Chen and Beattie, 1982).

None of the immunoprecipitates analyzed in Fig. 17 revealed the presence of polypeptides of molecular weights $< 15,000$ (Gellerfors et al, 1976; Larres and Slater, 1976; Weiss and Kolb, 1979; Beattie et al, 1980). This observation further supports our earlier suggestion that these low molecular weight species are absent from the native complex. The overall polypeptide pattern in the immunoprecipitates obtained from the various subunit-specific antisera, clearly demonstrates that these antisera, which were raised against the SDS-denatured subunit, were still capable of recognizing complex III solubilized in Triton X-100, suggesting a retention of the proper antigenic determinants in the SDS-denatured subunits.

The inability of the ^{35}S label to be incorporated into the core protein II polypeptide indicates the absence of either methionine or cysteine residues in this protein. Although earlier evidence had suggested that core protein II contained between 1-1.8 mol of methionine per 100 mol of amino acid residues (Silman et al, 1967; Bell and Capaldi, 1976; Katan et al 1976b), two of these analyses (Silman et al, 1967; Bell and Capaldi, 1976) were performed on mixtures of core proteins I and II isolated from beef heart complex III.

Very recently, Teintze et al (1982) also found that relatively little (^{35}S)sulfate was incorporated into core protein, thus confirming our results. Definitive proof of the absence or presence of the S-containing amino acids would require amino acid analysis to be performed on purified core protein II. Since it is well known that the first amino acid residue in the elongating polypeptide chain is formyl-methionine, our finding would suggest that core protein II is made in a precursor form, which is then processed into a mature form, following the removal of amino acids from the N-terminal. Indeed, Teintze et al (1982), have shown that a precursor for core protein II does exist in Neurospora cells.

3. LOCALIZATION OF THE SUBUNITS OF COMPLEX III

Proteolytic digestion of complex III: In order to elucidate the topology of the subunits of complex III, the enzyme was digested with trypsin and chymotrypsin. Mild trypsin digestion (20 μ g/mg of complex protein) of complex III in the oxidized and reduced state led to an accompanying decrease of enzymatic activity, being more rapid in the dithionite-reduced complex III (Fig. 19A). Subsequent analysis of the digested complex on 12.5% acrylamide gels revealed that subunits III, IV, V, and VI (cytochrome b apoprotein, cytochrome c₁, ISP, and the 17,500 dalton subunit, respectively) were most susceptible to trypsin digestion when the complex was in an oxidized form (Fig. 20, lanes 1-4). When the complex was in the reduced state, however, only subunits III and IV appeared to be digested (Fig. 20, lanes 5-8). When the complex was treated with antimycin A prior to trypsin digestion, the results obtained were identical with those obtained with the oxidized trypsin-digested complex III, except that subunit VI now appeared to be more resistant to trypsin digestion (Fig. 20, lanes 10-13). The two core proteins (subunits I and II) and subunit VII appeared to be completely resistant to trypsin in either the oxidized, reduced, or antimycin-treated complex III. These observations

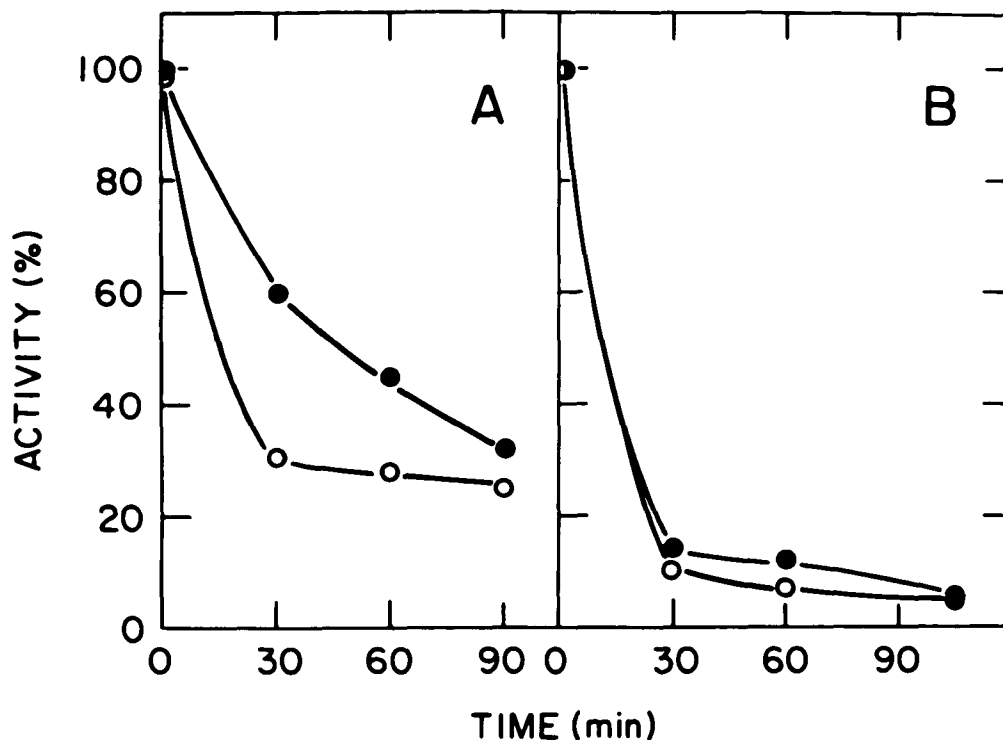


Fig. 19: Effect of proteolytic digestion on enzymic activity of complex III. Oxidized or reduced complex III (5 mg/ml) was digested with either 20 µg of trypsin/mg of complex III (Fig.12A) or 30 µg of chymotrypsin/mg of complex III (Fig.12B) for 30, 60, and 90 min, as described under "Experimental Procedures". The DBH₂:cytochrome *c* reductase activity remaining was calculated as the percentage of activity obtained by incubating complex III under identical conditions but in the absence of protease. (o—o), oxidized complex; (●—●), reduced complex.

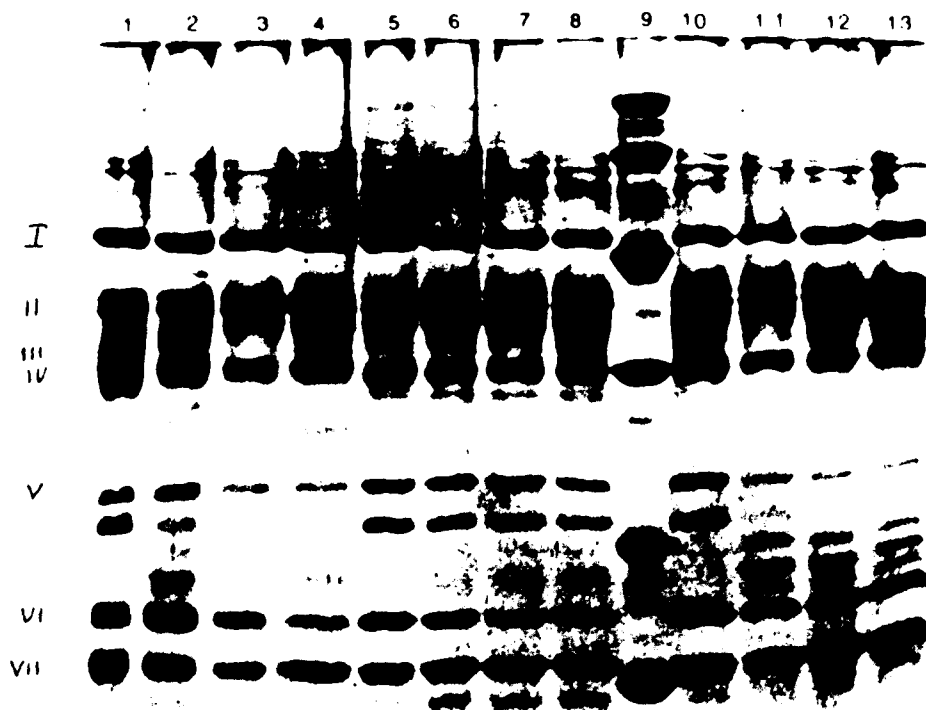


Fig. 20: Gel electrophoresis of trypsin-digested complex III. Oxidized, reduced, or antimycin A-treated complex III (5 mg/ml) was digested with trypsin (20 μ g/mg of complex III) for 30, 60, and 90 min, as described under "Experimental Procedures." The complex was dissociated in dissociation buffer and 50 μ g were loaded on each lane. Lanes 1 to 4, 5 to 8, and 10 to 13 contained the oxidized, reduced, and antimycin A-treated complex III, respectively. Lane 9 contained molecular weight standard proteins. The numbering of the complex III subunits is as described in the legend to Fig. 8

were further confirmed when the gel was subsequently scanned and the areas under the peaks calculated for the oxidized and reduced complex III (Fig. 21). At the end of 90 min, 29 and 37% of subunits IV and V, respectively, remained undigested by trypsin in the oxidized complex, while 85-90% of these subunits remained undigested when the complex was reduced. By contrast, 45 and 58% of cytochromes b and c₁ remained undigested in the oxidized complex, and 35% of these polypeptides remained undigested in the reduced complex.

Chymotrypsin digestion (30 μ g/mg of complex protein) of oxidized and reduced complex led to a rapid loss of enzymatic activity, with less than 15% remaining after 30 min (Fig. 19B). Similar results were obtained when the complex was digested with lower amounts of chymotrypsin (up to 5 μ g/mg of complex). Electrophoresis of chymotrypsin-digested complex III indicated a rapid digestion of subunits III, IV, V, and VII, both in the oxidized and reduced state (Fig. 22). The core proteins, along with subunit VI, were resistant to chymotrypsin digestion whether the complex was in the reduced or the oxidized form.

Immunoinhibition studies: Immunoinhibition curves were obtained by incubating either submitochondrial particles, mitoplasts, or cholate-solubilized mitoplasts, with IgG obtained from the different antisera, as described under "Experimental Procedures". Initially, immunoinhibition curves were obtained using antiserum. However, under these conditions

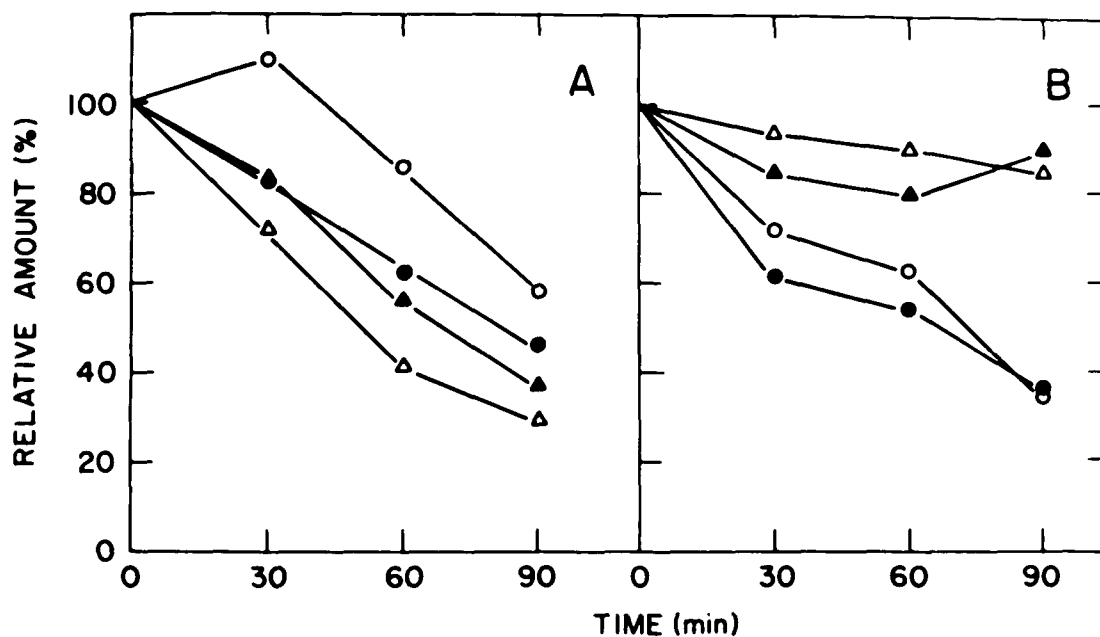


Fig. 21: Trypsin digestion of the individual subunits of complex III. Densitometric scans of the gel in Fig. 20 were obtained, and the percentage of subunits remaining, relative to the amount present in the absence of trypsin, was calculated by measuring the area under each peak. A, the oxidized and B, the reduced form of complex III. ●—●, cytochrome b apoprotein; ○—○, cytochrome c₁; △—△, iron-sulfur protein; ▲—▲, 17,500 subunit.

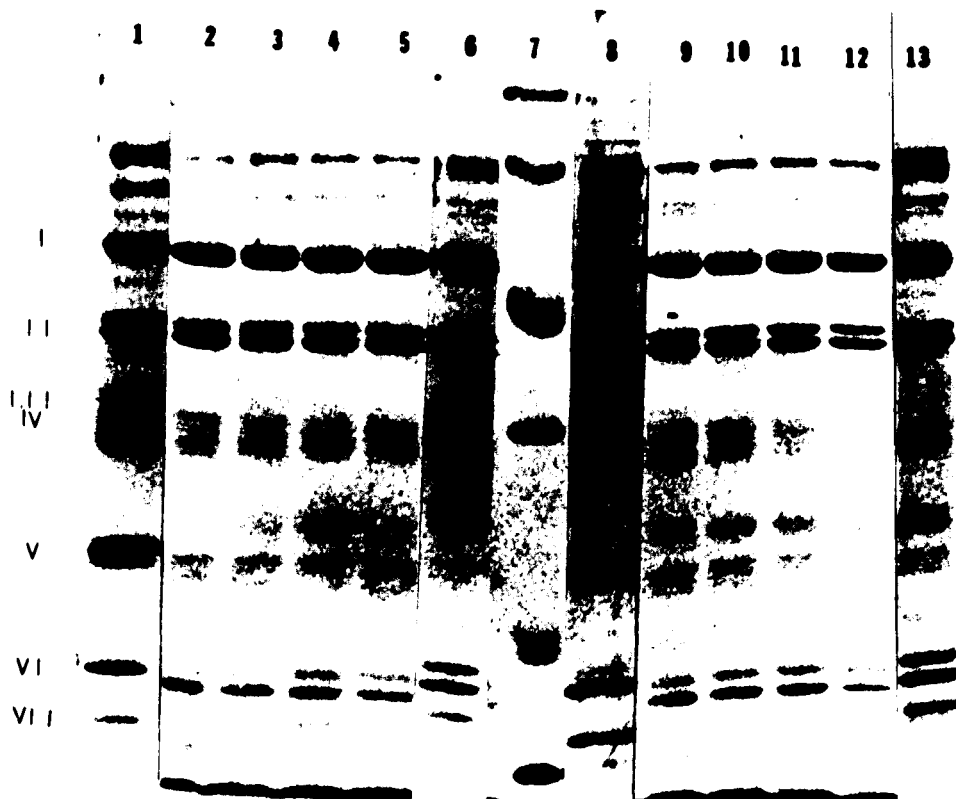


Fig.22 : Gel electrophoresis of chymotrypsin-digested complex III. Oxidized or reduced complex III (5mg/ml) was digested with 5,10,15,20 and 30 µg of chymotrypsin/mg of complex III for 30 min, as described under "Experimental Procedures". The complex was dissociated in dissociation buffer and 50 µg were loaded on a 12.5% acrylamide gel. Lanes 1 to 6 contain the oxidized complex III; lane 7, molecular weight standards; and lanes 8 to 13 contain the reduced complex III.

control serum also inhibited the enzyme activity by 60-70%. To overcome this non-specific inhibition, an IgG fraction was purified and used in subsequent studies, since control IgG did not inhibit the activity. The maximum inhibition of enzyme activity by the various IgG fractions were calculated and are presented in Table IV. Thus, the IgG fraction isolated from complex III inhibited the succinate:cytochrome c reductase activity by 88% in mitoplasts (Fig. 23 and Table IV), while only 51% inhibition of this activity was seen when submitochondrial particles were used (Fig. 24 and Table IV). When the mitoplasts were solubilized with cholate, the IgG from complex III inhibited 94% of the enzyme activity (Fig. 25 and Table IV). Moreover, this inhibition was seen at much lower concentrations of IgG (0.96 mg per 15 μ g of protein). The IgG fraction from the iron-sulfur protein inhibited only 57% of the enzyme activity in mitoplasts (Fig. 24 and Table IV). A dramatic increase in the maximum inhibition of reductase activity was seen in cholate-solubilized mitoplasts (Fig. 25 and Table IV), where the IgG from ISI inhibited 98% of the activity at an IgG concentration of 0.96mg per 15 μ g of protein. In submitochondrial particles however, no inhibition was observed with the ISP IgG fraction (Fig. 24). The IgG fractions from the two core proteins did not inhibit the activity of either solubilized or intact membrane preparations to any significant extent (Table IV).

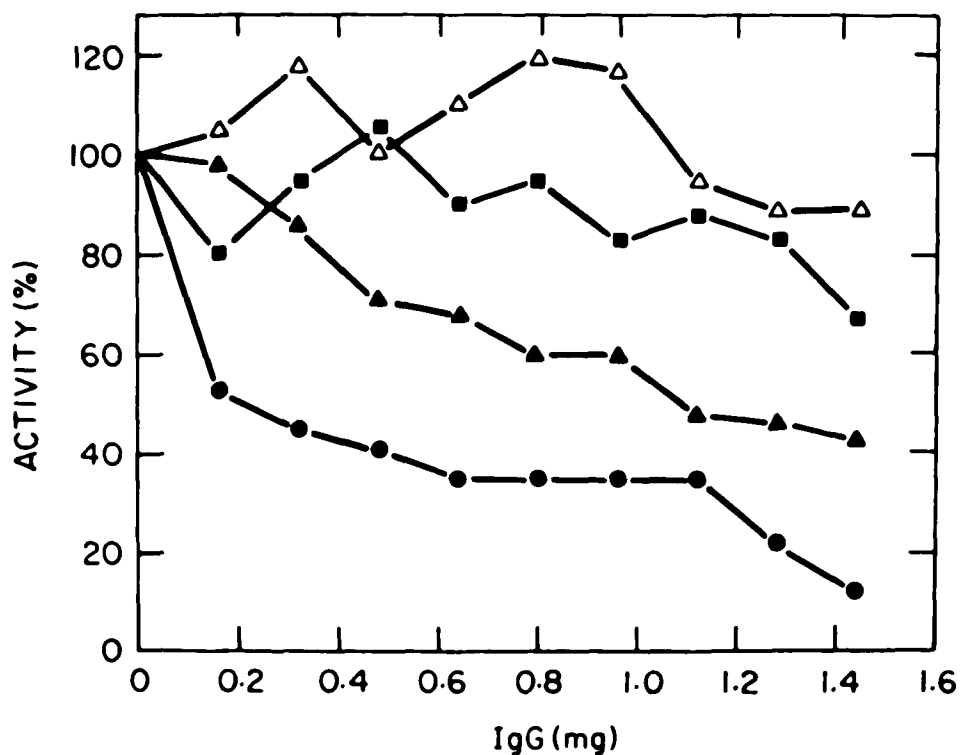


Fig. 23: Immunoinhibition of succinate:cytochrome c reductase activity in mitoplasts. Intact mitoplast protein (15 μ g) was incubated with varying amounts of IgG obtained from antisera against core protein I (Δ — Δ), core protein II ($\blacksquare$ — $\blacksquare$), iron-sulfur protein ($\blacktriangle$ — $\blacktriangle$) and complex III ($\bullet$ — $\bullet$). Succinate:cytochrome c reductase activity was calculated as the activity obtained in the presence of immune IgG relative to the activity obtained in the presence of an identical amount of pre-immune IgG.

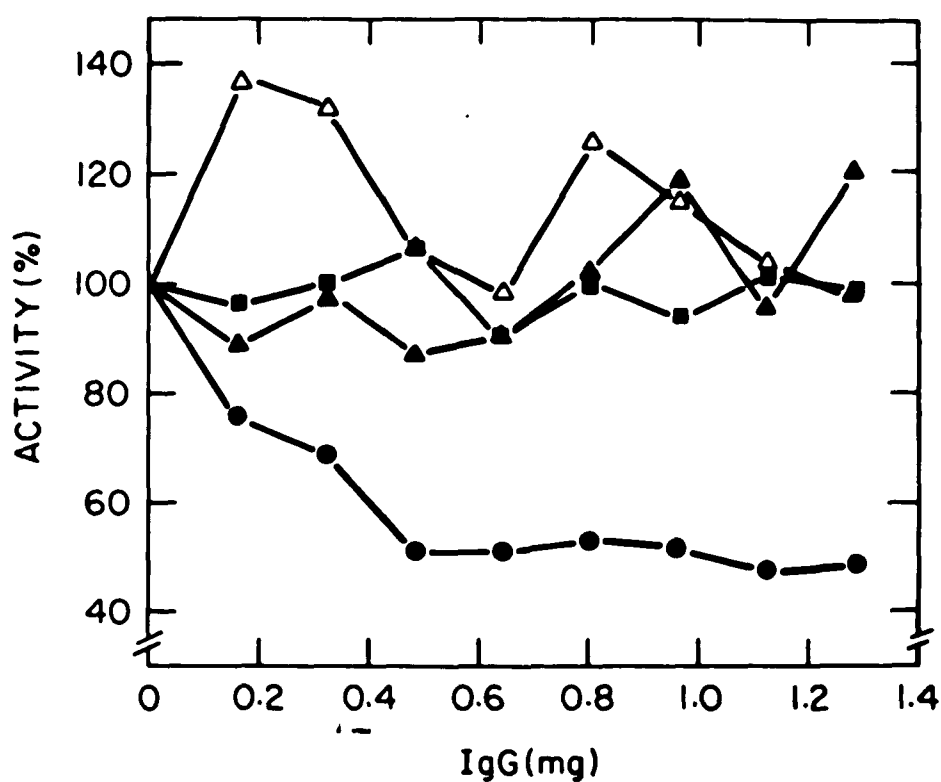


Fig. 24 : Immunoinhibition of succinate:cytochrome c reductase activity in SMF. The experimental conditions were identical to that described in the legend to Fig. 23, except that 15 μ g of submitochondrial particles were incubated with IgG obtained from antisera against core protein I (Δ — Δ), core protein II ($\blacksquare$ — $\blacksquare$), iron-sulfur protein ($\blacktriangle$ — $\blacktriangle$), and complex III ($\bullet$ — $\bullet$).

TABLE IV

Maximum Inhibition of Enzyme Activity by Immune IgG Fractions

From the immunoinhibition curves obtained in Figs. 23-25, the maximum inhibition of cytochrome c reductase activity by the various IgG fractions was calculated.

IgG fraction	Mitoplasts	Submitochondrial particles	Mitoplasts + cholate
Complex III	88%	51%	94%
Core protein I	33%	2%	0%
Core protein II	11%	2%	10%
Iron-sulfur protein	57%	4%	98%

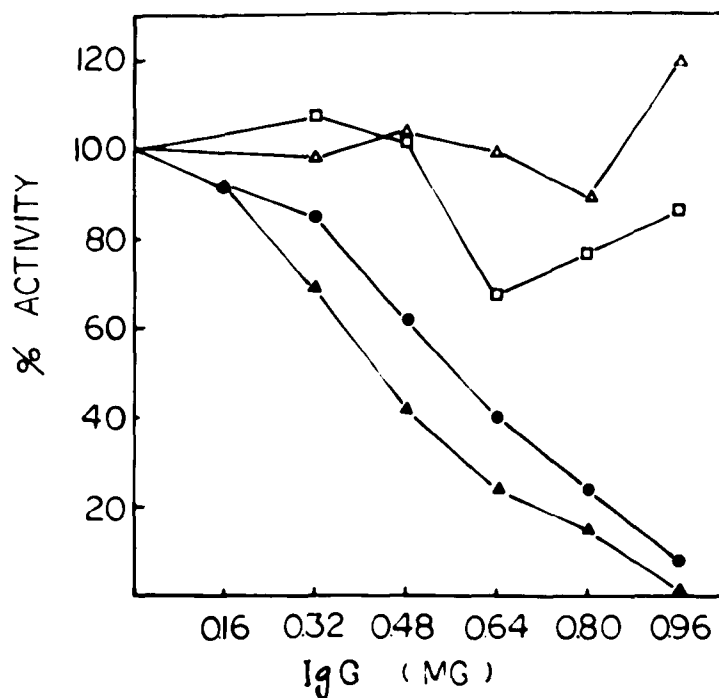


Fig. 25: Immunoinhibition of ubiquinone:cytochrome c reductase activity in solubilized mitoplasts. The experimental conditions were as described in the legend to Fig. 23, except that mitoplasts were solubilized with 1% Lubrol WX and 2% cholate, and DBH₂ was used as substrate. The solubilized protein (15 μ g) was incubated with varying amounts of IgG obtained from antisera against core protein I (Δ — Δ), core protein II ($\blacksquare$ — $\blacksquare$), iron-sulfur protein ($\blacktriangle$ — $\blacktriangle$) and core complex III ($\bullet$ — $\bullet$).

Effect of SDS on the associations between the subunits of complex III: protein-protein associations between the subunits of complex III were studied by dissociating mitochondrial proteins with a low concentration of SDS. Yeast cells were labeled in vivo with (^{35}S)sulfate and mitochondria were prepared. One-half of the mitochondria were solubilized in 1% Triton X-100 and the other half were solubilized by the addition of boiling SDS (1 mg per mg mitochondrial protein). The solubilized and centrifuged extracts were subjected to immunoprecipitation using antisera against complex III, core protein I and iron-sulfur protein. The autoradiograms of the immunoprecipitates were scanned. Complex III antiserum immunoprecipitated intact complex III, when mitochondria were solubilized in 1% Triton, and the six subunits of the complex can be clearly seen (Fig. 26A, upper curve). In the SDS-solubilized membrane fraction, however, a different pattern is seen. Thus, subunits III and IV are the predominant polypeptides seen, along with decreased amounts of subunits I and VII. The amount of subunit V present is dramatically decreased, while subunit VI is completely absent from the immunoprecipitate. These findings suggest that there are strong associations between subunits I, III, IV and VII. It is not known if this association is due to protein-protein or lipid-protein interactions, although the former seems to be more likely. In any case, these associations appear to be tighter and more resistant to disruption by SDS. Subunits V

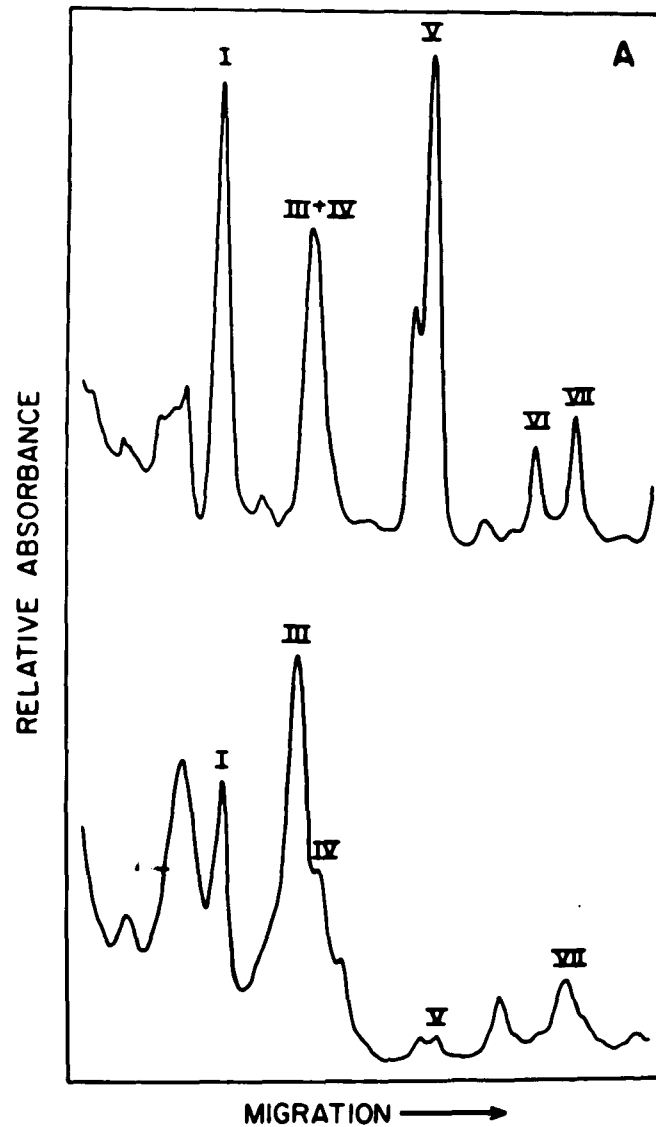
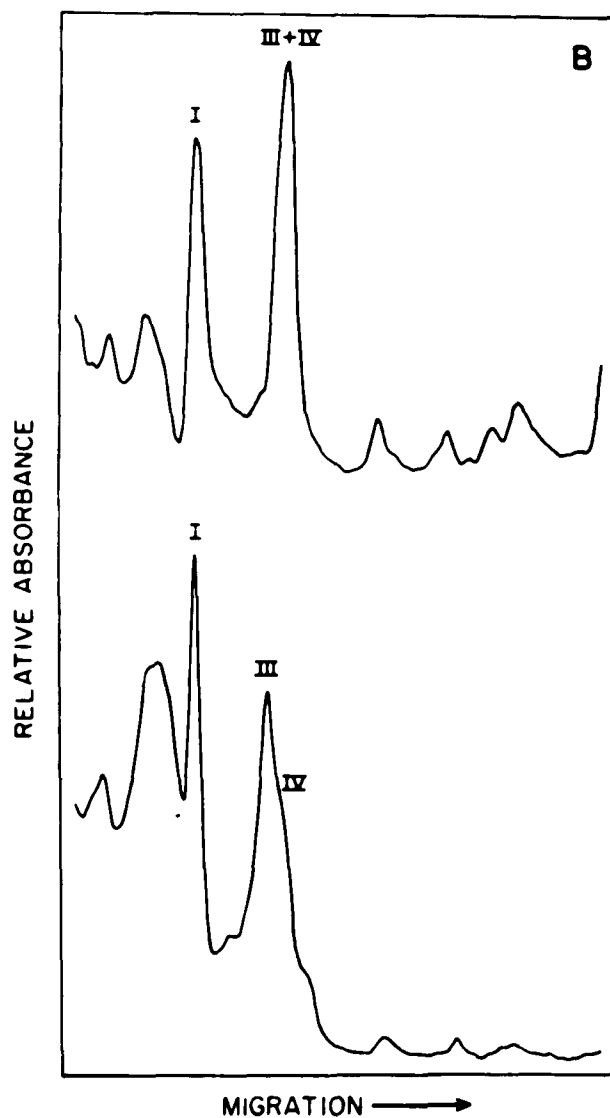
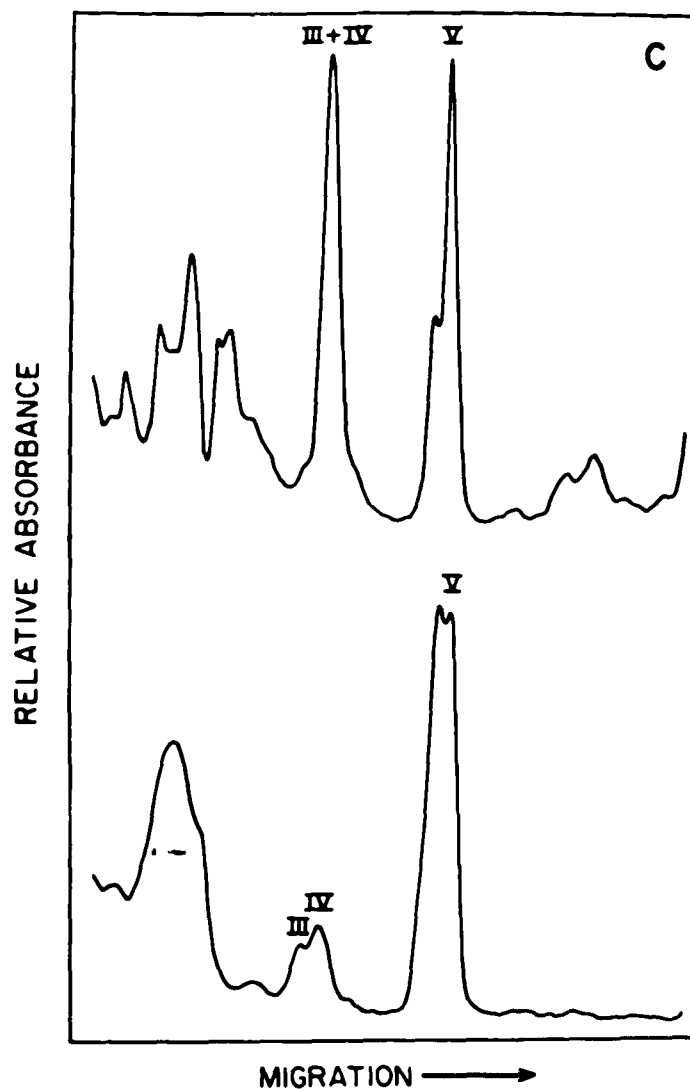


Fig. 26: Gel profile of immunoprecipitates obtained from mitochondria solubilized in either Triton X-100 or SDS. Yeast cells were labeled with (^{35}S) sulfate and mitochondria prepared as described under "Experimental procedures". (contd..)



(Fig.26 contd.) The labeled mitochondria were then solubilized with either 1% Triton or with boiling SDS (1mg/mg protein). Immunoprecipitation was performed on the solubilized extracts using antiserum directed against complex III(A), core protein I (B), and iron-sulfur protein (C); and the immunoprecipitates (contd..)



(Fig.26 contd.) were analyzed on a 12.5% gel. The autoradiograms were scanned in a Canalco microdensitometer. The upper graph in each figure is the scan of the immunoprecipitates obtained using Triton-solubilized mitochondria, while the lower graph represents the scan of the immunoprecipitates from SDS-solubilized mitochondria.

and VI appear to be more loosely attached to the rest of the complex, since they are easily removed from the complex upon dissolution of the proteins in SDS.

The immunoprecipitate obtained by using core protein I antisera (Fig. 26B, upper curve) shows the presence of predominantly subunits I, III, and IV in Triton-solubilized mitochondrial extracts. This coimmunoprecipitation of subunits III and IV with subunit I again suggests a strong interaction between cytochromes b-c₁ and core protein I. Moreover, this interaction between these three subunits is not susceptible to SDS, since even in the presence of this detergent, the same three subunits are immunoprecipitated by anti-core protein I antiserum (Fig. 26E, lower curve).

The immunoprecipitate obtained using the ISF antiserum seems to confirm the suggestion that the iron-sulfur protein is more loosely attached to the rest of the complex. The immunoprecipitate from the Triton-solubilized mitochondria revealed the presence of subunit V in association with subunits III and IV (Fig. 26C, upper curve). However, upon the addition of SDS, the iron-sulfur protein was easily dissociated from the rest of the complex and appeared as the major polypeptide immunoprecipitated (Fig. 26C, lower curve).

It should be noted that at higher concentrations of SDS, the protein-protein interactions between all the subunits are disrupted. Thus complex III antiserum cannot

immunoprecipitate any of the subunits of complex III (Fig. 29, lanes 2-4), while the core protein I antiserum can recognize only core protein I (Fig. 29, lanes 5-7). Similar results were obtained with the ISI antiserum, which immunoprecipitated a single band corresponding to the ISI, when mitochondria were dissociated in a high concentration of SDS (data not shown). The exact SDS:mitochondrial protein ratio is not known, but these experiments were performed by dissociating approximately 2 mg of cellular protein in a 4.5% SDS solution. The results of Fig. 29 taken together with those listed above (Fig. 26), strongly suggests that the immunoprecipitate pattern obtained in the presence of SDS is a reflection of protein-protein type associations between the various subunits of complex III. Thus, the disruption of a strong association between certain subunits would require higher concentrations of SDS, while weaker associations would be more susceptible to disruption by SDS at lower concentrations of the detergent. The logical conclusion of this observation would be that the protein-protein interaction is a direct function of the proximity of the polypeptides involved. Thus, the closer a pair of subunits, the more likely are they to be held together by protein-protein type interactions than are a pair of subunits situated far away from one another.

3. DISCUSSION

The topology and subunit interaction of complex III have been extensively studied, using both hydrophilic and hydrophobic probes (Bell et al, 1979; Smith et al, 1978; Gutweniger et al, 1981). A model for the orientation of the subunits in complex III can also be deduced from the proteolytic digestion, the immunoinhibition studies and the immunoprecipitation data presented in this paper. The rapid rate at which the apo-protein of cytochrome b and cytochrome c₁ were digested by both trypsin and chymotrypsin in the oxidized and reduced state suggests that these subunits are exposed on the surface of the complex in agreement with previous observations (Bell et al, 1979; Gutweniger et al, 1981). Indeed, the decline in enzymatic activity of complex III during proteolytic digestion appears to go in parallel with the loss of the two cytochromes. In addition, the immunoinhibition of the enzyme activity observed with the IgG against complex III in intact and solubilized mitoplasts, coupled with the partial inhibition seen in submitochondrial particles, suggests an external location of the proteins of complex III on the cytoplasmic side of the mitochondrial inner membrane.

The iron-sulfur protein and the 17,500 dalton polypeptide appear to undergo a conformational change in the reduced form,

rendering them inaccessible to trypsin digestion. Such a conformational change had been previously reported for the iron-sulfur protein upon reduction of complex III from beef heart (Baum, et al, 1967). The immunoinhibition data indicates that the iron-sulfur protein is substantially exposed on the outer surface of the mitochondrial inner membrane, with some shielding of the protein by the membrane. Very little, if any, of the iron-sulfur protein appears to be exposed to the mitochondrial matrix. These observations are in contrast to the model recently suggested after x-ray crystallographic analysis, where the iron-sulfur protein is reported to be totally buried in the mitochondrial inner membrane (Hovmoller et al, 1981).

The resistance of the two core proteins and the 15,500 dalton subunit to proteolytic digestion indicates some shielding of these polypeptides in the isolated complex. An alternative possibility is that these polypeptides lack the necessary amino acids for either trypsin or chymotrypsin digestion. However, amino acid analysis of the core proteins indicates that these proteins do indeed contain the requisite amino acids, making this possibility unlikely (Bell and Capaldi, 1976; Yu et al, 1977). Thus it seems highly unlikely that the two core proteins are exposed to the surface to the extent reported earlier, where it had been suggested that these polypeptides extend from the matrix side of the membrane (Hovmoller et al, 1981). Rather, the data presented here along

with the labeling studies with either DABS(Beattie et al,1981) or photoreactive lipid analogs(Gutweniger et al,1981) support the suggestion that the two core proteins are buried within the lipid bilayer. The absence of inhibition of enzyme activity in either solubilized or intact membranes, by the IgG from the two core proteins, suggests that these core proteins do not contain any redox centers and are not involved in the transfer of electrons. This observation supports an earlier report in beef heart mitochondria, whereby antisera against the core proteins did not inhibit NADH oxidation or duroquinol:cytochrome c reductase activity(Bendol-Hartvig and Nelson,1981).Alternatively, the antibody-binding sites in these proteins may be different from the catalytic sites.

The effect of SDS on the associations between proteins, can also provide some useful insight on the subunit-interaction between the polypeptides of complex III.When proteins are immunoprecipitated in the absence of prior SDS treatment, the antibody will recognize,primarily,its corresponding antigen. Some other proteins will also coimmunoprecipitate and will not be removed from the immune complex if the association between these proteins and the antigen is strong. The use of SDS to solubilize the proteins, gives an indication of the strength of the association between the antigen and proteins that coimmunoprecipitate with the immune complex.A high concentration of SDS will disrupt all antigen-

protein associations, and only the antigen will be precipitated. Thus, based on these conclusions, it appears that cytochromes b and c₁ are tightly associated and appear to be in close proximity to one another, since they always appear together in the presence and absence of prior SDS treatment. In addition, the appearance of core protein I with cytochromes b-c₁ in the presence and absence of SDS, also suggests a close association between core protein I and these cytochromes. Indeed, nearest neighbour analysis of the polypeptides of complex III by cross linking, indicates that core protein I lies close to cytochrome b apoprotein (Smith and Cavaldi, 1977). These cross linking studies also suggested that core protein I and the iron-sulfur protein were closely associated. Our immunoprecipitation data, however, indicates otherwise, since neither of these proteins appeared to coimmunoprecipitate when antisera from either core protein I or iron-sulfur protein were used. Instead, the iron-sulfur protein appears to be weakly associated to cytochromes b and c₁, since it is easily removed from the cytochromes at low concentrations of SDS.

The data presented in this section supports the model suggested by Beattie et al (1981), whereby an asymmetric localization of complex III in the mitochondrial membrane is proposed. A schematic diagram of this model is presented below.

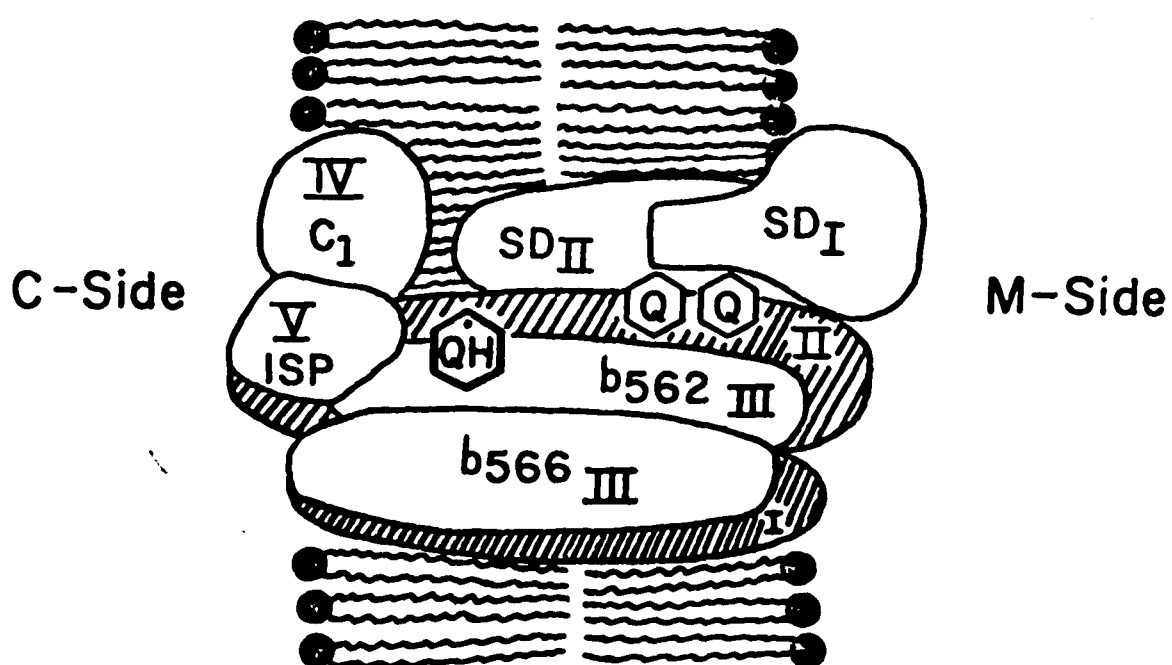


Fig. 27: A model for the orientation of complex III in the mitochondrial membrane. I and II are core proteins I and II, respectively; b-562 and b-566 are the two proposed forms of cytochrome b; IV is the cytochrome c_1 and V is the iron-sulfur protein. SD_I and SD_{II} are the subunits of succinate dehydrogenase. C and M refer to the cytoplasmic and matrix side of the inner membrane.

4. BIOGENESIS OF COMPLEX III

Site of translation of the subunits of complex III: To identify proteins of complex III synthesized on mitochondrial ribosomes, yeast cells were labeled in vivo in the presence of cycloheximide (1 mg/ml), a specific inhibitor of cytoplasmic protein synthesis. Electrophoresis of the immunoprecipitate formed with antisera against complex III revealed three radioactive proteins with molecular weights of 47,000, 32,000, and 25,000 (Fig. 28, lane 4). The major peak at 32,000 probably represents cytochrome b, a known mitochondrial product which is present in immunoprecipitates formed with specific antiserum to cytochrome b from yeast (Chen and Beattie, 1981). The band at 47,000 is probably due to cytoplasmically made core protein I and is seen in the gel since the inhibition of cycloheximide on cytoplasmic protein synthesis is not 100%. The band at 25,000 daltons is of unknown origin, and is sometimes absent from the gels.

Yeast cells were also labeled in the presence of 4 mg/ml chloramphenicol to block mitochondrial protein synthesis (Fig. 28, lane 3). The apoprotein of cytochrome b is not seen, while core protein I, cytochrome c₁ (M_r = 29,000), the ISF (M_r = 24,000), the 17,500 and the 15,500 dalton subunits are clearly seen, indicating that these polypeptides are synthesized on cytoplasmic ribosomes. The contaminant at

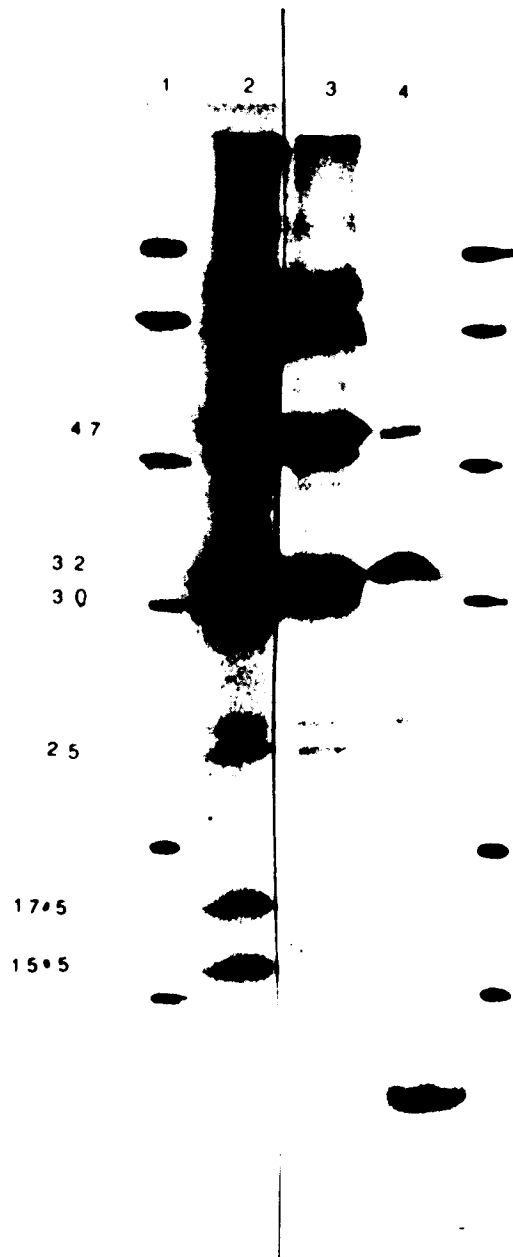


Fig. 28: Labeling of complex III in the presence of cycloheximide and chloramphenicol. Yeast cells were labeled with (^{35} S)methionine (200-300 μ Ci/ml) for 30 min in the absence of inhibitors (lane 2) and in the presence of either 1 mg/ml cycloheximide (lane 4), or 4 mg/ml chloramphenicol (lane 3). The label was chased for 5 min with 10 mM unlabeled methionine, mitochondria were prepared, and solubilized in 1% Triton and subjected to immunoprecipitation using anti-complex III antiserum. Immunoprecipitates were analyzed on a 12.5% gel. Lane 1 contained the molecular weight standards.

25,000 is clearly seen in this lane and in the immuno-precipitate obtained in the absence of inhibitor (Fig. 28, lane 2). Core protein II is not seen in the absence or presence of the chloramphenicol, since these cells were labeled with (^{35}S)methionine, and it has been previously shown that this label is not incorporated into the polypeptide (Figs. 17, 18 and 30). In control cells, labeled in the absence of inhibitors, six subunits of complex III can be clearly seen. It had been reported earlier (Beattie *et al*, 1980), that when cells were labeled in the presence of chloramphenicol, a prominent band with an apparent molecular weight of 100,000 daltons was seen, along with the subunits of complex III. No such band, however, could be detected in this (Fig. 28, lane 3) and other experiments.

Pulse-chase experiments: In order to detect the presence of the 100,000 dalton subunit and to investigate possible precursor-product relationships, the experiments of Beattie *et al* (1980) were initially repeated. **Spheroplasts** were labeled with (^{35}S)methionine in the presence of chloramphenicol (CAP). After 5 min, aliquots were removed and lysed in 2 vol of boiling 4.5% SDS solution, and the boiled lysates were then diluted with 3% Triton buffer and subjected to immuno-precipitation using complex III antiserum. Unfortunately, it was not possible to detect the presence of the 100,000 dalton subunit, even though this experiment was repeated many times with several modifications. Since spheroplasts

did not incorporate (^{35}S)methionine very efficiently, there was a possibility that due to the low incorporation of the label, the 100,000 dalton species, although present, was not detected by fluorography. To test this suggestion, whole yeast cells were pulsed with (^{35}S)methionine in the presence and absence of CMI. An aliquot of the CMI-treated cells was then mixed with 20% TCA to stop the reaction. The control cells (containing no inhibitor) and the remainder of the CMI-treated cells were then chased with unlabeled methionine. The cells were processed as described under "Experimental Procedures" and immunoprecipitation was performed using antisera against complex III (Fig. 29, lanes 2-4) and antisera against core protein I (Fig. 29, lanes 5-7). Again, it was impossible to detect the 100,000 dalton subunit. The anti-core protein I antiserum showed a single band corresponding to core protein I in the pulsed, pulse-chased and control cells, while the anti-complex III antiserum barely showed the presence of any subunit of complex III.

Assessment of precursor pools of the subunits of complex III: Since the 100,000 dalton subunit could not be detected, we decided to study, initially, the kinetics of synthesis and processing of complex III. Such experiments would indicate the presence or absence of precursor pools for the individual polypeptides of complex III.

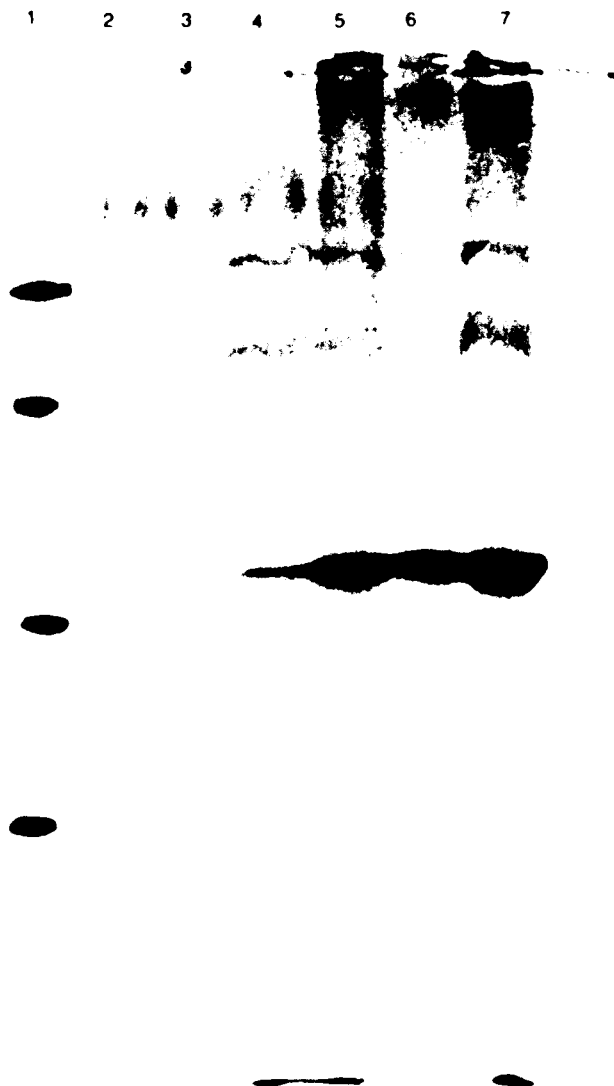


Fig. 29: Gel electrophoresis of proteins immunoprecipitated from yeast cells pulse-labeled with (^{35}S)methionine. Yeast cells were pulsed with (^{35}S) methionine for 5 min in the absence (lanes 2 and 5) and presence (lanes 3, 4, 6 and 7) of prior incubation with CAP. Cells were then lysed with 20% TCA without a chase (lanes 3 and 6) and with a chase of unlabeled methionine (lanes 2, 4, 5 and 7). The cells were processed as described under "Experimental Procedures" and immunoprecipitates were obtained using antisera against complex III (lanes 2 to 4) and core protein I (lanes 5 to 7). Lane 1 contains the molecular weight standard. The immunoprecipitates were analyzed on a 10% polyacrylamide gel.

The method of Werner (1974) was used, whereby, initially, spheroplasts were uniformly labeled with (^3H)methionine and then pulsed for 1-3 min with (^{35}S)methionine, followed by a chase with unlabeled methionine. At various time intervals after the addition of the (^{35}S) label, aliquots of the cultures were removed and mitochondria isolated. Although the spheroplasts were chosen because of the ease in which they can be broken, the incorporation of the (^{35}S) label was very low. The experiments were then subsequently repeated using whole yeast cells, which were then broken by agitation with glass beads. While this method greatly improved incorporation of the (^{35}S) label, it appeared that both methionine labels were being chased by the unlabeled methionine. To overcome this problem, cells were grown overnight in (^3H)leucine and then pulsed for 3 min with (^{35}S)methionine, followed by a chase with unlabeled methionine, so that only the second label would be selectively chased. Aliquots of the cell culture were removed 3, 6, 13 and 28 min after the addition of (^{35}S)methionine, mitochondria prepared and solubilized in 1% Triton, and then subjected to immunoprecipitation with complex III antiserum. The washed immunoprecipitates were analyzed by SDS-gel electrophoresis after loading constant amounts of (^3H) counts in each lane. As seen in Fig. 30, the distribution

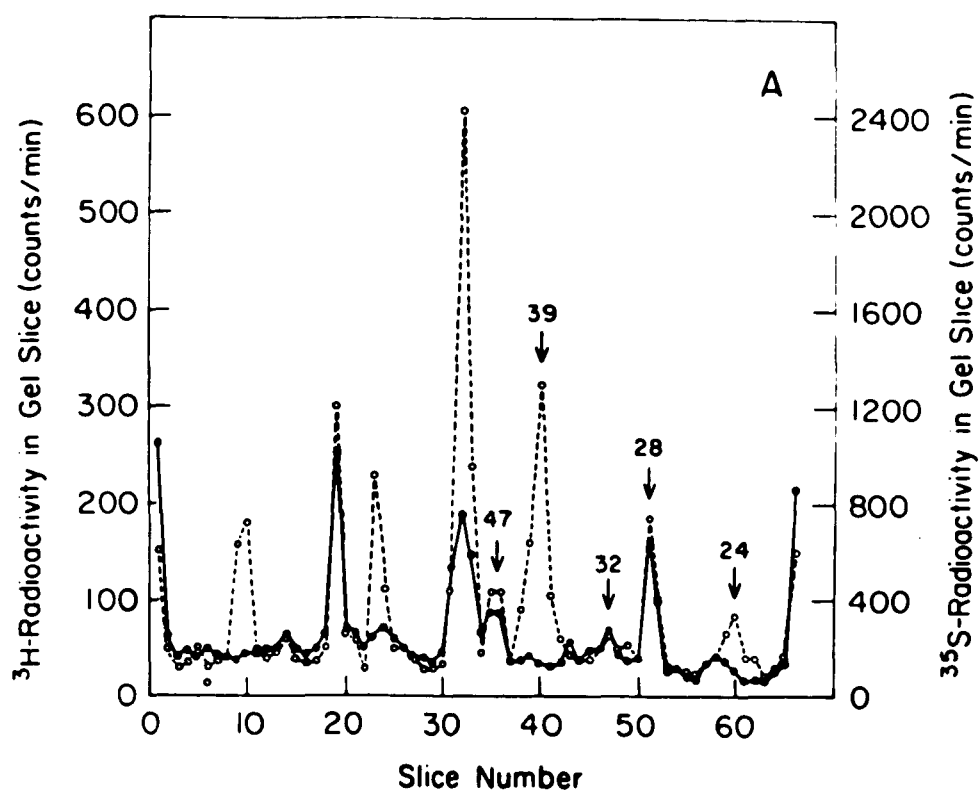
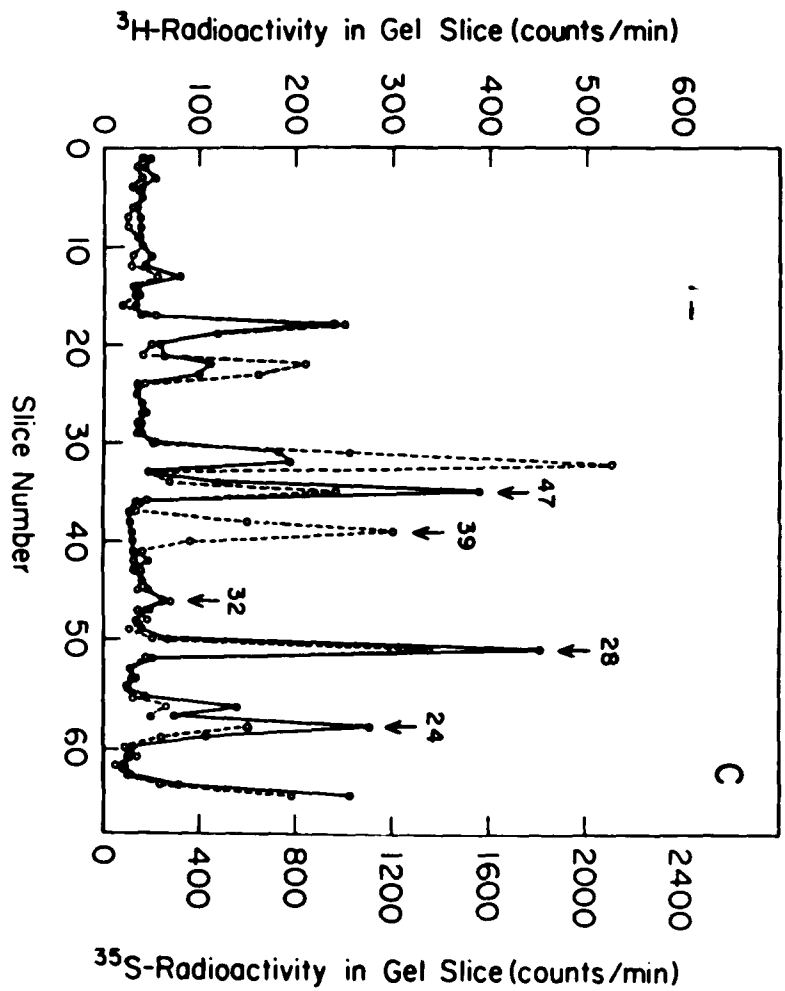
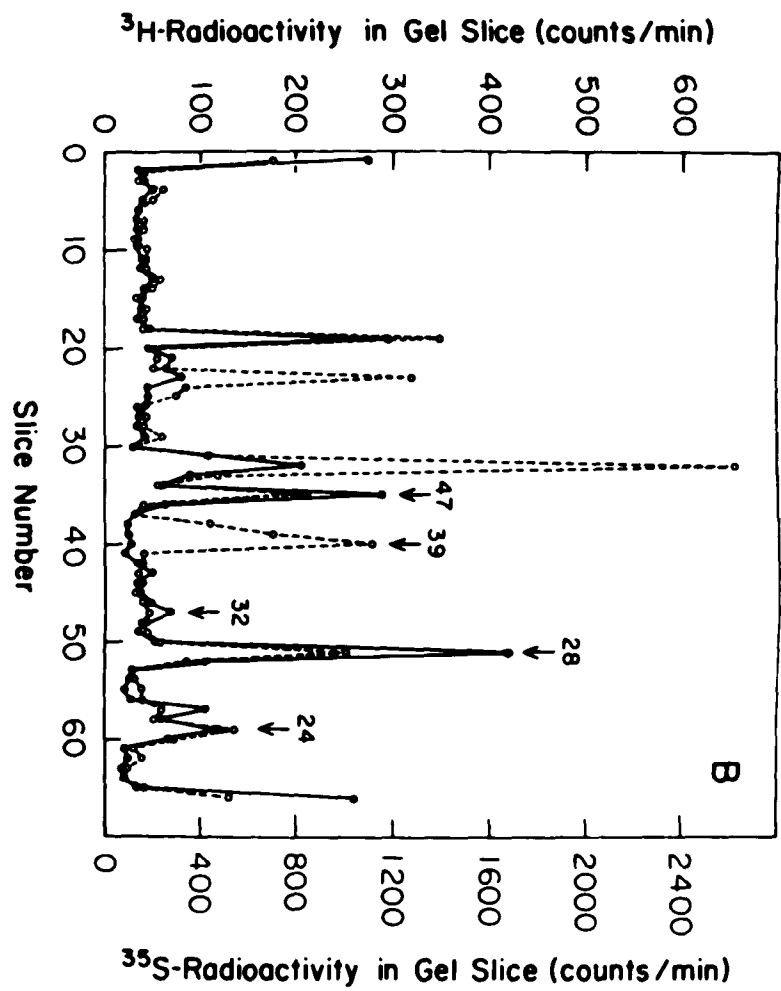
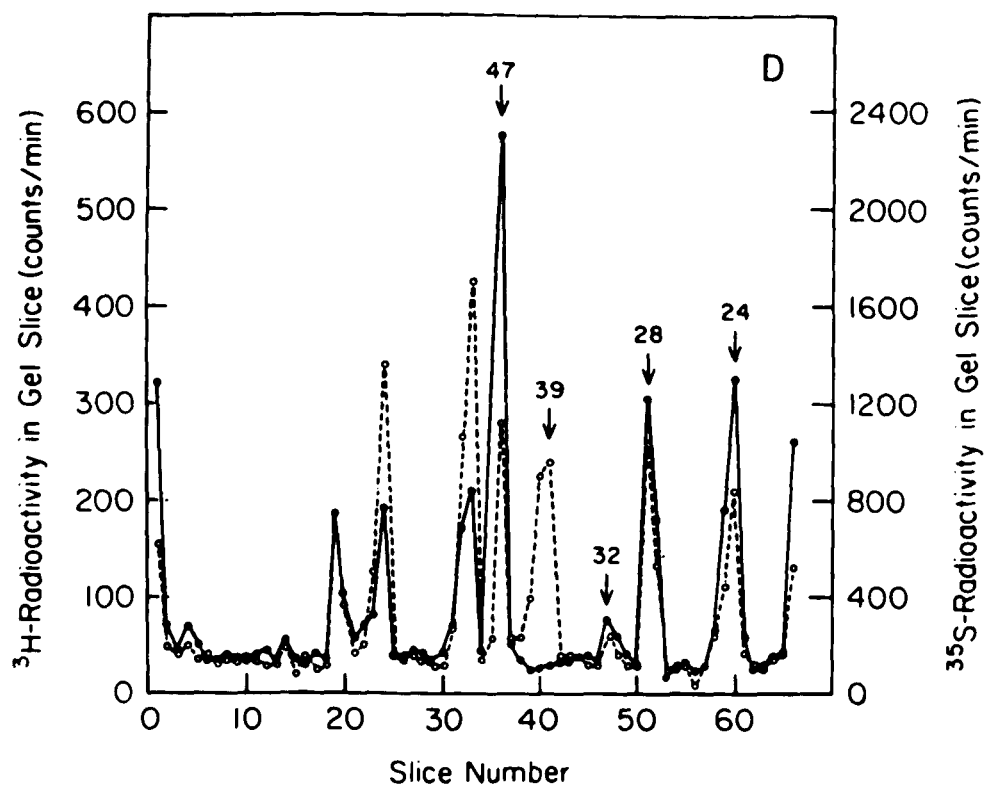


Fig. 30: Gel electrophoresis of complex III precipitated from double-labeled mitochondria. Yeast cells were grown overnight in the presence of (^3H)leucine. They were then pulsed with (^{35}S)methionine. Three min later, the culture was chased with 10 mM unlabeled methionine, samples of the





culture were harvested, mitochondria prepared and solubilized with 1% Triton, and treated with complex III antiserum. Immunoprecipitates were analyzed on a 10% polyacrylamide gel, which was then sliced and the radioactivity present in the slices was determined. Four of the immunoprecipitates are shown: (A) 3 min, (B) 6 min, (C) 13 min and (D) 28 min after the addition of (^{35}S) methionine. (o---o) ^3H , (●—●) ^{35}S radioactivity.

patterns of the (^3H) radioactivity were fairly constant, and five of the seven subunits of complex III could be seen. The two low molecular weight polypeptides could not be seen since it was necessary to perform this experiment on a 10% acrylamide gel in order to obtain maximal separation of cytochromes b and c₁.

The kinetics of incorporation of the (^{35}S)methionine label into the five subunits of complex III varied considerably. Thus, 3 min after the addition of (^{35}S)methionine (Fig. 30A), only the 28,000 dalton subunit appeared to be substantially labeled with methionine. This subunit was determined to be apocytochrome b based on its anomalous migration pattern on 10% polyacrylamide gels (Fig. 9). The amount of methionine incorporated into cytochrome b apoprotein increased 6 min after the addition of the label (Fig. 30B), followed by a slight decrease 13 and 28 min after the addition of the label (Figs. 30C and 30D, respectively). Core protein I, the 47,000 dalton subunit showed a delayed labeling with (^{35}S)methionine. Initially, only a small incorporation of the (^{35}S) label was seen in core protein I (Fig. 30A). The incorporation of the label increased upon further incubation and the core protein appeared to be maximally labeled 28 min after the addition of the methionine (Fig. 30D).

The ISP, the 24,000 dalton subunit, also showed an

extremely delayed labeling. Thus, 3 min after the addition of methionine, the (^{35}S) label could not be detected in the ISP. Instead, the (^{35}S) label was rapidly incorporated into a polypeptide having an apparent molecular weight of 25,500 daltons (Fig. 30A). The incorporation of the (^{35}S) label into this 25,500 dalton species increased upon further incubation (Figs. 30B and 30C), with a concomitant increase in the incorporation of the methionine label into the ISP. 28 min after the addition of (^{35}S)methionine, the (^{35}S) radioactivity was seen only in the ISP and the 25,500 dalton species could not be detected. This suggests a possible precursor-product relationship between the ISP and the 25,500 dalton polypeptide, especially since it had been reported earlier, that the precursor to the ISP is only 2,000 daltons larger than the mature form (Cote et al, 1979). Unfortunately, the 32,000 dalton subunit, cytochrome c_1 , was not clearly resolved in these gels. Nonetheless, incorporation of the (^{35}S)methionine label into the cytochrome c_1 polypeptide can be seen and the polypeptide appears to be labeled rapidly. As discussed earlier, the (^{35}S)methionine failed to be incorporated into core protein II, the 39,000 dalton subunit. Indeed, even 28 min after the addition of methionine, the (^{35}S)methionine label was completely absent from core protein II (Fig. 30D).

Fig. 31 demonstrates the time course of (^{35}S) labeling

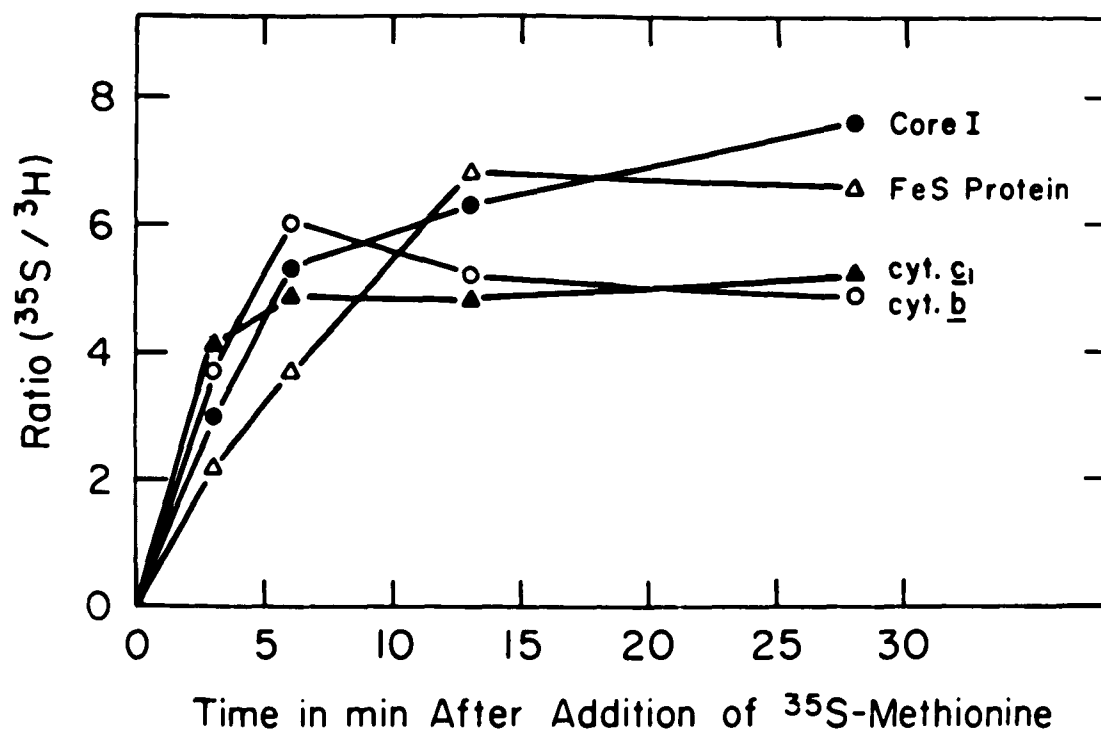


Fig. 31: Incorporation of (^{35}S)methionine in the subunits of complex III. The total ^3H and ^{35}S radioactivity present in each subunit of complex III was calculated from the gels in Fig. 27, and the ($^{35}\text{S}/^3\text{H}$) ratio was determined.

of the individual polypeptides of complex III. The increase in specific radioactivity in the subunits, as a function of time, is reflected by the $^{35}\text{S}/^3\text{H}$ ratios present in these subunits. Thus, apocytochrome b is rapidly labeled with a maximal labeling time of 6 min after the addition of (^{35}S)methionine, followed by a slight decrease in the (^{35}S) label present in this subunit. Although cytochrome c₁ appeared to be rapidly labeled (Fig. 30), maximal labeling of this polypeptide occurred 28 min after the addition of the label. On the other hand, labeling of both the ISF and core protein I with (^{35}S)methionine appeared to be a slower process with a maximal labeling time of 28 min after the addition of the methionine.

From Fig. 31, the half-maximal labeling time, $t_{1/2} \text{ max}$, for the individual subunits of complex III were calculated and are presented in Table V. These half-maximal labeling times can be interpreted to: 1. suggest the presence or absence of large pools of the polypeptides, possibly in the form of precursor proteins (Werner and Sebald, 1981); or 2. indicate the turnover rates of these polypeptides within the membrane. Thus, the $t_{1/2} \text{ max}$ of 276 s and 315 s calculated for core protein I and the ISF, respectively, suggests that either large pools of these proteins are present in the cells, possibly in the form of precursors, or that the turnover rates of these proteins are low. Similarly, the $t_{1/2} \text{ max}$ value of 144 s for cytochrome b

TABLE V

Half-Maximal Labeling Time of the Subunits of
Complex III.

The time required for half-maximal labeling, $t_{1/2}$ max, was calculated from Fig. 31, for the individual polypeptides of complex III. The $t_{1/2}$ max represents the rate at which the (^{35}S)methionine label was incorporated into the proteins.

Molecular Weight	Subunit	$t_{1/2}$ max in s
47,000	Core protein I	276
32,000	Cytochrome <u>c</u> ₁	105
28,000	Cytochrome <u>b</u>	144
24,000	Iron-sulfur protein	315

and 105 s for cytochrome c₁, suggests an absence of large pools of these polypeptides and a high turnover rate. The high turnover rate could be true for cytochrome b apoprotein, especially since a rapid incorporation of the (³⁵S) label was followed by a decrease in this label in the apoprotein (Fig. 31).

4. DISCUSSION

Earlier studies on the sites of translation of the constituent polypeptides of complex III performed in both Neurospora (Weiss and Ziganke, 1977) and yeast cells (Katan et al, 1976b; Beattie et al, 1980) suggested that only cytochrome b apoprotein was synthesized on mitochondrial ribosomes, while all the other polypeptides had a cytoplasmic origin. However, since some of these studies were performed with enzymatically inactive complexes that might have lost key proteins (Katan et al, 1976b; Weiss and Ziganke, 1977), the possibility that other proteins of complex III were synthesized by mitochondria could not be ruled out. It, thus, seemed pertinent to repeat these experiments using antibodies raised against an enzymatically active complex. Our results, however, confirmed all the earlier findings and only cytochrome b apoprotein appeared to be synthesized by mitochondria.

Our study failed to reveal the presence of a 100,000 dalton subunit, postulated to be the apparent precursor of the two core proteins (Beattie et al, 1980), despite several attempts, suggesting that the 100,000 dalton subunit seen was present only as an artifact. The possibility that precursors to the core proteins do exist within the yeast cell, cannot be ruled out. Indeed the data presented here on the kinetics of labeling of core protein I indicates a

slow labeling of the core protein due either to large pools of the protein in, possibly, the form of precursors, and/or alternatively, due to a slow turnover of the protein. Very recently, and subsequent to these findings, studies on the biogenesis of complex III support both of the above suggestions postulated to explain the labeling kinetics of core protein I. Thus, Teintze et al (1982) have demonstrated, by in vitro translation of Neurospora mRNA, the presence of a precursor protein to core protein I, with a molecular weight 1,500 daltons larger than the mature core protein I. Additionally, a very low turnover rate for core protein I was calculated, using hepatoma cultures (Hare and Hodges, 1982). These authors calculated a half-life of over 100 h for core protein I.

The delayed labeling of iron-sulfur protein is probably due to the presence of large precursor pools. Indeed, earlier studies have indicated that the precursor to ISP is 2,000 - 3,000 daltons larger than the mature form (Cote et al, 1979; Teintze et al, 1982), and the 25,500 dalton species seen in Fig. 30 may represent the ISP precursor.

The rapidity at which cytochrome b was labeled was also reported earlier by Clejan et al (1980), in studies performed on yeast cells. The rapid labeling of the cytochrome b apoprotein is, thus, probably due to an absence of large

pools of the apoprotein in the mitochondrial matrix, coupled with a rapid turnover rate. Indeed, Hare and Hodges (1982) calculated a half-life of 41 h for cytochrome b in hepatoma cells, and this apocytochrome b appeared to turnover more rapidly relative to the other polypeptides of complex III.

The $t_{1/2}$ max of 105 s for cytochrome c₁ would suggest that either a small pool of cytochrome c₁ exists or that this protein has a high turnover rate. However, recent evidence from several laboratories does not support either of these suggestions. Thus the presence of precursor pools for cytochrome c₁ has been established (Neupert and Schatz, 1981; Ohashi et al, 1982; Gasser et al, 1982; Teintze et al, 1982), and the protein was found to turnover slowly at the same rate as core protein I (Hare and Hodges, 1982). It is probable, that due to the poor resolution of cytochrome c₁ during SDS-gel electrophoresis, an erroneous $t_{1/2}$ max was obtained, and that the actual value of $t_{1/2}$ max for cytochrome c₁ is much higher.

5. BIOCHEMICAL ANALYSIS OF THE DIURON-RESISTANT MUTANTS OF YEAST

Enzymic and spectral properties: The cytochrome c reductase activity of the diuron resistant mutants was examined with the decyl analog of coenzyme Q, DBH₂, as substrate (Brown and Beattie, 1977). Comparable rates of cytochrome c reduction were observed in mitochondria (Table VI) isolated from all four strains. Upon the addition of cholate, to solubilize the membrane, a higher enzymic activity was observed, especially in the wild-type, KL14-4A. The activity present in the mutant strains was much lower than in the wild-type upon the addition of 1% cholate to mitochondria. A significantly higher rate of reduction was obtained in the submitochondrial particles in all four strains, as compared with the rates seen in the mitochondria. Again, upon solubilization of the SMP with cholate, a higher rate of enzyme activity was observed, and the percent increase was comparable with that observed in the mitochondria. With the SMP, a higher enzymic rate was observed in two of the mutants, Diu^r-740 and Diu^r-752. This higher enzymic rate was also reflected in a higher $V_{\max}$ when calculated by Eadie-Hofstee plots in intact SMP (Table VI). All four strains had a similar apparent K_m for DBH₂.

Spectral analyses on the four strains revealed that

TABLE VI

DBH₂:Cytochrome c Reductase Activity in Intact or Cholate-solubilized Mitochondria and Submitochondrial Particles in Wild Type and Diu^r Mutants.

Mitochondria and submitochondrial particles were prepared and used as such, or were solubilized with 1% cholate (per 1.5 mg protein), and the assay performed as described in "Experimental Procedures", using 10-20 µg of protein in each assay. Values of K_m and V_{max} were determined using submitochondrial particles, in the absence of cholate.

Strains	<u>Mitochondria^a</u>		Percent increase	<u>Submitochondrial particles^a</u>		percent increase	V _{max} ^a	K _m ^b
	+ 1% cholate			+ 1% cholate				
KL14-4A	231	377	63%	419	673	61%	400	60
Diu ^r -740	185	237	28%	555	629	13%	700	44
Diu ^r -742	244	261	7%	465	524	13%	480	50
Diu ^r -752	218	281	29%	636	859	35%	780	59

^a nmol/min/mg of protein.

^b µM DBH₂

two of the mutants, $\text{Diu}^{\text{F}}-742$ and $\text{Diu}^{\text{F}}-752$, had decreased levels of cytochrome a, while $\text{Diu}^{\text{F}}-740$ contained only trace amounts of this cytochrome (Table VII). Moreover, $\text{Diu}^{\text{F}}-740$ exhibited much lower amounts of cytochromes b and c₁, while the other two mutants had similar levels of these cytochromes as the wild-type.

In vitro resistance to drugs: The resistance of the three mutants to diuron was determined in vitro by examining cytochrome c reductase activity with the decyl analog of coenzyme Q, DBH_2 , as substrate (Brown and Beattie, 1977). Mitochondria obtained from all three mutant strains were equally sensitive to diuron during the assay of DBH_2 :cytochrome c reductase (Table VIII), despite their resistance to comparable concentrations of diuron in assays of NADH oxidation in submitochondrial particles (Briquet and Goffeau, 1981). By contrast, significantly higher amounts of diuron were needed to attain 50% inhibition of cytochrome c reductase activity in intact submitochondrial particles from two of the mutants, $\text{Diu}^{\text{F}}-742$ and $\text{Diu}^{\text{F}}-752$, as compared to particles from the parental strain. Upon solubilization of the submitochondrial particles with cholate, the resistance to diuron of the two mutants, however, was completely abolished. Enzymic activity in submitochondrial particles from the other mutant studied, $\text{Diu}^{\text{F}}-740$, was as equally sensitive to diuron as the wild-type. $\text{Diu}^{\text{F}}-740$, however, was determined to be resistant to diuron during growth, since this mutant grew on glycerol

TABLE VII

Cytochrome Content in Cytochrome c-depleted Submitochondrial Particles of KL14-4A and Diu^R-mutants.

Cytochrome c-depleted submitochondrial particles were suspended in 0.1M phosphate buffer at a protein concentration of 3-5 mg/ml. Difference spectra were obtained by scanning the dithionite-reduced sample against the ferricyanide-oxidized reference.

Strains	Total cytochrome content (nmol/min/mg protein)		
	Cytochrome <u>a</u> ^a	Cytochrome <u>b</u> ^b	Cytochrome <u>c</u> ₁ ^c
KL 14-4A	0.33	0.60	0.34
Diu ^R -740	0.02	0.29	0.19
Diu ^R -742	0.11	0.49	0.33
Diu ^R -752	0.20	0.62	0.39

Extinction coefficients used were: ^a12 mM/cm, ^b25.6 mM/cm and ^c20.9 mM/cm for cytochromes a, b and c₁, respectively.

TABLE VIII

The Effect of Diuron on DBH_2 :Cytochrome c Reductase Activity in the Wild Type and Diu^F Mutants.

Mitochondria and submitochondrial particles were prepared and solubilized with 1% cholate. The solubilized and intact membranes were incubated for 4 min with varying concentrations of diuron (methanolic solution) prior to addition of the substrate, and the specific activities were determined according to the method described under "Experimental Procedures". Diuron resistance is expressed as mM of diuron/mg of protein giving 50% inhibition of specific activity.

Strains	<u>Mitochondria</u>		<u>Submitochondrial Particles</u>	
	+ 1% cholate		+ 1% cholate	
KL14-4A	2.8	2.7	2.0	2.2
Diu^F -740	2.9	1.9	2.0	2.9
Diu^F -742	3.0	1.3	5.3	1.8
Diu^F -752	2.0	1.1	5.2	2.1

medium supplemented with 130 μM diuron, while the growth of the parental strain was completely blocked by 25 μM diuron (Clejan et al, 1982).

As seen in Table IX, the three mutants showed no cross-resistance to either antimycin or UHDBT, the synthetic analog of ubiquinone (Bowyer and Trumpower, 1980). This suggests that the binding and/or mechanism of action of diuron is different from antimycin and UHDBT. Moreover, the kinetics of inhibition of these three drugs appear to be different. Thus, from spectral studies, antimycin caused the "red-shift" of cytochrome b in the wild-type and the mutants, while diuron did not cause a similar shift (data not shown). Additionally, in the inhibition curves obtained with antimycin, the wild-type and the mutants exhibited a sigmoidal response to antimycin, while non-sigmoidal curves were obtained with diuron and UHDBT (data not shown).

Extra-reduction of cytochrome b: In order to detect functional changes in the behaviour of cytochrome b which might have resulted as a consequence of the mutation conferring resistance to diuron, the extra-reduction of cytochrome b was studied in submitochondrial particles. Thus, the amounts of total cyto-

TABLE IX

The Effect of Antimycin A and UHDBT on DBH₂:Cytochrome c Reductase Activity in the Wild Type and Diu^R Mutants.

Mitochondria and submitochondrial particles were used intact or were solubilized with 1% cholate. The membranes were incubated for 4 min with antimycin (ethanolic solution) prior to addition of substrate. Cholate solubilized mitochondria were not pre-incubated with UHDBT. The enzymic activity and concentration of antibiotic giving 50% inhibition of the activity were calculated as described under "Experimental Procedures".

Strains	<u>Antimycin Resistance</u>				<u>UHDBT Resistance</u>
	<u>Mitochondria</u> ^a + 1% cholate		<u>Submitochondrial Particles</u> ^a +1% cholate		<u>Mitochondria</u> + 1% cholate ^b
KL14-41	1.7	2.7	2.7	2.2	1.8
Diu ^R -740	1.3	2.1	2.2	4.3	3.5
Diu ^R -742	1.0	2.3	1.5	2.3	2.6
Diu ^R -752	1.5	2.0	1.9	2.9	1.9

^aμM of antimycin/mg of protein giving 50% inhibition.

^bmol of UHDBT/mol of cytochrome c, giving 50% inhibition.

chrome b reduced by succinate was compared with that reduced in the presence of either diuron or antimycin plus succinate (Roberts et al, 1980). In these experiments, the antibiotic was preincubated with the particles prior to the addition of the substrate, succinate. Table X indicates that 68-77% of cytochrome b in the wild-type was reduced in the presence of succinate alone, and upon the addition of antimycin and diuron an extra-reduction of cytochrome b occurred to 80% and 83%, respectively. No extra-reduction of cytochrome b was observed in the presence of either antibiotic in particles from the mutants.

TABLE X

Extra-reduction of Cytochrome b in Submitochondrial Particles of KL14-4A and Diu^R Mutants.

Submitochondrial particles were suspended at 1.5-2 mg/ml in 0.1M phosphate buffer, pH 7.5. Spectra were recorded 15 min after the addition of 60 mM succinate. For the extra-reduction assays, antimycin A (5µg/mg protein) or diuron (500 mM) were incubated with submitochondrial particles 2 min before the addition of succinate (60 mM). Total cytochrome b was obtained by reduction with a few grains of dithionite.

Cytochrome <u>b</u> reduced (% of total) ^a				
Strains	Experiment 1		Experiment 2	
	Succinate	Antimycin	Succinate	Diuron
KL14-4A	68	80	77	83
Diu ^R -740	79	79	79	79
Diu ^R -742	54	54	60	56
Diu ^R -752	61	61	65	65

^a In experiment 1, the total cytochrome b reduced by dithionite was 0.61, 0.29, 0.46 and 0.68 nmol/mg protein; and in experiment 2, the total was 0.64, 0.29, 0.41 and 0.60 nmol/mg protein in strains KL14-4A, Diu^R-740, Diu^R-742 and Diu^R-752 respectively.

5. DISCUSSION

The results of this study suggest that the mutations conferring resistance to diuron are manifested as subtle changes in the biochemical functions of cytochrome b in the mutants. These modifications are not sufficient to alter gross properties of cytochrome b, and indeed the molecular weights of the apoprotein of cytochrome b in the mutants and wild-type were found to be the same (Clejan et al, 1982). Instead, the mutations caused changes in biochemical function that suggest that these modifications occur in regions which are related to activity of cytochrome b. Thus the functional changes in cytochrome b included resistance to diuron at the level of the respiratory chain, measured as DBH_2 :cytochrome c reductase activity, and no "extra-reduction" of cytochrome b when either antimycin or diuron was allowed to bind to submitochondrial particles in the oxidized state prior to addition of succinate. Moreover, all the mutants exhibited a greatly reduced oxidant-induced extra-reduction when succinate was added before the addition of diuron or antimycin, and a diminished reversed electron flow induced by ATF in KCN and antimycin treated submitochondrial particles reduced with fumarate:succinate (Clejan et al, 1982). Generally these changes in cytochrome b behaviour were observed in all three mutant strains; however, the observed differences in the three diuron-resistant mutants confirm the genetic studies indicating that each mutation maps in a different

locus on the mitochondrial DNA (Colson and Wouters, 1980; Lazowska et al, 1980). Thus $\text{Diu}^{\text{R}}-740$, which appears to have a mutation mapping in the first intron locus of cytochrome b gene, was not resistant to diuron in vitro; it had the lowest levels of the three cytochromes and had a similar V_{max} as the $\text{Diu}^{\text{R}}-752$ mutant and the wild-type, but with a lowered K_{m} . The $\text{Diu}^{\text{R}}-742$, which maps in the first exon of the cytochrome b gene, had a higher cytochrome content than $\text{Diu}^{\text{R}}-740$, but lower than $\text{Diu}^{\text{R}}-752$, which maps in the first intron locus of the cytochrome b gene. The absence of cross-resistance of the mutants to antimycin and UHDBT suggests that these mutations that confer diuron-resistance occur at sites different from those that confer resistance to antimycin or UHDBT.

Interestingly, no resistance to diuron was observed in mitochondria when cytochrome c reductase activity was assayed with DBH_2 as substrate. Likewise, the resistance to diuron in submitochondrial particles was less pronounced when DBH_2 :cytochrome c reductase was assayed than in previously reported studies in similar diuron-resistant mutants in which NADH and succinate oxidation were measured with an oxygen electrode (Briquet and Goffeau, 1981). Perhaps this lack of sensitivity to diuron during cytochrome c reduction with the coenzyme Q analog may reflect the pattern of reduction of this compound in the respiratory chain. Previously, it had been reported that DBH_2 , when added to either mitochondria, mitoplasts or submitochondrial particles achieved a very different pattern

of cytochromes b and c₁ reduction during anaerobiosis as compared to succinate (Beattie et al, 1981). For example, addition of DBH₂ and succinate to mitoplasts resulted in the reduction of 29% and 72% of the total cytochrome b, while their addition to submitochondrial particles resulted in the reduction of 60% and 70% respectively. One explanation for this observation might be that the decyl analog of coenzyme Q, which is less hydrophobic than the natural coenzyme Q₆, may not equilibrate completely with the coenzyme Q pool but may directly reduce the cytochrome or other component of the chain.

The diuron-resistant mutants described in this study should be useful for studies of both the bioenergetics and the biogenesis of complex III in the respiratory chain. These mutants may be especially useful in elucidating the role of cytochrome b in energy transduction either by the Q cycle or by the proton pumping models.

CONCLUDING REMARKS

Complex III of the mitochondrial respiratory chain was isolated and purified to homogeneity. The enzymatically active complex was found to contain only seven subunits present in stoichiometric amounts. In addition, polypeptides having molecular weights lower than 15,000 were not observed in the purified complex. From the immunological studies, complex III labeled in vivo was found to have an identical polypeptide composition as the isolated complex obtained from bakers' yeast.

The data obtained from proteolytic digestion and immunological studies of complex III suggest that cytochromes b and c₁, the iron-sulfur protein and the 17,500-dalton subunit are substantially exposed on the cytoplasmic side of the mitochondrial inner membrane. The two core proteins appear to be embedded in the mitochondrial inner membrane. The two core proteins were not found to contain any prosthetic groups and they do not appear to be involved in the transport of electrons through complex III.

From the biogenesis studies, core protein I and the iron-sulfur protein appear to have either large precursor pools and/or low turn-over rates within the mitochondrial inner membrane. An apparent precursor-polypeptide was seen for the iron-sulfur protein. Cytochrome b apoprotein, the only subunit of complex III found to be synthesized on

mitochondrial ribosomes, turned-over rapidly.

Characterization of complex III in diuron-resistant mutants of yeast revealed that the mutation conferring diuron resistance did not result in gross alterations in the functioning of complex III. Instead, the mutation caused only subtle changes in cytochrome b, resulting in an absence of "extra-reduction" of cytochrome b.

LIST OF ABBREVIATIONS

ATP:	adenosine triphosphate
CAI:	chloramphenicol
CCCI:	carbonyl cyanide m-chlorophenyl hydrazone
CHI:	cycloheximide
DABS:	diazobenzene sulfonate
DBH ₂ :	reduced form of decyl analog of coenzyme Q; 2,3-dimethoxy-5-methyl-6-n-decyl,1,4- benzoquinone
DCCD:	dicyclohexylcarbodiimide
DCPIP:	2,6-dichlorophenolindophenol
Diuron:	3-(3,4-dichlorophenyl)-1,1-dimethylurea
EDTA:	ethylenediaminetetraacetic acid
EGTA:	ethyleneglycol-bis-(α -amino-ethyl ether) N,N'-tetraacetic acid
FAD:	flavin adenine dinucleotide
ISI:	iron-sulfur protein
LSM:	low sulfate medium
NADH:	reduced nicotinamide adenine dinucleotide
PAGE:	polyacrylamide gel electrophoresis
PMS:	phenazine methosulfate
PMSF:	phenylmethylsulfonyl fluoride
SDS:	sodium dodecyl sulfate
SMP:	submitochondrial particles

(contd...)

LIST OF ABBREVIATIONS (contd...)

TCA:	trichloroacetic acid
TENED:	N,N,N',N'-tetramethylethylenediamine
TLCK:	N ^a -p-tosyl-L-lysine chloromethyl ketone
TPCK:	L-1-tosylamido-2-phenyl ethyl chloromethyl ketone
UHDBT:	5-(n-undecyl)-6-hydroxy-4,7-dioxobenzo- thiazole

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