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PURIFICATION AND CHARACTERIZATION OF TWO FORMS OF APOCYTOCHROME B FROM YEAST MITOCHONDRIA

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PURIFICATION AND CHARACTERIZATION OF TWO FORMS OF APOCYTOCHROME b FROM YEAST MITOCHONDRIA

by

Yu-Shiaw Chen

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

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ABSTRACT

Purification and Characterization of Two Forms of Apocytochrome b from Yeast Mitochondria

By

Yu-shiaw Chen

Adviser: Professor Diana S. Beattie

Cytochrome b in a partially purified preparation from yeast mitochondria showed normal migration behavior during dodecyl sulfate electrophoresis in different concentrations of acrylamide. This protein migrated at a molecular weight of 31,000 when the preparation was heated in dissociation medium at 20°, 37°, 70° or 100°C when phenylmethanesulfonyl fluoride, the protease inhibitor, was present or absent and in dodecyl sulfate urea gels. By contract, cytochrome b in the intact mitochondrial membrane displayed anomalous migration behavior in gels of different acrylamide concentrations.

Two forms of apocytochrome b were purified by a procedure involving ammonium sulfate fractionation, Ultrogel filtration, minicon concentration, polyacrylamide gel separation and acetone extraction. Both proteins showed one single band with distinct molecular weight, protein I of 31,000 dalton and protein II of 29,00 dalton, upon polyacrylamide gel electrophoresis. Purified protein I and protein II showed similar antigenical response and identical fingerprints after limited proteolytic digestion. So far, this is a report about two species of cytochrome b, for the first time, purified to homogeneity.

Antisera against apocytochrome b-I and b-II were raised individually. The specificity of these antisera was established by 1) ouchterlony double diffusion test, 2) counter

immunoelectrophoresis, 3) immunotitration of labeled mitochondrial extract with antiserum, 4) electrophoretic analysis of immunoprecipitates. Both apo-cytochrome b-I and b-II were demonstrated to be translated on mitochondrial ribosomes by labeling yeast cells in the presence of cycloheximide and immunoprecipitating with antiserum.

Both IgG fractions purified from antisera against apocytochrome b-I and b-II inhibited cytochrome c reductase activity of a mitoplast preparation, an inner membrane - matrix fraction and a reverse - sided submitochondrial particles preparation suggesting the transmembraneous occurance of both apocytochromes b in the mitochondrial inner membrane. Radioautographical analysis of immunoprecipitates from diazotized (^{125}I)iodosulfonate, the membrane non-penetrating reagent, labeled mitochondria and submitochondrial particles also revealed that both apocytochromes b span the mitochondrial inner membrane.

To

My Father (陳晉祿先生),

My Husband (謝有焜先生)

and

My Daughter (謝舜如)

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I want to express my sincere thanks to Dr. Diana S. Beattie for her superb guidance throughout the course of my graduate study, and for the scientific perspective that she helped shape on me. I also want to thank Dr. Heng Chun Li, who offered valuable advice from time to time.

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1. MITOCHONDRIAL ELECTRON TRANSPORT CHAIN

The mitochondrion is a unique energy-transducing organelle which provides energy mostly in the useful chemical form, ATP (adenosine triphosphate), by passing electrons through the respiratory chain. The respiratory chain of mitochondria has been dissected into four distinct lipid-protein complexes, complexes I, II, III, IV, each complex possessing a catalytic role in the oxidation reduction reactions of the over-all electron transfer process (Green and Hatefi, 1961). The sequence of electron transfer in the respiratory chain is determined by the oxidation-reduction midpoint potentials of the electron carriers in each complex (Chance, 1956). Complex V, the fifth designated 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 $\Delta\mu H^+$, 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 97,000. The enzyme is composed of two subunits: a large subunit of 70,000 daltons 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 b_{560} 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 QH_2-cytochrome *c* reductase, catalyzes the reduction of cytochrome *c* by coenzyme QH_2 and constitutes the second site of oxidative phosphorylation. Several methods have been developed for isolation of Complex III by ammonium salt fractionation of membrane proteins

that have been previously solubilized by bile acids in the presence of high salt concentrations. The molecular weights of this multienzyme complex were within the range of 200,000 - 250,000 (Marres and Slater, 1977; Siedow et al, 1978; von Jagow et al, 1978a). Complex III isolated from either beef heart or Neurospora was present as a dimer in an aggregated state as determined by ultracentrifugal studies (von Jagow et al, 1977; Weiss & Kolb, 1979). So far, only two groups have been able to isolate a functionally active complex III from yeast mitochondria (Siedow et al, 1978; Beattie et al, 1980). The isolated b-c_1 complex from our laboratory contains 10 component polypeptides. These 10 components include two "core" proteins (50,000 and 40,000), one cytochrome c_1 (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 analogue of coenzyme Q, 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 a_3. Only heme a_3 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). It is still unknown which subunit(s) of yeast cytochrome c oxidase is linked to heme a or a_3. Previous reports have suggested that subunits I, II, IV (Freedman et al, 1979) or V (Tanaka et al, 1979) might be associated with heme in cytochrome oxidase from beef heart mitochondria.

Poyton and McKemmie (1979) claimed that the four small subunits of cytochrome oxidase are synthesized in the cytoplasm as a single large precursor. Subsequently, this polyprotein 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 mitochondrial made subunit II is also synthesized as a precursor form which is processed posttranslationally (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[1]. The purified complex from yeast contained 10 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 (Bossord, Vik and Schuster, 1980). Fo, or the membrane factor, composed of at least three hydrophobic subunits (Tzagoloff *et al*, 1973) functions as a proton conducting channel and contains the site for oligomycin and DCCD[1] 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 then mature subunits, and then processed in the mitochondrion posttranslationally (Maccecchini et al, 1979; Lewin et al, 1980).

2. PURIFICATION OF CYTOCHROME b IN COMPLEX III

Cytochrome b of Complex III in the mitochondria respiratory chain has received special interest, since it is intimately involved in the energy conversion process at the second phosphorylation site (Chance et al,1970). It has been purified mainly from beef heart and Neurospora mitochondria by solubilization with cholate, deoxycholate or Triton X-100, and ammonium sulfate fractionation, followed by column chromatography such as DEAE cellulose for beef heart cytochrome b (Ohnishi, 1966), Sephadex G-150 for beef heart cytochrome b (Yu, Yu, and King, 1975), hydroxyapatite for beef heart cytochrome b (von Jagow et al, 1978b), and oleylpolymethacrylic acid resin for Neurospora cytochrome b (Weiss and Ziganke, 1974; 1978). The reported molecular weight of the isolated cytochrome b varies from 21,000 to 38,000 depending upon the purification procedure and method of determination even when it has been purified from the same source - beef heart mitochondria. One agreement on the molecular weight of cytochrome b was obtained in different preparations where a value of 33,000 and 31,000 was reported for von Jagow's preparation (1978b) from beef heart and a value of 30,000 and 32,000 for Weiss's preparation (1974) from Neurospora. Both values were based on the pyridine hemochromogen content and on sodium dodecyl sulfate gel electrophoresis (Jagow and Sebald,1980).

However, the isolated cytochrome b from Neurospora crassa yielded a molecular weight of 58,000 when it was subjected to gel filtration on Sephadex G-75 in a bile salt and potassium chloride medium suggesting a dimeric structure of cytochrome b (Weiss and Ziganke, 1974).

In yeast, Lin and Beattie (1978) have purified cytochrome b by a procedure involving detergent solubilization, ammonium sulfate fractionation, mild pronase digestion and sucrose gradient centrifugation. The purified cytochrome b was homogenous, spectrally pure and yielded a single band on both sodium dodecyl sulfate polyacrylamide gel electrophoresis and gel isoelectric focusing. The molecular weight of the purified cytochrome b polypeptide was 28,000 based on dodecyl sulfate gel electrophoresis and 28,800 based on sucrose gradient centrifugation. This value, however, may be an underestimation of the true molecular weight of cytochrome b, since proteolytic digestion to solubilize the proteins was a necessary step in the overall purification scheme. One indication of this possibility occurred when the immunoprecipitates obtained from labeled yeast mitochondria treated with the specific antiserum against cytochrome b in the presence of phenylmethylsulfonyl fluoride (PMSF), the protease inhibitor, were analyzed by gel electrophoresis (Lin et al, 1978). The major labeled band in the immuno-

precipitate migrated with a molecular weight of 31,000. Alternatively, the differences in molecular weight of the purified protein and the major band of the immunoprecipitate may have occurred because of the various conditions used for electrophoresis. Several groups have reported that the electrophoretic migration of cytochrome *b* in *b*-c_1 complexes isolated from either beef heart (Bell and Capaldi, 1976; Marres and Slater 1977) or yeast (Groot *et al*, 1978) mitochondria varied depending on the gel conditions used. Similarly, the molecular weight of cytochrome *b* varies when total yeast mitochondrial translation products were examined on polyacrylamide gels of different concentrations (Groot *et al*, 1978; Cabral *et al*,1978).

Our first goal in the present study was to investigate the electrophoretic behavior of cytochrome *b* and to evaluate its actual molecular weight under various conditions of gel electrophoresis and solubilization using both intact mitochondria and a partially purified preparation of cytochrome *b*.

3. THE PROPERTIES AND FUNCTION OF CYTOCHROME *b*

Cytochrome *b* of Complex III is an electron-transferring, heme-containing protein which contains 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. However, there are two (even three) spectrally identified cytochromes *b*, i.e., classical cytochrome *b*, b_k (named after Keilin,1925) or b_{562}, with an Em=+40 mV and energy-transducing cytochrome *b*, b_t or b_{566} (and b_{558}) with an Em=-30 mV (Wikstrom, 1973). The relative amount of these different species of cytochrome *b* in rat liver mitochondria has been estimated to be 558:562:566=1:4:2 (Wikstrom, 1971). The existence of two species of cytochrome *b* is widely supported by observations such as the biphasic response of the kinetics of cytochrome *b* oxidation (Chance *et al*, 1970), the red shift and increased reduction of cytochrome *b* caused by antimycin (Pumphrey, 1962; Dutton *et al*, 1972; Wikstrom and Berden, 1972) and the different oxidation-reduction potentials (Dutton *et al*, 1970; Weiss, 1976). On the contrary, Wainio reviewed the components of cytochrome *b* and argued against the presence of an energy-transducing cytochrome *b* because (i) the values for the

redox potential were all underestimations and influenced by the redox state of other components, (ii) the measurements of cytochrome b at one wavelength in the kinetic experiments offered equivocal evidence, (iii) the red shift in the α peak of cytochrome b suggests that there may be more than one component of cytochrome b: one is reduced while the other is oxidized (Wainio, 1977). However, the fact that electron paramagnetic resonance spectroscopy and circular dichroism spectroscopy distinguish two components: a b_{562} and a b_{566} in the complex III of beef heart mitochondria (Dervartanian et al, 1973; Orme-Johnson et al, 1974) provides more convincing evidence for the existence of two components of cytochrome b.

Nevertheless, these two different species of cytochrome b, upon isolation from the surrounding membrane, became indistinguishable (Weiss, 1976). The absorbance spectra of various cytochrome b preparations either from beef heart, Neurospora or from yeast show only one cytochrome b with an α-absorbance maxima at 560-562 nm. The amino acid composition of beef heart cytochrome b and Neurospora cytochrome b are comparable, having a high content of leucine and isoleucine and a low content of lysine and arginine (Weiss and Ziganke, 1978). Furthermore, partial amino acid sequences of the proteins from Neurospora and beef heart, including the carboxyl- and amino terminals, showed only one unique sequence (von Jagow

et al, 1978b; Weiss and Ziganke, 1976).

The isolation of a dimeric form of cytochrome b from Neurospora (Weiss and Ziganke, 1974) correlates with the occurrence of two cytochromes b per cytochrome c_1 in complex III (Rieske, 1976; Beattie et al, 1980). Cytochrome b in a dimeric form was also isolated from a beef heart preparation of complex III (von Jagow et al, 1978b). Attempts to demonstrate a difference between the two monomers have failed so far (Weiss, 1976). Weiss and Ziganke (1976) claimed to have separated two cytochrome b subunits by hydroxylapatite chromatography but the two subunits were indistinguishable by gel filtration. These two cytochrome b subunits have similiar amino acid compositions and end terminal groups, but little else has been reported about them.

At present, it remains difficult to describe the function of cytochrome b; however, it is clear that its function is intimately interconnected with other subunits of complex III. In the literature, numerous hypothetical schemes have been outlined for the passage of electrons through the cytochrome b-c_1 complex (Papa, 1976). Recently, von Jagow and Sebald (1980) offered a preliminary scheme for the arrangement of the electron carriers in complex III:

$$\text{-----Q} \rightleftharpoons \begin{matrix}\text{cytochrome b}\\ \text{cytochrome b}\end{matrix} \rightleftharpoons (Q) \rightleftharpoons \text{Fe/S} \rightleftharpoons \text{cytochrome } c_1 \rightleftharpoons \text{cytochrome c}$$

The presumptive direct electron donor for the reduction of cytochrome b is ubiquinone-10 (Klingenberg, 1968; Kroger and Klingenberg, 1970), while a second ubiquinone 10 has been suggested as the immediate electron acceptor of cytochrome b. Recent experiments have indicated that the iron-sulfur protein may be the electron acceptor (Trumpower and Edwards, 1979a, 1979b). Electron flow from succinate to cytochrome c only occurred when the iron-sulfur protein was present in a preparation containing both complexes II and III. In the absence of the iron-sulfur protein only cytochrome b was reduced by succinate, but not cytochrome c_1 (Trumpower and Edwards, 1979a, 1979b).

Recently, a model for the cytochrome b dimer of the ubiquinol - cytochrome c oxidoreductase as a proton translocator has been proposed (von Jagow and Engel, 1980). Their model assumes that a group R on one amino acid of cytochrome b is subject to a pk change depending on the redox state of the heme b center (von Jagow and Engel, 1980).

Before attempting to prove this hypothesis and to study the exact function of cytochrome b, a detailed understanding of the structure of the two components of cytochrome b is essential. In addition, it is an intriguing question, whether the dimeric cytochrome b represents a homo- or a hetero- protein, since the existence of the two functionally

different cytochromes b has generally been postulated. Our second goal in the present study was to purify two cytochromes b with distinct molecular weights from yeast mitochondria by a new purification scheme and to investigate the structural relationship between them.

4. BIOGENESIS OF CYTOCHROME *b*

The mitochondrion has an autonomous genome distinct from nuclear DNA and is capable of synthesizing some of its own polypeptides on its own ribosomes. Mitochondrial DNA(mtDNA) are replicated and transcribed into the mitochondrial DNAs, mRNAs and rRNAs, which are distinct from their nuclear counterparts (Berk and Clayton, 1974; Locker and Rabinowitz, 1979). Most of the mtDNA's seem to be closed circular duplexes of about 10^7 daltons (Lock and Rabinowitz, 1979) and with contour lengths of 4 to 6 μm in animal species (Borst, 1972). By contrast, mtDNA of *Saccharomyces Cerevisiae* is considerably larger, with a contour length of 25 μm and a molecular weight of 5×10^7 daltons, and contains about 75,000 base pairs.

The identification of mitochondrial translational products was 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 our laboratory have shown that both chloramphenicol and cycloheximide block the increase of the activities of coenzyme QH_2-cytochrome *c* reductase, cytochrome oxidase as well as oligomycin-sensitive ATPase in yeast undergoing glucose

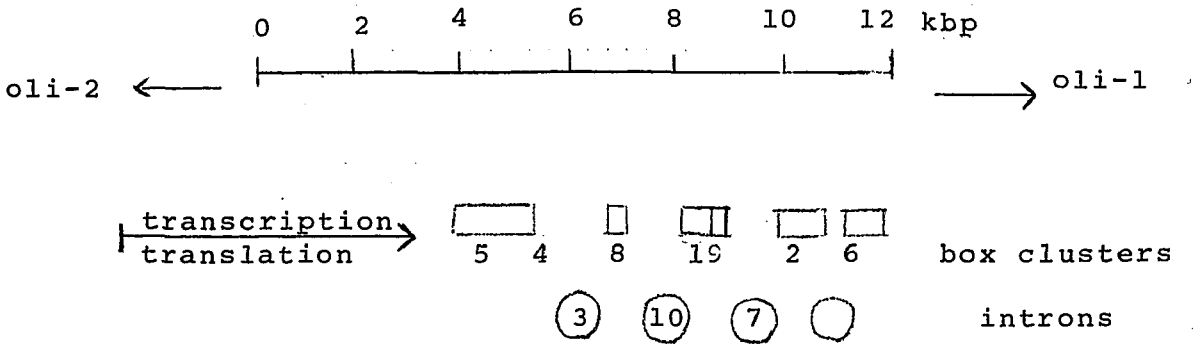
derepression, suggesting that the formation of complexes III, IV, and V requires products of both mitochondrial and cytoplasmical protein synthesis. From similar experiments, they demonstrated that the development of NADH-coenzyme Q reductase activity of complex I and succinate dehydrogenase activity 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 the cytochrome b-c_1 complex of the respiratory chain was also observed to require the coordinated synthesis of proteins in the two genetic systems by other groups (Weiss and Ziganke, 1974; Perlman and Mahler, 1974).

Cytochrome b in the cytochrome b-c_1 complex was shown in Neurospora crassa 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). Furthermore, Lin et al (1978) using a specific antiserum raised against a highly purified cytochrome b demonstrated that the incorporation of [^{3}H]leucine into the apoprotein of cytochrome b isolated by immunoprecipitation followed by gel electrophoresis was insensitive to cycloheximide and sensitive to acriflavin, erythromycin and chloramphenicol. The translation of cytochrome b on mitochondrial ribosomes was further confirmed by the observation

that a yeast petite mutant which lacked mitochondrial protein synthesis was not able to synthesize the cytochrome b apoprotein (Lin et al, 1978). All the other polypeptides of complex III including cytochrome c_1, have been shown to be products of cytoplasmical translation in experiments with yeast Saccharomyces cerevisiae (Katan et al,1976; Beattie et al, 1980) and Neurospora crassa (Weiss and Ziganke, 1977). Accordingly, three large subunits of cytochrome oxidase, two to three subunits of ATPase complex, together with cytochrome b belong to the small number of hydrophobic proteins that are translated on mitochondrial ribosomes.

The isolation and genetic analysis of various yeast mutants with altered function of complex III (Dujon et al, 1977) allowed the identification of the structural gene of apocytochrome b on mitochondrial DNA (Dujon, 1979). The region of mtDNA which codes for apocytochrome b (usually referred to as the cob-box region) is located between the oli-1 and oli-2 loci. In certain strains of Saccharomyces cerevisiae the cob-box region extends over 8 kilobases, or ten times the size required for the encoding of cytochrome b protein (about 30,000 daltons) (Borst and Grivell, 1978). This segment of the mitochondrial genome is now believed to exhibit a mosaic organization, in which five coding sequences (or exons, proposed by Gilbert, 1978) for the polypeptide product

are interspersed among four non-coding (intron or intervening) sequences (Dujon, 1979).



Genetic and physical Map of the Mitochondrial cytochrome b region in Strain ID41-6/161 (from Alexander et al, 1980)

There are five exons in the box clusters 5/4, 8, 1, 2 and 6 responsible for the primary structure of the polypeptide. Mutations in these sites produce a single, novel protein homologous to apocytochrome b but with the same or smaller molecular weight. The latter probably results from mutations causing premature chain termination (Solioz and Schatz, 1979; Tzagoloff et al, 1979). Intron mutations located in box clusters 3, 10 and 7 lack apocytochrome b and synthesize one or more polypeptides with no homology to the wild type cytochrome b (Haid et al, 1979; Hanson et al, 1979). Not only the expression of cytochrome b but also that of subunit I of cytochrome oxidase (molecular weight 42,000) is affected in these intron mutants (Haid et al,1979). These results suggest

that these intron genes may act in the regulation of the expression of cytochrome b and cytochrome oxidase subunit I (Alexander et al, 1980). Recently, by using double mutants in the cytochrome b region, Alexander et al (1980) suggested that the majority of novel proteins accumulating in the intron mutants are hybrid products in which the polypeptides are from RNA containing part of apocytochrome b exon sequences.

These genetic and biochemical studies provide the evidence for the existence of only one structural gene for apocytochrome b albeit a mosaic gene. Considering the presence of two functionally different cytochromes b in Complex III (described in the preceding section) two possibilities exist: (i) two structurally similiar cytochromes b undergoing a reversible conformational change which is responsible for their two different functions, (ii) two structurally different cytochromes b result from posttranslational modification of the primary product (suggested by von Jagow and Engel, 1980). Our data favors the latter possibility since two species of cytochrome b with distinct molecular weights of 29,000 and 31,000 daltons have been purified from one source - yeast Saccharomyces cerevisiae. The third goal in this thesis was to examine the biogenesis of these two purified cytochromes b and to investigate their structural differences with the aid of both antisera raised against these two forms of cytochrome b.

5. LOCALIZATION OF CYTOCHROME *b* IN MEMBRANE

The topography of the inner membrane of rat liver mitochondria was first investigated by Tinberg *et al* (1974) who used the probe, [^{35}S] diazobenzene sulfonate ([^{35}S]DABS) which has been employed to study the orientation of structural proteins of red cells in the membrane. This reagent is impermeable to intact human erythrocytes and reacts with amino acid residues on the surface of the membrane (Berg, 1969). Based on enzyme inactivation as a consequence of DABS labeling, Tinberg *et al* (1974) suggested that the ATPase complex and succinate dehydrogenase are both located on the matrix side of the inner membrane while the cytochrome *b*-c_1 complex is located on the outer surface of the membrane. Sodium dodecylsulfate gel analysis revealed that [^{35}S]DABS labeled two polypeptides (66,000 and 26,000) on the outer surface and five polypeptides (80,000, 66,000, 51,000, 48,000 and 26,000) on the inner surface, indicating an asymmetric distribution of proteins of the inner membrane. Different patterns of protein labeling with DABS were also observed in beef heart mitochondria (Schneider *et al*, 1972; Eytan *et al*, 1975). The identities of the labeled peptides was not established.

DABS does not inhibit cytochrome *c* oxidase activity either of submitochondrial particles or mitochondria from

rat liver (Tinberg et al, 1974), or of an isolated complex from beef heart (Eytan and Schatz, 1975). However, it inhibits duroquinol-cytochrome c reductase and labels nearly all the peptides of isolated complex III from beef heart mitochondria (Gellerfors and Nelson, 1977). Mendel-Hartvig and Nelson (1978) immunoprecipitated complex III from DABS incorporated mitochondria and submitochondrial particles using antiserum against complex III and found that the two core proteins were labeled from the matrix surface and a peptide of 29,000 dalton was heavily labeled from the cytoplasmic surface and weakly labeled from the matrix side. The latter correlates with the results obtained using lactoperoxidase plus ^{125}I to label mitochondria in which a peptide of 29,000 molecular weight was primarily labeled (Boxer, 1975; Brdiczka and Schumacher, 1976). These workers concluded that this 29,000 polypeptide is most likely cytochrome c_1 but did not rule out the possibility that this protein is cytochrome b.

Recently, the orientation of the polypeptides in beef heart complex III was determined by surface labeling intact mitochondria and submitochondrial particles separately with [^{35}S] diazobenzene sulfonate (Bell et al, 1979). This experiment, together with low levels of trypsin digestion to cleave the surface components (Ball et al, 1977) and cross-

linking studies (Smith and Capaldi, 1977), suggested a model for the arrangement of seven subunits of complex III (Bell *et al*, 1979). In this model, the two molecules of cytochrome *b* plus the core protein 2 span the mitochondrial membrane protruding from both sides, whereas, the iron sulfur protein, cytochrome c_1 and two small subunits VI, VII, face the cytosolic side of the membrane. Cytochrome c_1 is less peripheral than the small subunit VI. The two small polypeptides VIII and IX seem to be buried in the membrane, since they were not labeled with DABS from either side.

Considering the localization of the two functionally different cytochromes *b*, Bell *et al* (1979) claimed that the isolated beef heart mitochondria contain two cytochrome *b* of similiar molecular weight, both of which contribute to one band. These two cytochromes *b* could be localized on different sides of the membrane or both could span the membrane. The results obtained in the experiments with DABS labeling could not differentiate between these two possibilities. Nevertheless, electron paramagnetic resonance studies (Case *et al*, 1976) suggested that both b_{562} and b_{565} are close to the cytoplasmic side of the membrane. DiJeso *et al* (1978) reported that a specific antibody raised against cytochrome *b* from pig heart mitochondria inhibited electron transport of the respiratory chain in intact mitochondria, but had no effect

on submitochondrial particles again indicating that cytochrome b is located on the outer surface of the inner membrane. The relationship between this purified pig heart cytochrome b and two functional distinct cytochromes b is not clear. In contrast to the above findings, cytochrome b from Neurospora integrated into mitochondrial membrane was inaccessible to ^{125}I plus lactoperoxidase either from the matrix side or from the cytoplasmic surface suggesting that cytochrome b is buried in the interior of the mitochondrial inner membrane (Weiss and Ziganke, 1976) or inaccessible to the lactoperoxidase enzyme.

In the present thesis, two forms of cytochrome b have been purified from one source, yeast mitochondria, with distinct molecular weights, similar antigenic properties and identical peptide maps obtained after limited proteolysis. Moreover, both of the purified cytochromes b are mitochondrial translational products. The fourth goal in this dissertation was to localize these two species of cytochrome b from Saccharomyces cerevisiae by two approaches: first, the inhibition of cytochrome c reductase activity of mitochondria and submitochondrial particles by the two specific antibodies raised against these two types of cytochrome b, respectively, was studied. Secondly, the surface labeling from both sides of the inner membrane with [^{125}I] diazobenzene iodosulfonate followed by immunoprecipitation with the two antisera was

used to identify the localization of each cytochrome b in both mitochondria or submitochondrial particles.

CHAPTER II: MATERIALS AND METHODS

Growth of Yeast and Preparation of Mitochondria

Purification of Cytochrome b

Proteolytic Digestion

Immunological Studies

Labeling Cells and Electrophoresis

Enzymatic Assays

Labeling of Mitochondria and Submitochondrial Particles with [^{125}I]DABS

Materials

Growth of Yeast and Preparation of Mitochondria.

The strain of yeast used in the molecular weight determination and purification procedure was a diploid strain of Saccharomyces cerevisiae which has been used in our laboratory for several years. In the remaining studies a haploid strain, KL14-4A, was used.

Cells were grown aerobically at 30°C with 3% galactose as carbon source, under the conditions described previously (Brown and Beattie, 1977). Growth medium contained 0.3% yeast extract, 0.04% $CaCl_2$, 0.05% NaCl, 0.07% $MgSO_4.7H_2O$, 0.1% KH_2PO_4, 0.12% $(NH_4)_2SO_4$, 0.5 mg% $FeCl_3$ and 3% galactose.

Isolation of mitochondria by cell-breakage with glass-beads. Yeast cells were harvested by centrifugation for 5 min at 5000g, washed with distilled water, and suspended with STE buffer (0.25 M sucrose, 0.02 M Tris-HCl, and 1 mM EDTA containing 1 mM PMSF, pH 7.5). Cells were then broken by shaking with glass beads two times for 20 sec in a Bronwill shaker, as described by Kim and Beattie (1973). The suspension was centrifuged for 10 min at 1000g to remove unbroken cells and debris. After sedimenting at 18,000g for 20 min, mitochondria were washed by suspension with a glass-glass homogenizer in STE buffer and centrifuged for 20 min at the same speed.

Isolation of mitochondria from spheroplasts. The procedures for preparation of spheroplasts and isolation of

mitochondria were modifications of the procedure described by Schatz and Kovac (1974). Cells were harvested in late log phase, washed with distilled water and centrifuged at 1200g for 5 min before an incubation in 20 mM Tris-Cl, 20 mM EDTA, 50 mM β-mercaptoethanol, pH 9.0, for 15 min at 30°C to soften the cell wall. This buffer was removed by washing the cells three times with distilled water and recentrifuging at 1200g for 5 min. The cells were then suspended in 1 M sorbitol containing 20 mM KH_2PO_4, pH 7.4, at a concentration of 0.25 g cells per ml, and lysed with 2 mg Zymolyase 5000 per g cells at 30°C for 1 hr in a metabolic shaker. After centrifugation at 1200g for 5 min, the sedimented spheroplasts were washed and resuspended in 0.6 M minnitol, 1 mM EDTA, 20 mM Tris-Cl, 1 mM PMSF, 0.1% bovine serum albumin, pH 6.8, at a ratio of 3 g cells per 10 ml buffer. The spheroplast suspension was shaken in 10 to 20 ml aliquots in a mini-Waring blendor for 20 to 25 sec at high speed. The broken suspension was centrifuged 5 min at 1200g to remove unbroken spheroplasts. After sedimenting at 18000g for 20 min, mitochondria were resuspended in a glass-glass homogenizer in 0.6 M mannitol, 10 mM sodium phosphate, pH 7.0, and centrifuged for 20 min at the same speed.

Mitoplast preparation. Mitoplasts were prepared by the digitonin fractionation procedure of Velours et al. (1977) with some modifications. Mitochondria, isolated from

spheroplasts as described above, were suspended in 0.6 M mannitol, 10 mM sodium phosphate, pH 7.0, at a protein concentration of 10 mg per ml. A stock solution of 35 mg digitonin per ml water, prepared freshly by gently heating in a hot water bath, was added to the mitochondrial suspension to a final concentration of 3 mg digitonin per 10 mg mitochondrial protein. After a 5 min incubation at 4°C, the mitoplasts were sedimented by a 10 min centrifugation at 12000g.

Preparation of submitochondrial particles. The mitochondrial pellet was resuspended in sodium phosphate buffer (0.1 M Na_2HPO_4, NaH_2PO_4, 0.5 mM EDTA, and 1 mM PMSF, pH 7.5) and sonicated for 2 min in 15 sec bursts in a Branson sonifier at a power setting of 4.5. The suspension was centrifuged for 10 min at 4300g in a Sorvall SS 34 rotor. The pellet was discarded and the supernatant was centrifuged for 30 min at 100,000g in a Spinco type 40 rotor. The pellet of submitochondrial particles was resuspended in sodium phosphate buffer and the protein concentration adjusted to 10 mg per ml.

Purification of Cytochrome b

Step 1: KCl/cholate extraction - To a suspension of submitochondrial particles, at a protein concentration of 10 mg per ml, was added a 20% solution of sodium cholate, pH 8.0, to give a final concentration of 3.5% cholate. After

75 mg of KCl per ml was added, the suspension was stirred for 1 to 3 hr at 4°C and then centrifuged 10 min at 12000g. The supernatant was kept at 4°C overnight and centrifuged again at 35000g for 10 min.

Step 2: Ammonium sulfate fractionation - Solid ammonium sulfate was added to the supernatant to a final concentration of 16% saturation while stirring. The suspension was maintained in an icebath for 90 min with constant stirring and centrifuged 10 min at 35000g. The yellowish green supernatant was discarded and the red pellet containing cytochrome b was homogenized in sodium phosphate buffer and the protein concentration adjusted to approximately 3 mg per ml. Sodium cholate at a final concentration of 3.5% was added to dissolve the protein and to reduce the turbidity. Difference absorption spectra were obtained with a Cary Model 15 at room temperature with dithionite in the sample cuvette and potassium ferricyanide in the reference cuvette. The heme b content of the preparation was calculated as described by Lin and Beattie (1978).

Step 3: Ultrogel filtration - The spectrally pure cytochrome b preparation precipitated by 16% saturated ammonium sulfate was solubilized in 0.1 M Tris-acetate, 5% sodium dodecyl sulfate, 5% 2-mercaptoethanol at room temperature for 2 hr and subjected to gel filtration through a 1 m long ultrogel ACA 44 (LKB, Bromma) column as described

by Weiss and Juchs (1978). The column was equilibrated with the gel buffer which contained 0.1% sodium dodecyl sulfate, 2 mM dithioerythritol, 50 mM Tris-acetate, pH 7.0, and 0.02% sodium azide and the sample was eluted with the same buffer. The flow rate was kept at 0.2 ml per min at room temperature. After the sample was loaded onto the column, approximately 300 fractions(1.6ml per fraction)were collected and the column was washed with 2 gel bed volumes (530 ml each) of gel buffer. Several fractions containing the heme of cytochrome b, as determined by the optical density at 418 nm, were pooled and concentrated in a minicon B15 concentrator.

Step 4: Preparative gel electrophoresis - After analysis by preliminary sodium dodecyl sulfate polyacrylamide gel electrophoresis, the fraction containing proteins with molecular weights in the 30000 dalton range was subjected to further electrophoresis on a super 10% acrylamide gel (25 cm x 12 cm x 0.4 cm thick). Super gels were stained with 0.25% Coomassie brilliant blue in 45% methanol, 9.2% acetic acid and destained by shaking in 5% methanol, 7.5% acetic acid. Two major bands with molecular weights of approximately 30000 were excised from the super gels and soaked in 0.1% dodecyl sulfate, 1 mM EDTA, 0.125 M Tris-HCl, pH 6.8 and 1 mM dithiothreitol for 1 hr. These gel slices were further subjected to a preparative gel electrophoresis consisting of a modified funnel-shaped 10% acrylamide gel with its bottom attached by

a close-ended dialyzing spectra/por 1 membrane tubing (molecular weight cut off 6-8000) (Fig. 1). The preparative gel electrophoresis was performed in the cold room at 4°C for 24 hr until the dye front had entered the dialysis tubing.

Step 5: Acetone extraction - The two samples, each containing a single polypeptide thus eluted from the gel slices, were present in a mixture of Coomassie brilliant blue and 50 mM Tris-glycine reservoir buffer containing 0.1% sodium dodecyl sulfate. To remove the dye, protein samples were extracted once with acidic acetone and twice with iced acetone according to Chua and Blomberg (1979).

The purification procedure is summarized in the following scheme. (Figs. 2,3)

Proteolytic Digestion.

Limited proteolytic digestion of purified protein I and protein II and labeled cytochrome b was performed by the method of Cleveland et al (1977). To obtain samples of protein I and II for digestion, the spectrally pure cytochrome b preparation (purification Step 2) was separated on a super gel (25 cm x 12 cm x 0.4 cm). Two major Coomassie blue stained bands, one corresponding to protein I of 31000 dalton and the other corresponding to protein II of 29000 dalton were excised, trimmed to slices of 10 mm x 4 mm x 1 mm and soaked

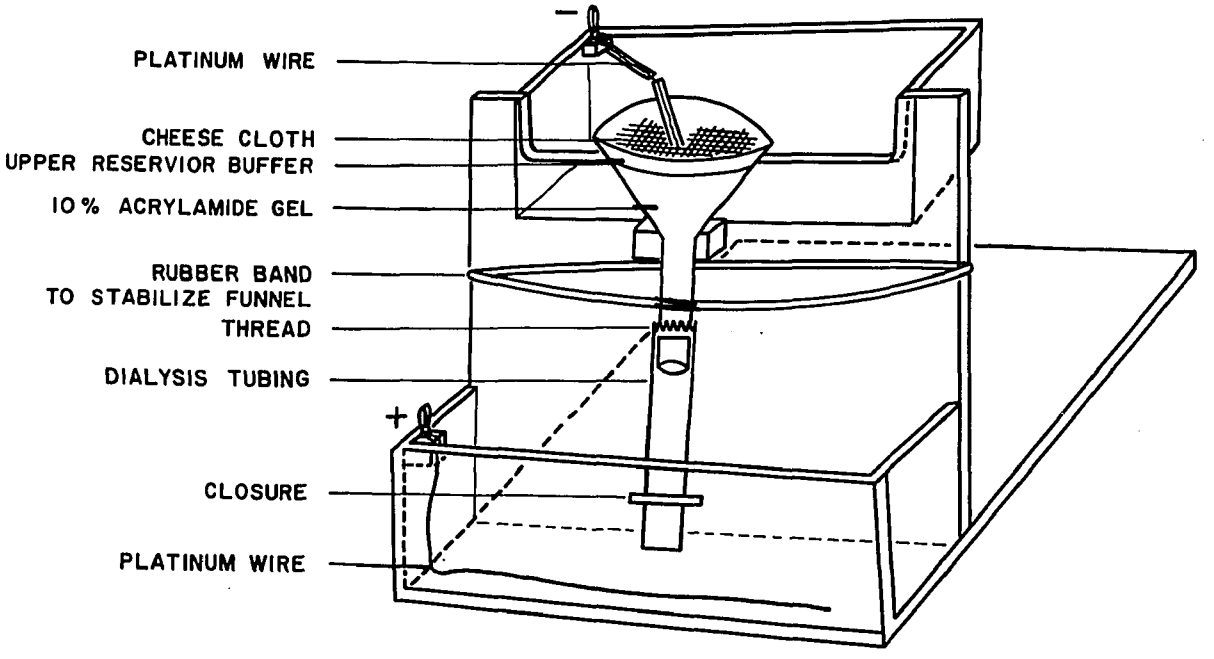


Fig. 1. Modified funnel-shaped acrylamide gel electrophoresis apparatus for protein elution from Coomassie brilliant blue stained gel slices.

Fig. 2. Purification Scheme of Cytochrome b from Growing Yeast Cells.

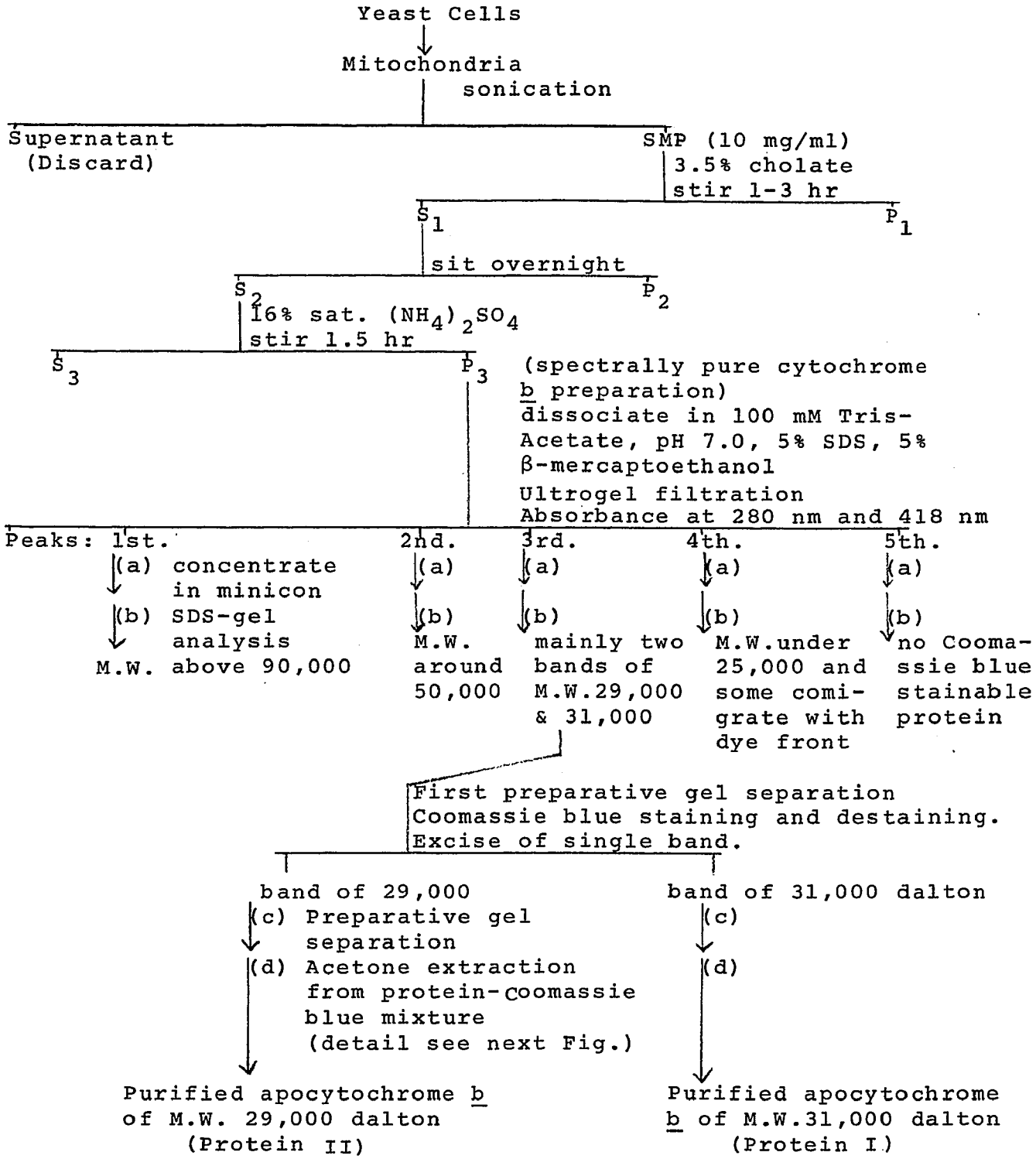
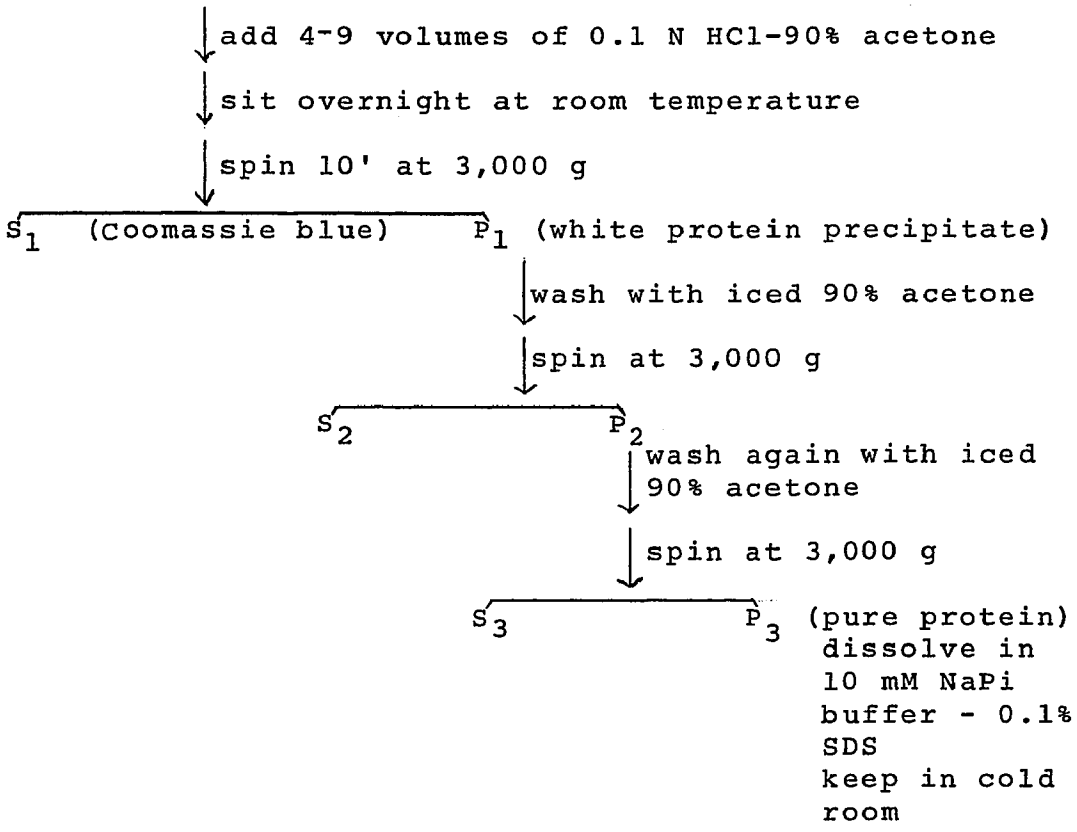


Fig. 3. Acetone extraction of protein from protein-coomassie blue mixture



in buffer containing 0.1% dodecyl sulfate, 1 mM EDTA, 0.125 M Tris-HCl, pH 6.8 and 1 mM dithiothreitol for 30 min prior to transfer to the sample well for digestion. Digestion was performed in a tall slab gel (14 cm long, 1 mm or 2 mm thick) containing 15% acrylamide with a stacking gel 4 cm long. S. aureus V8 protease (25 or 50µg) was loaded in each sample well together with the gel slice to be analyzed. Sample proteins were digested in the stacking gel for 40 min at 37°C, while the remaining electrophoresis was performed at 4°C to avoid further digestion.

For the digestion of labeled cytochrome b, mitochondria extracted from cells labeled in vivo with [^{35}S] methionine in the presence of cycloheximide were dissociated in 5% sodium dodecyl sulfate, 2 mM EDTA, 5% 2-mercaptoethanol, 50 mM Tris-HCl, pH 6.8, 10% glycerol and subjected to electrophoresis on a 10% acrylamide gel. Identification of the labeled cytochrome b band was made possible by exposing a dried stripe of the gel for 4 days on an x-ray film. This identified cytochrome b band was excised from the nondried part of the same gel which had been kept in the destaining solution and subjected to electrophoresis on the 15% acrylamide digestion gel. Fingerprints obtained after limited proteolysis were stained with Coomassie blue.

Immunological Studies.

Preparation of antibodies. Purified protein I suspended in 0.2 ml of 10 mM sodium phosphate buffer, pH 7.0, containing 0.1% sodium dodecyl sulfate was adjusted to 1.0 ml with physiological saline. Two rabbits were injected with a total of 225 µg protein in two equal doses intravenously in the ear. The second injection was 40 days after the first injection. Samples of blood were collected on the 40th day, the 47th day and the 64th day after the first injection. Sera were obtained by centrifugation at 1500g for 20 min.

Antiserum against protein II was raised by the same procedure except that the total dose of 212 µg of purified protein was solubilized in 10 mM sodium phosphate, pH 7.0, containing 0.5% sodium dodecyl sulfate prior to mixing with saline.

In some experiments an IgG fraction was further purified from both antisera as described by Chan and Schatz (1979). Antisera were incubated 90 min with 34% saturated ammonium sulfate at 4°C and centrifuged at 3000g for 25 min. The ammonium sulfate precipitates were suspended in 10 mM 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 concentrated IgG fraction in phosphate buffer containing 0.1% sodium azide was dialyzed against the same buffer at 4°C for at least 24 hr to remove the ammonium salt and then stored at 4°C. The protein concentration of dialyzed IgG fraction

was adjusted to 8.5 mg per ml.

Control serum was obtained from non-immunized rabbits. The same ammonium sulfate fractionation procedure was performed with control serum.

Immunodiffusion and immunoelectrophoresis. The method for the Ouchterlony diffusion test was essentially derived from Ouchterlony (1953). The diffusion plates contained 1% agarose, 150 mM NaCl, 1% Triton X-100, 0.1% NaN_3 and 38 mM Tris-glycine, pH 8.6. Samples of the ultrogel fractions containing protein I and protein II were solubilized in 5% Triton X-100, 1% sodium dodecyl sulfate, 150 mM NaCl, 38 mM Tris-glycine, pH 8.6 at a protein concentration of 8 mg per ml. An aliquot of 20 microliters of the solubilized sample was placed in the central well, while aliquots of 20 microliters of antisera collected on different days and control serum were placed in peripheral wells. Six days were required for the proteins to diffuse at room temperature and another six days were needed to rinse out the non-precipitated material in rinsing buffer (150 mM NaCl, 1% Triton, 38 mM Tris-glycine, pH 8.6). The plates were stained and destained as were the slab gels except that the Canalco destainer was not used here.

Counter immunoelectrophoresis was performed according to Lin et al (1978). Agarose slides were made of 1% agarose, 150 mM NaCl, 1% Triton X-100 and 0.1% NaN_3 in

38 mM Tris-glycine, pH 8.6. The latter was also used as the running buffer. An aliquot of 10 to 20 microliters of purified protein I (1.2 mg per ml), purified protein II (1.2 mg per ml) or the suspension of spectrally pure cytochrome b preparation (8.5 mg per ml) was placed in each cathodic well while an aliquot of 10 microliters of antisera was placed in the anionic well. A minimum of 3 hr was needed for electrophoresis when a current of 4 milliamperes per slide was applied. For the big agarose slide containing 6 sample wells on each side, a 40 milliampere current was applied for 3 hr to complete the electrophoresis. Subsequently, agarose slides were pressed for 30 min to remove the buffer, and rinsed, stained and destained by the same method as used for the Ouchterlony agarose plates.

For immunoprecipitation, labeled mitochondria, mitoplasts or submitochondrial particles were solubilized by the following techniques:(1) 1% Lubrol WX and 1% sodium deoxycholate at 4°C for 1 hr;(2)1 M KCl and 1% Triton X-100(Bell et al,1979)by shaking at 37°C for 2 hr, and (3) 5% Triton X-100, 0.1% sodium dodecyl sulfate (Nelson et al,1977) by shaking at 37°C for 2 hr. The solubilized extracts were then centrifuged at 12000g for 10 min to remove any unsolubilized material.

An immunotitration curve was obtained by incubating a fixed amount of solubilized membrane extract (10 microliters, 10 mg per ml) with varying amounts of antiserum. The

mixtures of extract and serum were incubated at 4°C for 14 hr and then centrifuged for 2 min in an Eppendoff centrifuge. The immunoprecipitates were washed 3 times prior to addition of dissociation buffer (5% sodium dodecyl sulfate, 2 mM EDTA, 5% 2-mercaptoethanol, 50 mM Tris-HCl, pH 6.8, 10% glycerol). The samples were incubated at 37°C for 2 hr or at room temperature overnight. The dissociated precipitates were either counted in Liquiscint (National Diagnostics, a liquid scintillation fluid) or further analyzed by polyacrylamide gel electrophoresis.

Labeling of Cells and Electrophoresis.

Cells were grown in growth medium containing 0.67% yeast nitrogen base, 0.2% yeast extract, 1% glucose and 5 mCi per liter of [^{3}H]leucine for 10 generations prior to harvest. Cells were also labeled with [^{35}S]methionine or [^{3}H]leucine under non-growing condition in the presence of cycloheximide as described previously by Beattie et al (1979). Cells were harvested in early stationary phase and washed twice with 100 ml of water. The cell pellet was weighed and resuspended in incubation medium containing 2% glucose and the inorganic salts described by Saltzgaber and Schatz (1978) to a concentration of 200 mg of wet cells per ml. The cell suspension was shaken for 5 min in a water bath at 30°C, after which cycloheximide, to a final concentration of 1 mg per ml, was added and the incubation continued for 5 min. Labeled [^{35}S]methionine

(75-100 μCi per 100 mg of cells) was then added and the incubation was continued for the times indicated. Labeling was stopped by the addition of 10 mM unlabeled methionine. The cells were then harvested and washed with 20 ml of water containing 0.5 mg of cycloheximide per ml and mitochondria prepared.

Electrophoresis. Slab gels (10 cm long, 1.2 mm thick) were prepared as described by Studier (1973) with minor modifications. The discontinuous sodium dodecyl sulfate-Tris buffer system (Laemmli, 1970) was used for electrophoresis. The stacking gels were 5% acrylamide and the running gels were 10% acrylamide except where indicated. In some cases disk electrophoresis in glass tubes of 12 cm length was performed in gels of different porosities (5-15% acrylamide) by using sodium dodecyl sulfate-phosphate buffer, according to Weber and Osborn (1969), and by using sodium dodecyl sulfate-urea buffer, according to Swank and Munkres (1971), for gels of 10% acrylamide.

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 (14,000) was used for molecular weight determination. The slab and disk gels were stained for 2 hr with 1.25% Coomassie brilliant blue in 45% methanol, 9.2% acetic acid and destained in a

Canalco destainer with destaining solution containing 5.0% methanol and 7.5% acetic acid. In order to eliminate the variations due to gel elongation and shrinkage during the staining and destaining processes, the length of the gel was determined before and after staining and the position of the tracker dye was marked with a small needle.

For radioactive samples, slab gels were dried for autoradiography and exposed to Kodak NS-5T x-ray film at -70°C. Disk gels were sliced every 1 mm or 2 mm thick with a Gilson gel slicer which automatically adds drops of hydrogen peroxide to each vial. In some cases, longitudinal strips from a slab gel were also sliced manually using a slicer made of a row of 100 razors. It was necessary to freeze the gel strips on a piece of flat dry ice prior to slicing. The gel slices were digested with a few drops of hydrogen peroxide at 70°C for a minimum of 3 hr. After addition of Liquiscint, they were counted on a Beckmann counter.

Sample preparations. The following dissociating systems were used to solubilize the proteins for electrophoresis: for disk gels prepared in sodium dodecyl sulfate-phosphate buffer, 0.01 M sodium phosphate, pH 7.2, 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, and 10% glycerol; for the sodium dodecyl sulfate-urea system, 0.01 M Tris-phosphate, pH 6.8, 1% sodium dodecyl sulfate, 8 M urea, 1% 2-mercapto-ethanol; for the discontinuous buffer system (slab gels),

0.05 M Tris-HCl, pH 6.8, 5% sodium dodecyl sulfate, 2 mM EDTA, 150 mM 2-mercaptoethanol, 10% glycerol. Samples treated with sodium dodecyl sulfate-urea buffer were heated for 10 min at 60°C and stored overnight at room temperature. For electrophoresis in the other buffers, the samples were treated either overnight at 20°C, for 2 hr at 37°C, for 20 min at 70°C, or for 3 min at 100°C. Bromophenol Blue (0.1%) in 25% glycerol was added as tracker dye to each sample, prior to electrophoresis.

Labeled mitochondria were adjusted to 2-6 mg of protein per ml and to 30000-40000 counts per 10-20 µl of dissociating medium.

Ferguson plots. The relative mobilities of cytochrome b, of other mitochondrial translational products, and of the standard proteins were plotted against the acrylamide concentration, according to Ferguson (1964). The "free mobilities" of the proteins were obtained by extrapolating the respective lines to "zero" concentration of acrylamide.

Enzymatic Assays.

Cytochrome c reductase activity was performed according to the method of Brown and Beattie (1977) except that sodium succinate and NADH were not used in the reaction mixture.

DBH_2^1, the reduced form of DB, 2,3-dimethoxy-5-methyl-6-n-decyl-1,4-benzoquinone, was used as a substrate. The reduction of DB to prepare DBH_2 was carried out by the method

of Rieske (1976). An aliquot (10 or 20 microliters) of mitochondria, submitochondrial particles or mitoplasts at a concentration of 1.5 mg protein per ml in STE buffer (0.25 M sucrose, 0.05 M Tris-HCl, 1 mM EDTA, pH 7.5) was used in each assay.

To investigate the effect of digitonin and various concentrations of detergent on the cytochrome c reductase activity of mitoplasts, a stock 3.5% digitonin solution, prepared as described above, was added to mitochondria, suspended at 10 mg protein per ml, to obtain a final concentration of 1 mg, 3 mg, or 10 mg digitonin per 10 mg protein, respectively. After 5 min incubation, the digitonin-treated mitochondrial fraction were centrifuged, washed and resuspended in 0.6 M mannitol, 10 mM sodium phosphate, pH 7.0, at 1.5 mg/ml protein concentration. To each mitoplast fraction, a 20% cholate solution was added just prior to assay to obtain a final concentration of 1%, 2% and 3% cholate, respectively.

For the inhibition experiments using 10, 20, or 40 µl of purified IgG fractions containing 8.5 mg protein per ml were incubated with 10 µl (15 µg protein) of 2% cholate solubilized submitochondrial particle preparation or a mitoplast suspension for 45 min at room temperature before each assay. The same amounts of IgG fraction purified from control serum were used in parallel as a control. All the enzyme assays were performed at room temperature.

Labeling of Mitochondria and Submitochondrial Particles with [^{125}I]DABS

[^{125}I]diazotized iodosulfanilic acid was prepared according to New England Nuclear product information. After addition of 45 nmoles of sulfanilic acid as a carrier, 1 mCi of [^{125}I]iodosulfanilic acid (2200 Ci per mmol) in 10 µl of water was diazotized by adding 5 µl of 50 mM sodium nitrate and 5 µl of 0.1 N hydrochloric acid at 4°C, followed by neutralization with 170 µl of 100 mM phosphate buffer (pH 8.3).

An aliquot of 100 µl of mitochondria were suspended at 2 mg protein per ml in 0.25 M sucrose and 10 mM sodium phosphate, pH 7.5, and a 100 µl aliquot of submitochondrial particles in 0.1 M sodium phosphate, 0.5 mM EDTA, pH 7.5, and incubated with 400 µCi or 8 µCi of [^{125}I]DABS at 4°C for 30 min or 60 min with frequent vortexing. Labeling was terminated by addition of 5 mM histidine and immediate centrifugation. Iodinated mitochondria and submitochondrial particles were washed twice with 4 ml of 100 mM Tris-HCl, 5 mM histidine, pH 7.5 and twice with 100 mM Tris-HCl, 2.5% bovine serum albumin and then solubilized either with 1% Lubrol - 1% sodium deoxycholate at 4°C for 1 hr or 1% Triton X-100 - 1 M potassium chloride at 37°C for 2 hr. After centrifugation at 12,000 g for 10 min to remove unsolubilized membranes, labeled mitochondria and submitochondrial particles were immunoincubated with the optimum amount of IgG fractions I and II and the control IgG fraction. Iodinated immunoprecipitates were analyzed on a

10% acrylamide gel electrophoresis and autoradiographed as described above.

Materials:

L-[^{35}S]methionine (1000 Ci.mmol^{-1}) and L-[4,5-^{3}H]leucine (55 Ci.mmol^{-1}) were obtained from Amersham. [^{125}I]iodo-sulfanilic acid (2200 Ci.mmol^{-1}) was purchased from New England Nuclear. Zymolyase 5000 was from Kirin brewery, Japan. Sodium cholate and sodium deoxycholate were from Calbiochem. Digitonin, cycloheximide, Coomassie brilliant blue, phenylmethanesulfonyl fluoride and β-mercaptoethanol were obtained from Sigma. Ultrogel AcA 44 was from LKB, Bromma; Minicon B15 concentrator was from Amicon. Acrylamide, bisacrylamide and TEMED (N,N,N',N'-tetramethylenediamine) were from Eastman. Protein A sepharose was purchased from Pharmacia, whereas staphylococcus aureus V8 protease was from Miles Laboratories. Other chemicals used were of the highest purity.

CHAPTER III: RESULTS

1. Molecular Weight Determination of Cytochrome *b*

2. New Purification of Cytochrome *b* from Growing Yeast

3. Identification of Protein I and Protein II as Apocytochrome *b*

4. Biogenesis of Apocytochrome *b*-I and Apocytochrome *b*-II

5. Localization of Apocytochrome *b*-I and *b*-II.

1. MOLECULAR WEIGHT DETERMINATION OF CYTOCHROME b

Yeast cells were labeled with [^{35}S]methionine in the presence of cycloheximide under non-growing conditions and the translation products present in intact mitochondria examined by dodecyl sulfate gel electrophoresis at concentrations of acrylamide varying from 7.5% to 15% (Fig.4). The anomalous migration behavior of cytochrome b at different concentrations of acrylamide as compared to standard proteins or to other mitochondrial products is clear. At low concentrations of acrylamide, the apparent molecular weight of cytochrome b using carbonic anhydrase as a 30,000 dalton standard, is less than 30,000, while at higher acrylamide concentrations, its molecular weight is significantly greater than 30,000. In addition, Figure 4 shows that VAR 1 (Perlman, et al. 1977) plus a minor product of mitochondrial protein synthesis with a molecular weight of 67,000 migrate at the same rate as do standard proteins in different concentrations of acrylamide, while VAR 3, presumably a subunit of the oligomycin-sensitive ATPase (Perlman et al. 1977), also displays anomalous behavior. We have confirmed the results of Cabral et al. (1978) that subunit I of cytochrome oxidase behaves abnormally under these conditions while subunits II and III behave normally (data not shown).

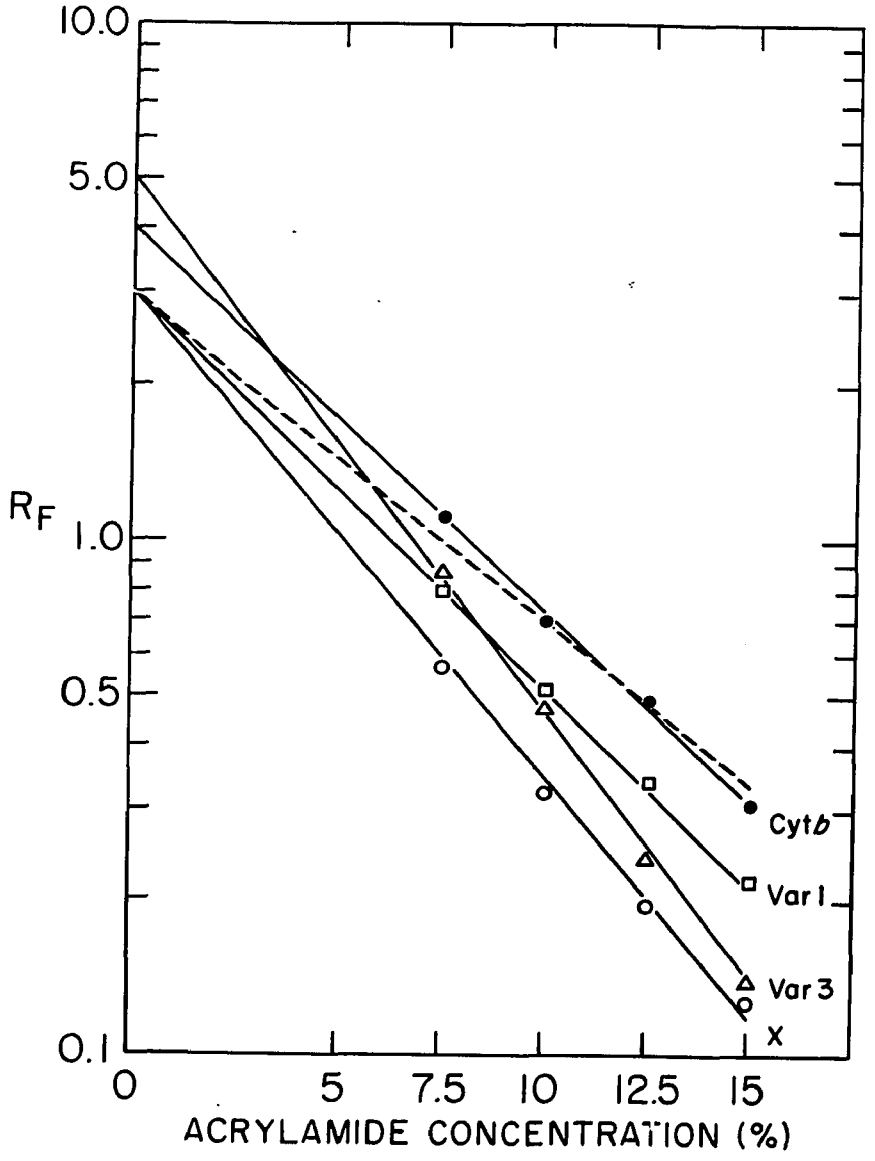


Fig.4. Ferguson plot of mitochondrial translation products. (□——□) VAR 1; (△——△) VAR 3; (●——●) cytochrome b; (○——○) , a mitochondrially synthesized polypeptide with a molecular weight of 67,000; (- - - -) carbonic anhydrase, 30,000-dalton standard.

All the data indicating that cytochrome b behaved anomalously during dodecyl sulfate gel electrophoresis were obtained with either intact mitochondria or a purified b-c_1 complex (Groot et al. 1978). We felt that it would be of interest to study the migration of cytochrome b in a partially purified preparation in which cytochrome a and c_1 had been completely removed as determined by spectral analysis (Fig.5). The specific heme content of this preparation obtained by ammonium sulfate fraction of cholate-solubilized submitochondrial particles was 7.1 nmoles/mg protein, a value comparable to that observed in the b-c_1 complex obtained from yeast mitochondria (Siedow et al, 1978). When analyzed on a 10% acrylamide gel in the presence of sodium dodecyl sulfate, the presence of 4 major polypeptides with apparent molecular weights of 31,000, 35,000, 48,000 and 50,000 was revealed as well as 4-5 minor bands (Fig.6). The identification of the band of 31,000 daltons as cytochrome b was made possible by comparison with an autoradiography of mitochondrial products (Fig.6), or with the labeled band present in the immunoprecipitate formed when labeled mitochondria were incubated with the specific antiserum against cytochrome b. The identity of the 35,000 dalton polypeptide is unknown; however, the higher molecular weight bands may be the so-called core proteins present in the b-c_1 complex as they have a similar molecular weight and comigrate with the core proteins of an isolated complex during gel electrophoresis (Fig.6).

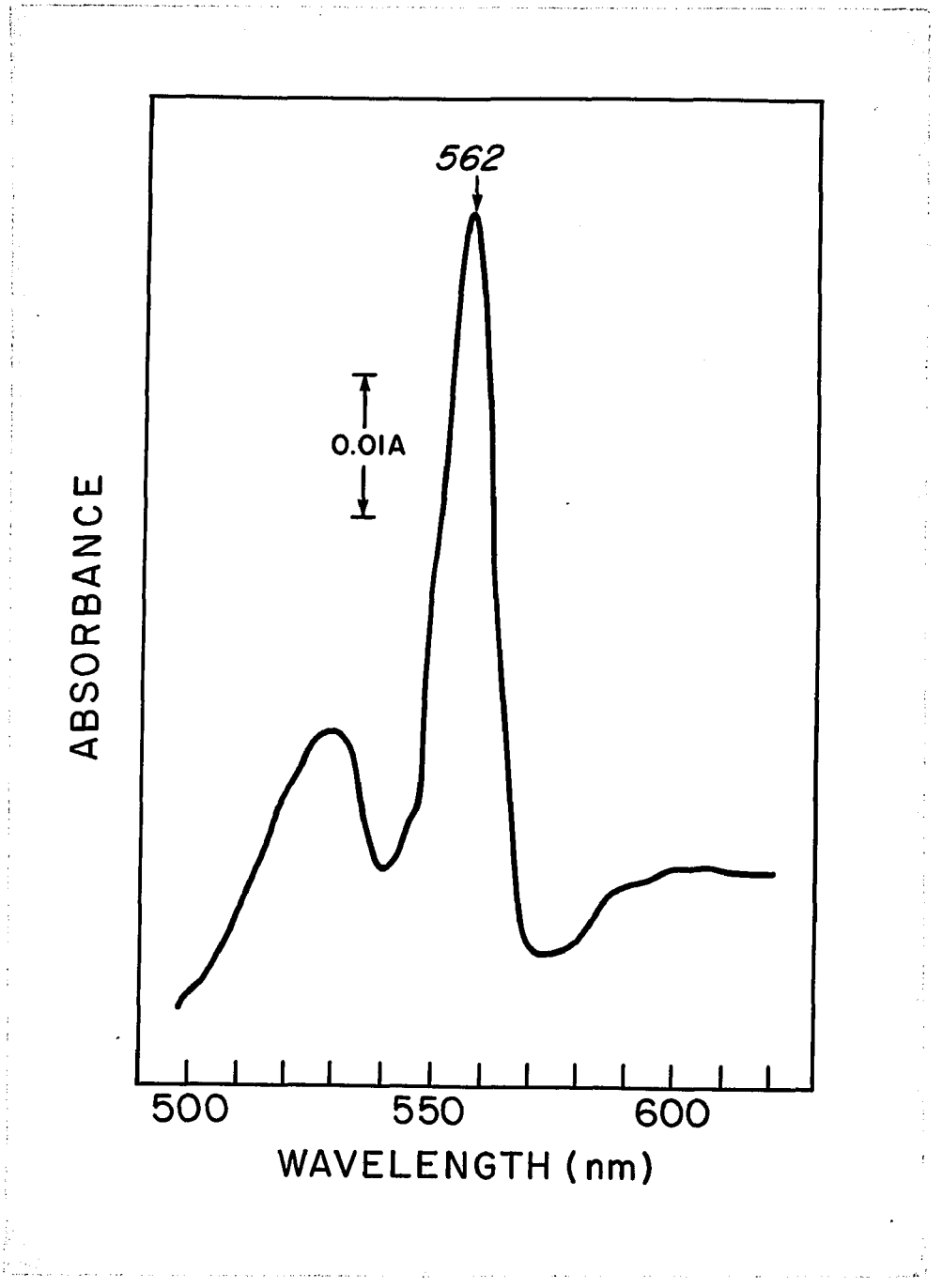


Fig. 5. Difference spectrum of the cytochrome b preparation from yeast mitochondria. Dithionite-reduced vs. ferricyanide-oxidized spectrum was recorded at room temperature. Sample was suspended in sodium phosphate buffer, pH 7.5, containing 3.5% cholate at a protein concentration of 2.9 mg/ml.

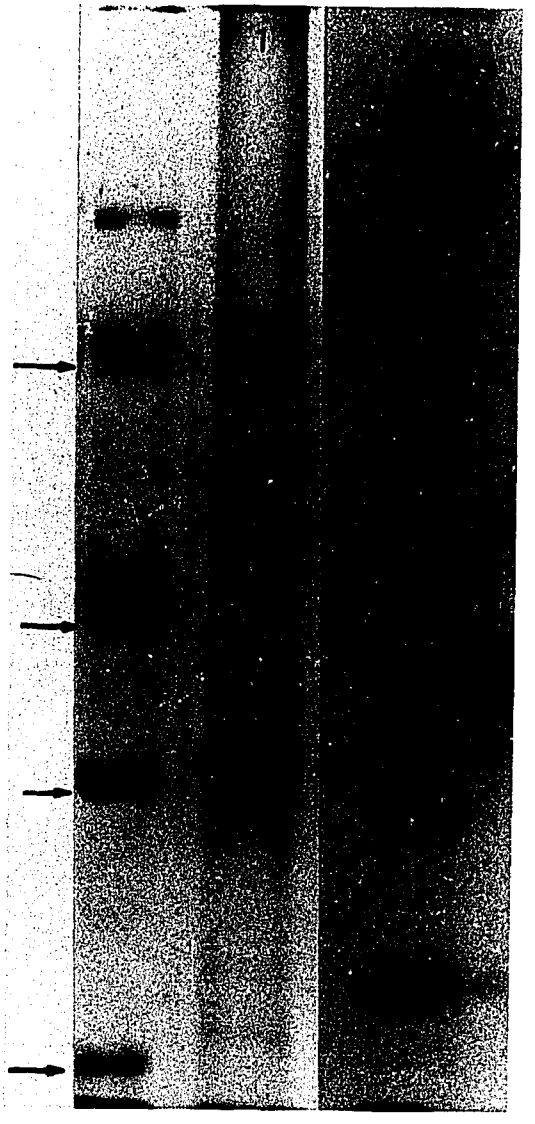


Fig. 6. Identification of the 31,000 dalton polypeptide in the partially purified cytochrome b preparation as cytochrome b. Labeled mitochondrial translation products were separated on the same 10% polyacrylamide slab gel with a partially purified cytochrome b preparation. The gel was stained with Coomassie Blue and then dried for autoradiography. Lane 1 contains a stained cytochrome b preparation and lane 2 the labeled mitochondrial translation products. The arrows indicate the

(contd...

positions of the standard proteins: bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), and myoglobin (17,200).

This preparation of cytochrome b was also subjected to electrophoresis at various acrylamide concentrations ranging from 5% to 15%. As seen in Figure 7, the band corresponding to cytochrome b migrates normally and the R_F values obtained when plotted against acrylamide concentration extrapolate back to the same point as do the standard proteins. The molecular weight of cytochrome b calculated as the mean of 4 determinations at each acrylamide concentration was 31,000 ± 600.

The determinations were all performed on a cytochrome b preparation obtained when PMSF was added to all solutions used after the yeast cells were harvested. We also determined the molecular weight of cytochrome b when the purification was performed in the absence of PMSF. As seen in Figure 8, the pattern of polypeptides obtained was identical when the preparation of cytochrome b was performed in the presence or absence of PMSF. Each polypeptide migrated with the same molecular weight. In addition, we varied the temperature used to solubilize the preparation prior to dodecyl sulfate gel electrophoresis: 100°C for 3 min, 70°C for 20 min, 37°C for 2 hr or 20°C overnight. Figure 8 indicates the similarity in migration behavior of the bands in these preparations treated at 100°C or 37°C with or without PMSF. Identical results were also obtained after the 70°C or 20°C treatment (data not shown). These results are in contrast to those of Capaldi et al (1977)

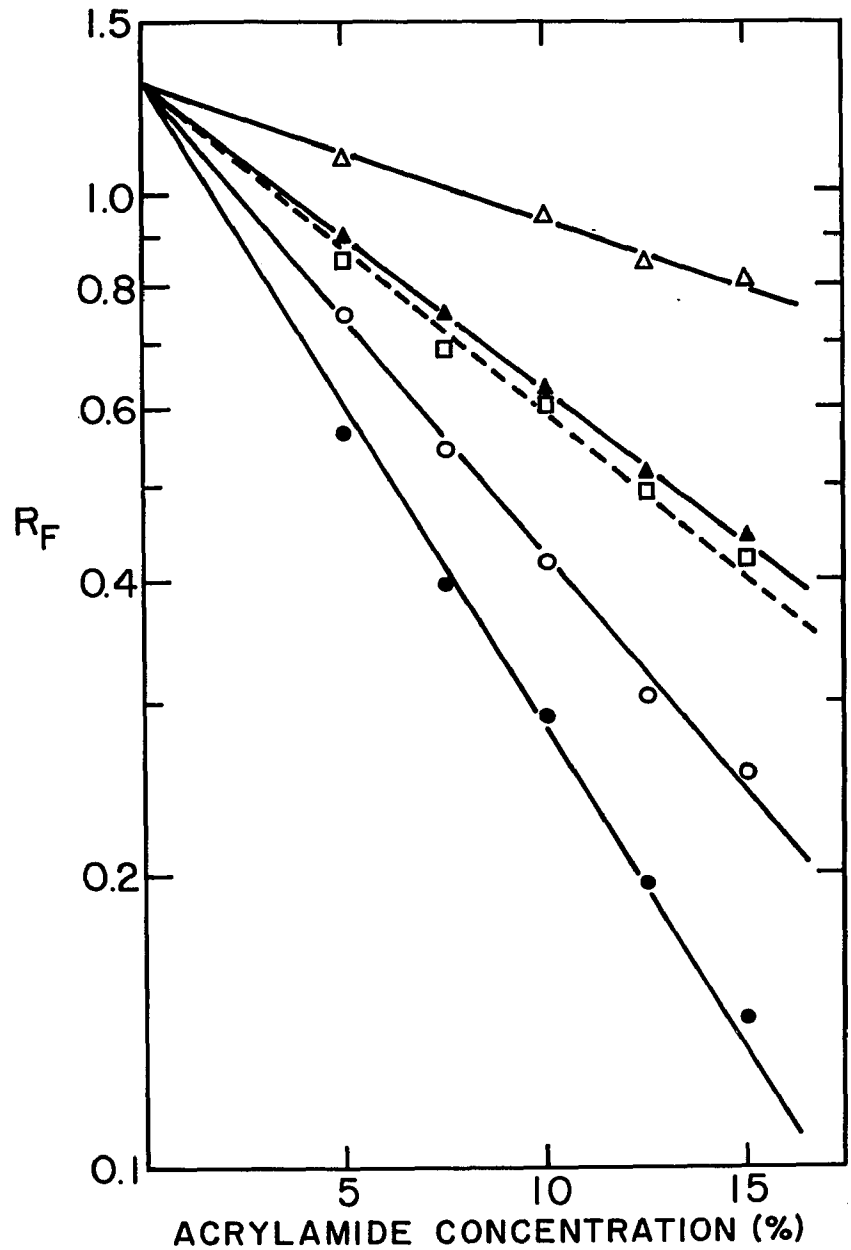


Fig. 7. Ferguson plot of cytochrome b in the partially purified preparation as compared to standard proteins. △—△ , myoglobin (17,200); ▲—▲ , carbonic anhydrase (30,000); o—o ovalbumin (43,000); ●—● , bovine serum albumin (68,000); □---□ , cytochrome b.

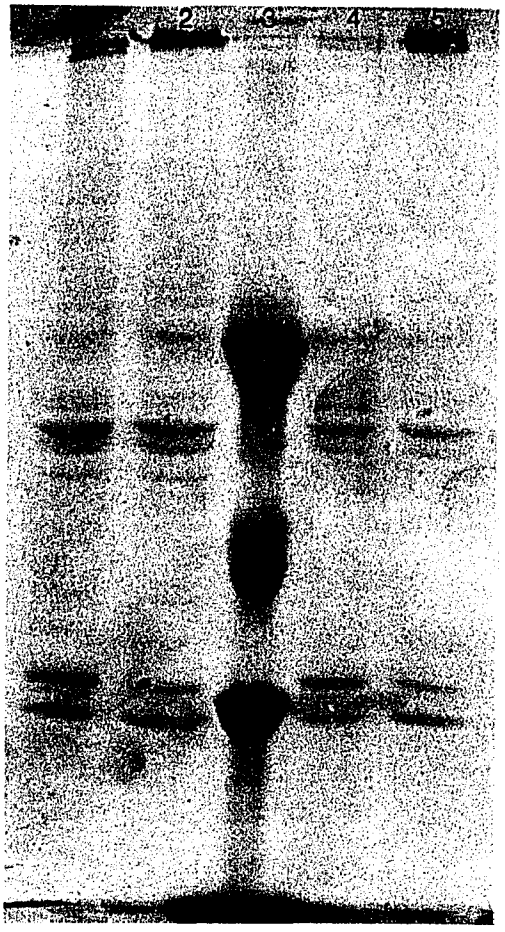


Fig. 8. Gel patterns of the cytochrome b preparation showing the effect of dissociation temperature and PMSF used during preparation. The lanes are numbered from left to right. Lane 1 contains a cytochrome b preparation obtained in the presence of PMSF and treated at 37°C for 2 hr. Lane 2 contains a cytochrome b preparation obtained in the presence of PMSF and treated at 100°C for 3 min. Lane 3 contains standard proteins includ-

(contd...

ing bovine serum albumin (68,000), ovalbumin (43,000), and carbonic anhydrase (30,000). Lane 4 contains cytochrome b preparation obtained in the absence of PMSF and dissociated at 37°C for 2 hr. Lane 5 contains a cytochrome b preparation obtained in the absence of PMSF and dissociated at 100°C for 3 min.

who reported that the polypeptides of Complex III, especially cytochrome b, had different migrations depending on the conditions used to dissociate the proteins. One major difference observed in the gel of Figure 8 was the increase in material remaining at the top of the gel in the samples which had been solubilized by heating at 100°C. This may reflect aggregation of cytochrome b, as a slight decrease in the staining intensity in the band corresponding to cytochrome b was observed. A comparison of the molecular weight of cytochrome b in this partially purified preparation subjected to electrophoresis at different gel concentrations and to solubilization at different temperatures is shown in Table 1. Also included in this Table are the molecular weight values obtained when the cytochrome b preparation was subjected to gel electrophoresis in the presence of sodium dodecyl sulfate and urea as described by Swank and Munkres (1971). The mean molecular weight of cytochrome b obtained from all these different methods of acrylamide gel electrophoresis is $31,000 \pm 600$.

The labeled mitochondrial products of higher molecular weight present in the immunoprecipitate (Lin et al,1978) migrate with a molecular weight (48-50,000) similar to the "core" proteins in the isolated cytochrome b preparation (Fig.6). It was of interest to learn whether these possible "core" proteins in the isolated cytochrome b preparation were mitochondrial translation products and possibly those present in the immunoprecipitate. Hence, cytochrome b was partially

TABLE 1

Molecular Weight of Cytochrome b

SDS - Gels

	Acrylamide Concentration	Condition	Temperature of Dissociation	Molecular Weight x 10^{-3}
Exp.1	5%	+PMSF	100°	31.1
	7.5%	"	100°	30.6
	10%	"	100°	31.7
	12.5%	"	100°	31.3
	15%	"	100°	31.3
Exp.2	10%	+PMSF	100°	32.5
	10%	-PMSF	100°	32.0
	10%	+PMSF	37°	32.0
	10%	-PMSF	37°	33.0
SDS - Urea Gels				
	12.5%	+PMSF	60°	31.0
		-PMSF	60°	31.0

A partially purified preparation of cytochrome b was solubilized with sodium dodecyl sulfate and either glycerol or urea and subjected to polyacrylamide gel electrophoresis as described under Methods.

Experiment 1. The molecular weight determined at each acrylamide concentration is the average of 4 separate experiments.

Experiment 2. The molecular weight determined for each condition is the average of 3 separate experiments.

SDS-Urea Gels: The molecular weight determined in the presence or absence of PMSF is the average of 3 separate experiments.

purified from yeast cells labeled with [^{35}S]methionine in the presence of cycloheximide and the labeled proteins studied by autoradiography. Considerable difficulties were encountered in achieving a pure preparation of cytochrome b when using small amounts of labeled mitochondria as starting material. Often contamination by other mitochondrial products, especially the subunits of cytochrome oxidase, was observed on the autoradiographs even when the proteins corresponding to those molecular weights were only barely detectable after staining with Coomassie blue. This problem was avoided by adding unlabeled mitochondria to the small amount of labeled mitochondria to yield sufficient starting material; however, the starting specific activity was considerably lowered. Figure 9 indicates that the purified cytochrome b preparation obtained in this manner contains a major labeled band with a molecular weight of 31,000, cytochrome b, plus a distinct band with a molecular weight of 67,000 which appears as a minor band when the total mitochondrial translation products are analyzed (cf Fig.6). It should be noted that none of the labeled bands in the cytochrome b preparation correspond to any of the subunits of cytochrome oxidase. In addition, the two "core" proteins associated with cytochrome b during its separation from b-c_1 complex are not labeled in the presence of cycloheximide. It thus appears that the mitochondrially synthesized proteins of 48-50,000 molecular weight present in the immunoprecipitate

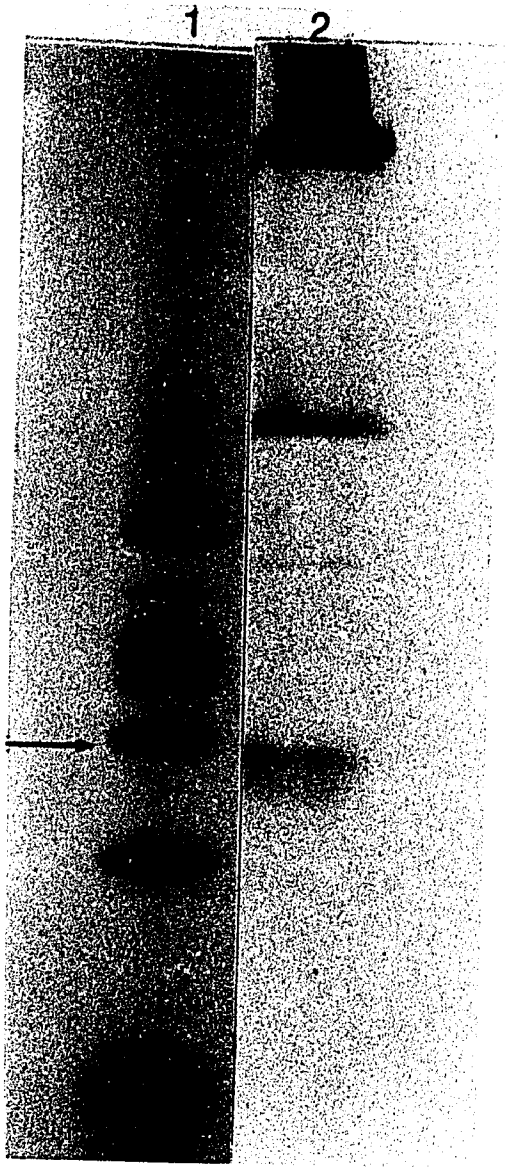


Fig. 9. Comparison of total mitochondrial translation products and a labeled preparation of cytochrome b. Cells were labeled in vivo with [^{35}S]methionine in the presence of cycloheximide and fractionated as described under Materials and Methods. Electrophoresis was in 10-15% gradient polyacrylamide gels. Lane 1 contains an extract of labeled mitochondria and lane 2 a partially purified cytochrome b preparation. The arrow indicates cytochrome b.

formed from the antiserum against cytochrome *b* are not the "core" proteins of the *b*-c_1 complex. Furthermore, these proteins are not associated with cytochrome *b* during its separation from the *b*-c_1 complex.

2. NEW PURIFICATION OF CYTOCHROME b FROM GROWING YEAST.

To investigate the properties of the native cytochrome b with a molecular weight of 31,000, we have developed a new purification scheme. It was impossible to use our previous preparation to obtain a purified cytochrome b, since proteolytic digestion was a necessary step during the purification (Lin and Beattie, 1978).

The first two steps in the new purification scheme (Fig.2) involve solubilization of submitochondrial particles with cholate followed by fractionation with 16% saturated ammonium sulfate. The red pellet precipitated from ammonium sulfate was shown to contain spectrally pure cytochrome b in which cytochromes $\underline{a}$-$\underline{a}_3$ and $\underline{c}_1$ had been completely removed (Fig.5). To further purify cytochrome b, this spectrally pure cytochrome b preparation was solubilized in 0.1 M Tris-acetate buffer containing 5% sodium dodecyl sulfate and 5% 2-mercaptoethanol and subject to gel filtration through a 1 m long ultrogel AcA 44 (LKB, Bromma) column as described by Weiss and Juchs (1978). For this particular cytochrome b preparation from yeast, ultrogel filtration was a crucial purification step whereas Sephadex G-150 which was used by Yu, Yu, and King (1977) to purify cytochrome b from beef heart failed to give an acceptable resolution of the various proteins. The absorbance at 418 nm, the Soret band of

oxidized cytochrome b, was used to measure the heme b content of each fraction immediately after collection from the ultrogel column. Because of the solubilization in sodium dodecyl sulfate and β-mercaptoethanol, the absorption at 418 nm was quite unstable and dissipated gradually within several days. Thus, the absolute heme b content of each fraction was impossible to determine. However,Fig.10 shows the relative value of 280 nm and 418 nm absorption profile obtained within 40 hr after sample loading onto the ultrogel column. Three to five peaks were resolved as indicated by the absorbance at both 418 and 280 nm. Fractions from those peaks were pooled and concentrated in a minicon B15 concentrator ten to twenty fold to minimize sample volume. Subsequently, sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed to reveal which polypeptides were present in each peak (Fig.11). As determined by the absorbance at 418 and 280 nm, the majority of cytochrome b was present in the first peak off the column. The cytochrome b in this fraction was aggregated and either remained at the top of a 10% acrylamide gel or migrated slowly with an apparent molecular weight above 90,000 daltons.

The second peak contained a prominent polypeptide with a molecular weight of 55,000 dalton. This protein may possibly be a dimeric form of cytochrome b, not only because its molecular weight was twice that of cytochrome b but also

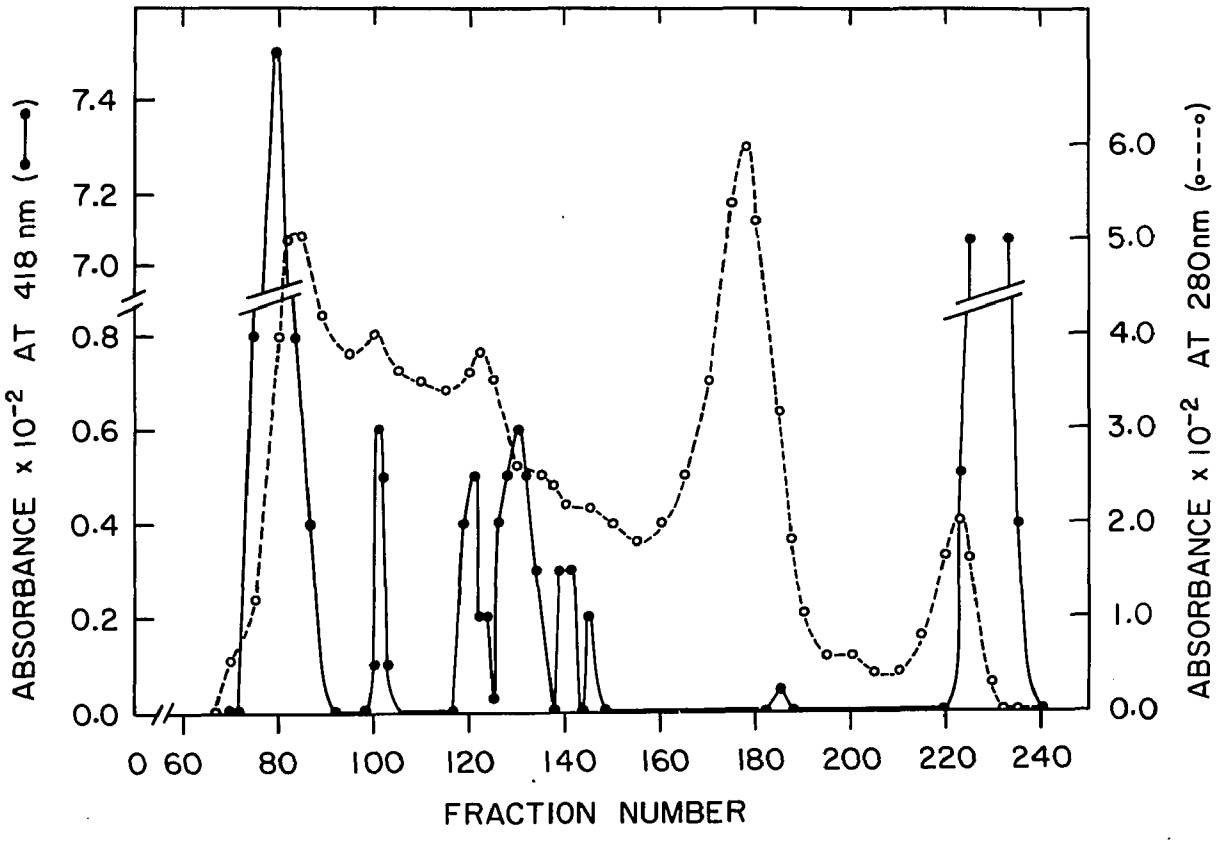


Fig. 10. Ultrogel chromatography in dodecyl sulfate of the spectrally pure cytochrome b preparation. (●—●), absorption at 418nm and (o---o), absorption at 280 nm.

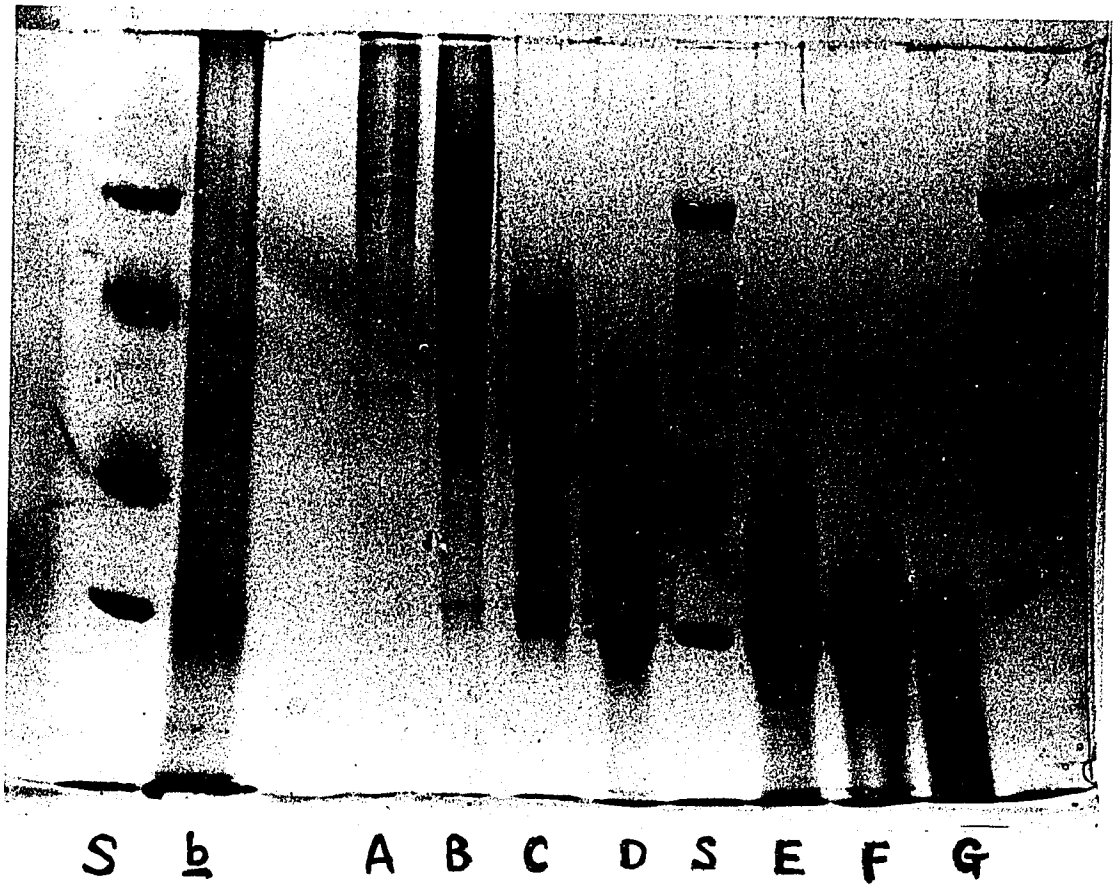


Fig. 11. Gel patterns of various ultrogel column fractions of partially purified cytochrome b preparation. Fractions were obtained by passing the cytochrome b preparation through an ultrogel column and concentrating them through a minicon concentrator. S, standard proteins with molecular weights, from top, 94,000; 67,000; 43,000 and 30,000 daltons, respectively. b, partially purified cytochrome b preparation, before loading to ultrogel column. A, is a fraction eluted from the first peak of the ultrogel AcA 44 column. B and C, are fractions eluted from the second peak off the ultrogel column. D, E, and F, are fractions eluted from the third peak off the column. G, is a fraction collected from the fourth peak off the column.

because this fraction absorbed light of 418 nm wavelength. This polypeptide was excised from a preparative gel and subjected to further gel electrophoresis and acetone extraction. From these serial procedures the sample, which was supposed to be a pure single polypeptide, surprisingly separated into two bands of molecular weights, 44,000 and 28,000 (Fig. 12). These two molecular weights do not add up to 55,000, the molecular weight of the starting material. This experiment excluded the possibility that this particular peptide was a dimer of cytochrome b. However, it did not rule out the possibility that a dimeric form of cytochrome b was present in this peak from the column. The 44,000 dalton band could be a protein tightly bound to a cytochrome b of 28,000 molecular weight which may not necessarily be a native cytochrome b. Alternatively, the 418 nm absorbance could be attributed to another lightly stained protein, possibly a dimeric cytochrome b.

The fourth peak, a major peak in terms of 280 nm absorption, consisted of polypeptides with molecular weights under 25,000 dalton and some small peptide comigrating with the dye front (Figs. 10 and 11). However, no cytochrome b was detectable spectrophotometrically. The fifth peak had a large absorption at 418 nm wavelength but contained no protein which was stainable with Coomassie blue. The heme b spectrum may be attributed

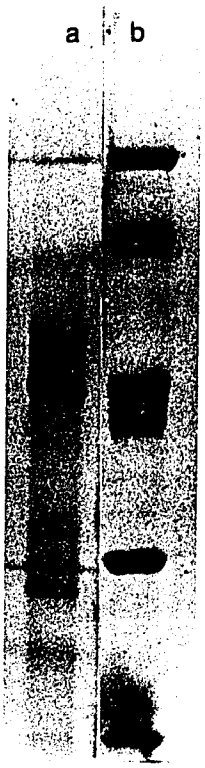


Fig. 12. Sodium dodecyl sulfate, 10% acrylamide gel electrophoresis of a protein of 55,000 dalton, isolated from the second 280 nm peak off the ultrogel column. (a) the split of the 55,000 dalton protein into two bands of 44,000 and 28,000 daltons. (b) standard proteins: from the top, 94,000; 67,000; 43,000; 30,000 and 20,000 daltons.

to some free heme molecules dissociated from cytochrome b.

Of major interest to us was the third peak which contained material absorbing at 418 nm. In this fraction, two major polypeptides were observed after gel electrophoresis with molecular weights around 30,000 (Figs. 10, 11), the molecular weight of native cytochrome b. In order to separate these two major bands, the fraction involving these proteins (Fig.11) was subjected to electrophoresis on super slab gels (25 cm x 12 cm x 0.4 cm thick). After staining and destaining with Coomassie brilliant blue, these two polypeptide bands were excised respectively from the tall gels and further subjected to a preparative gel electrophoresis consisting of a modified funnel-shaped acrylamide gel with its bottom attached by a closed-ended dialyzing Spectra/por 1 membrane tubing with a molecular weight cut off of 6-8,000 (Fig.1). The single polypeptide thus eluted from the gel bands was in a mixture of Coomassie brilliant blue and 50 mM Tris-glycine reservoir buffer containing 0.1% sodium dodecyl sulfate. To remove the dye, protein samples were extracted with acetone as described in Materials and Methods (cf Fig.3).

Two single polypeptides, namely protein I of 31,000 dalton and protein II of 29,000 dalton, were obtained by this purification procedure and showed one single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig.13). With 550 mg of mitochondrial protein as starting material,

Fig. 13. Sodium dodecyl sulfate-gel electrophoresis of two purified single polypeptides. S, standard proteins of molecular weights, from top, 94,000; 67,000; 43,000; and 30,000, daltons, respectively. b, partially purified cytochrome b preparation. E, one ultrogel eluted fraction containing two major bands. L, lower band (Protein II) with 29,000 dalton molecular weight. U, purified upper band (Protein I) with molecular weight 31,000 dalton.

0.5 mg of protein I and a 0.25 mg of protein II were obtained (Table 2). Although the yield was merely 0.09% for protein I and0.05% for protein II, protein I was 1,200 fold purified and protein II 2,200 fold purified as compared to the starting mitochondria.

Two major factors which cause aggregation of cytochrome b are heat and detergents such as Triton X-100 and sodium dodecyl sulfate (Lin et al, 1978). When the partially purified preparation of cytochrome b was applied, solubilization by 5% sodium dodecyl sulfate was necessary. In addition, during prolonged preparative gel electrophoresis, electrical heating was unavoidable. Under these circumstances we have been able to compromise to some degree the possible losses which may occur because of aggregation of cytochrome b. The final preparation in terms of both yield and purity were quite satisfactory.

TABLE 2

Purification of two apocytochrome b's from growing yeast

Steps		Heme b Content	Total Protein	Yield
		n mole b/mg protein	mg	%
1.	Mitochondria	-	554.	100
2.	Submitochondrial particles	0.224	148.	26.7
3.	Spectrally pure Cytochrome b preparation	7.0	46.	8.31
4.	Ultrogel fraction with two major Cytochrome b bands	-	15.1	2.73
5a.	Purified protein I - Apocytochrome b-I with 31,000 dalton M.W.	-	0.474	0.086
5b.	Purified protein II - Apocytochrome b-II with 29,000 dalton M.W.	-	0.255	0.046

Step 1 to 4: The values obtained at each step are the averages of 5 separate preparations.

Step 5a to 5b: The values obtained at these steps are the averages of 3 separate preparations.

3. IDENTIFICATION OF PROTEIN I AND PROTEIN II AS APOCYTOCHROME b

Antigenicity

To determine which of these two purified proteins was antigenically related to cytochrome b, counter immunoelectrophoresis of protein I and protein II was performed essentially according to Lin et al (1978). Surprisingly, both polypeptides reacted with the specific antiserum against cytochrome b obtained by Lin et al (1978) and revealed obvious precipitin lines (Fig.14). No precipitin line was observed when the same samples were electrophoresed against control serum. These results provided strong evidence that both protein I and protein II were antigenically similiar to cytochrome b.

The electrophoretic mobility of protein I and II during electrophoresis in various acrylamide concentrations indicated that both proteins displayed normal migration behavior in both 10% and 15% acrylamide gels. We have reported previously (Beattie et al, 1979) that cytochrome b when free from other membrane proteins migrates predictably during gel electrophoresis. Protein I migrates with an apparent molecular weight (31,000) identical to that of native cytochrome b which was immunoprecipitated by the cytochrome b antiserum from mitochondria pulse-labeled in the presence of cycloheximide or from cells grown in [^{3}H]leucine (Lin et al 1978). Protein II migrates

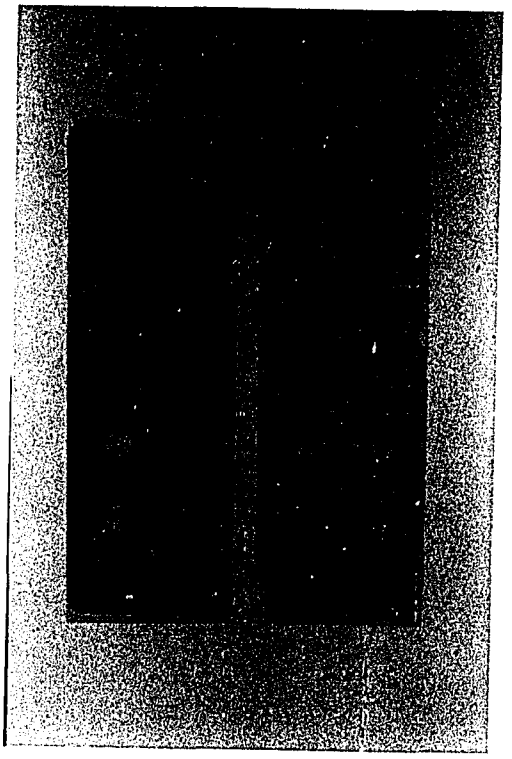


Fig. 14. Counter immunoelectrophoresis of purified protein I and protein II against antiserum to cytochrome b. Different sera were placed in the anodic wells (on the top) in the following order from left to right: (1,3) 7 µl, 10 µl of antiserum to cytochrome b, respectively, (2,4) 7 µl, 10 µl of control serum. Samples of purified proteins were placed in the cathodic wells (on the bottom) in the following order from left to right: (1,2) 21 µl of purified protein II at a protein concentration of 1.2 µg/µl, (3,4) 20 µl of purified protein I at a concentration of 1.2 µg/µl.

with the same electrophoretic mobility(29,000) as the cytochrome b band of the total mitochondrial translation products in a 10% acrylamide gel (Fig.6). These data together suggest that protein I and protein II are two forms of cytochrome b, sharing most of the peptide sequence including the antigenically active site(s). Since there was a 2,000 dalton difference in their molecular weights, protein II might contain twenty fewer amino acids than protein I. It would appear most likely that protein I, the 31,000 dalton protein is the native form of cytochrome b.

Proteolytic Digestion

As a further structural identification of protein I and protein II as apocytochrome b, a limited proteolysis of both proteins excised from a super gel, together with a cytochrome b band excised from mitochondria labeled in the presence of cycloheximide, was performed according to the method of Cleveland et al (1977). After digestion by 50 µg of S. aureus V8 protease, protein I, protein II and the labeled cytochrome b band all gave a similiar fingerprint pattern (Fig.15, lanes 3,2,1). The pattern differs completely from that obtained with protease alone (lane 4). Lanes 5 and 6 were loaded with the protein II and protein I bands, respectively without protease. Peptide maps of protein II in lane 2 and labeled cytochrome b in lane 1 are identical including a broad undigested protein band, with the same mobility as the

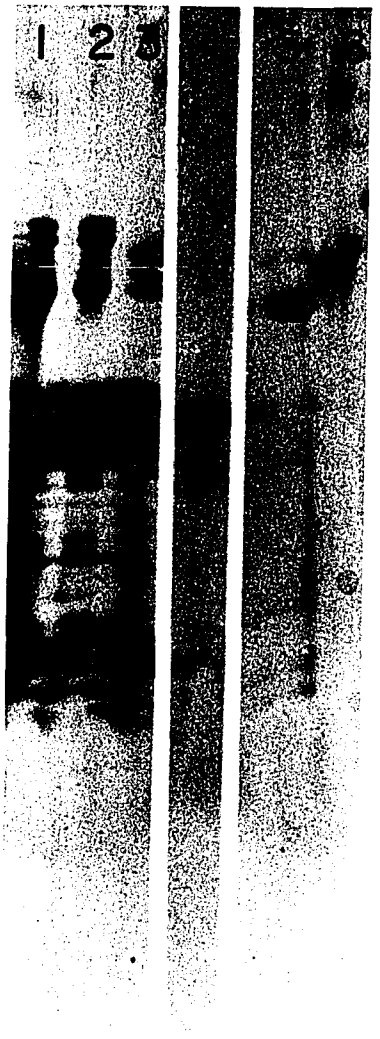


Fig. 15. Peptide map of limited proteolytic digestion of cytochrome b, protein I and protein II. Lane 1, labeled cytochrome b band excised from mitochondria labeled in the presence of cycloheximide, digested by 50 µg of S.aureus V8 protease. Lane 2, protein II, digested by 50 µg of the same protease. Lane 3, protein I digested by 50 µg protease. Lane 4, 50 µg protease only. Lane 5, undigested protein II. Lane 6, undigested

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protein I. Digestion was performed for 40 min at 37°C in the stacking gel without the current turned on. The remaining electrophoresis was performed at 4°C to prevent further digestion.

undigested band in lane 5, and several bands directly above it. Those polypeptides which are larger than the original protein may result from the binding of the protease to the original protein prior to digestion. These bound proteins may not dissociate during the subsequent electrophoresis. Limited proteolysis of protein I (lane 3) produced a protein with the same mobility as undigested protein II suggesting that protein II is a part of protein I. The fingerprint obtained from another proteolytic digestion (Fig.15-1) showed again that both proteins I and II yield a similiar digestion map after preliminary proteolysis. The binding of the protease to the original proteins during an early stage of the digestion was suggested by the presence of two broad bands with molecular weights greater than that of the original proteins.

This proteolytic digestion experiment provided another structural characterization of protein I and protein II. The identical antigenic response to the antiserum to cytochrome b, their electrophoretic mobility and the identical fingerprint pattern after limited proteolysis, provide a strong suggestion that both proteins I and II are two forms of cytochrome b sharing a major peptide sequence with the exception of a 2,000 dalton residue missing from protein II.

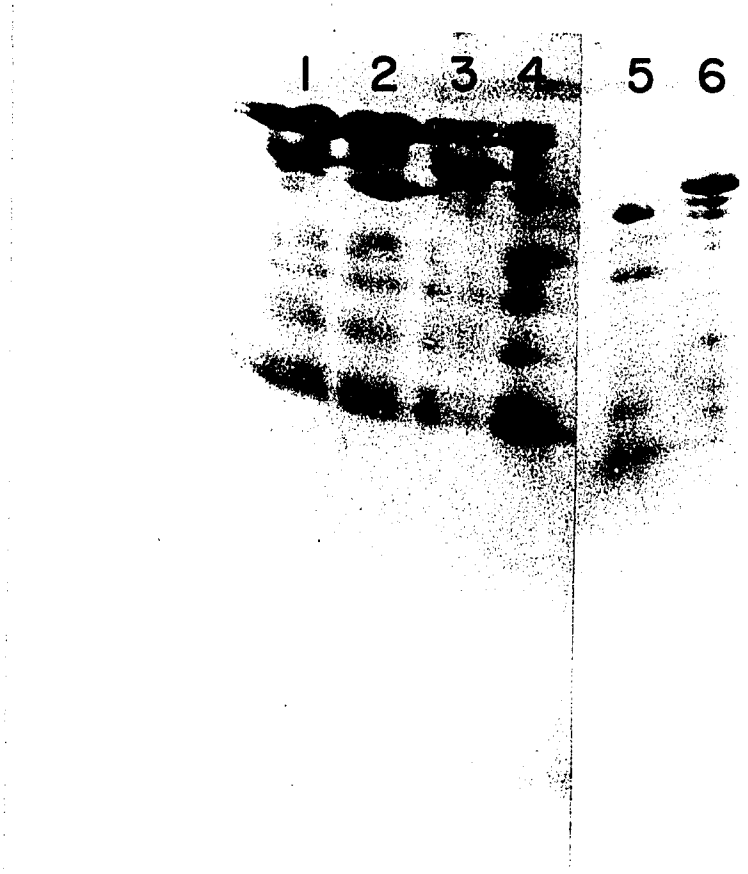

Fig. 15-1. Fingerprint of limited proteolytic digestion of protein I and protein II by Staphylococcus aureus V8 protease. Lane 1, protein I digested by 50 µg protease. Lane 2, protein II digested by 50 µg protease. Lane 3 and lane 4, protein I and protein II digested by 25 µg protease, respectively. Lane 5 and 6, undigested protein II and protein I, respectively. Digestion was performed for 40 min at 30°C in the stacking gel (5% acrylamide) with the interruption of current. After digestion, electrophoresis was performed at 4°C.

4. BIOGENESIS OF APOCYTOCHROME b-I AND APOCYTOCHROME b-II

Specificity of Antisera Against Apocytochrome b-I and Apocytochrome b-II.

In order to investigate the relationship between apocytochrome b protein I and protein II and to study the biosynthesis of cytochrome b, antisera against protein I and protein II were raised individually by injection of the proteins into rabbits as described under Materials and Methods . After an Ouchterlony double diffusion test, both antisera against protein I and protein II reacted with the third peak obtained from the gel chromatography (Fig. 10) leading to the formation of significant precipitin lines. Control serum from non-immunized rabbits failed to produce any precipitin lines (Fig.16).

Counterimmunoelectrophoresis of the purified protein I against antiserum to protein I and of the purified protein II against antiserum to protein II revealed obvious precipitin line (Fig 17 a&e). No precipitin lines were formed when either purified protein I or purified protein II was tested against control serum in the same test (Fig. 17 c&f). It was of interest to find out whether there was any cross reaction, first, between purified protein I and antiserum against protein II, and secondly between purified protein II and antiserum against protein I. To the latter the answer was

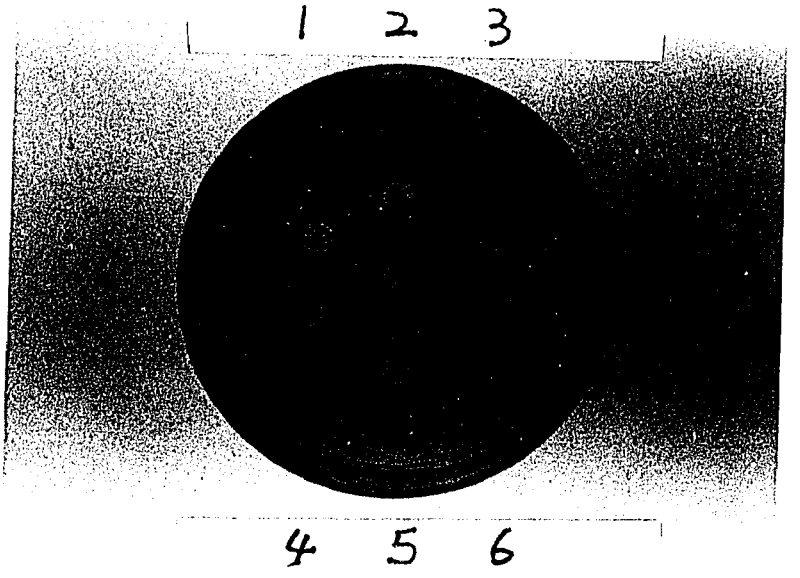

Fig. 16. Ouchterlony double diffusion test of antisera against protein I and protein II. Center well, 20 µl of an ultrogel fraction containing protein I and protein II at protein concentration of 8 µg/µl in 5% Triton X-100, 1% sodium dodecyl sulfate and 38 mM tris-glycine, pH 8.6. 20 µl of serum was placed in each peripheral well as follows: well 1, antiserum against protein I collected on the 64th day after the booster injection of the antigen; well 2, control serum; well 3, antiserum against protein I collected on the 40th day; well 4, antiserum against protein II collected on the 64th day; well 5, antiserum against protein I collected on the 47th day; well 6, antiserum against protein II collected on the 47th day. The plate was stained with 1.25% Coomassie brilliant blue in 45% methanol, 9.2% acetic acid and destained in 5% methanol and 7.5% acetic acid.

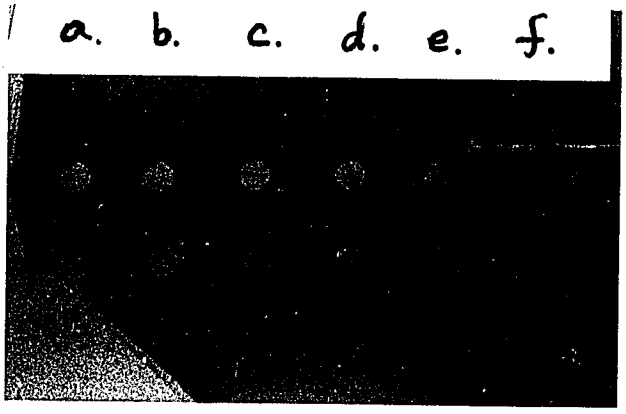

Fig. 17. Demonstration of the specificity of antisera against protein I and protein II by counter immunoelectrophoresis. An aliquct of 60 µl of serum was placed in each top well as follows: a and d, antiserum against protein I; b and e, antiserum against protein II; c and f, control serum. 8 µl of purified protein I or protein II at 1.2 µg protein/µl was added to each bottom well: a,b,c, purified protein I; d,e,f, purified protein II. After electrophoresis was performed for 3 hr with the application of 40 milliamperes current, the agarose slide was pressed and rinsed to remove buffer and unbound components, then stained and destained as described in the legend to Fig. 16.

positive since a precipitin line was observed between purified protein II and antiserum to protein I in the same counterimmuno-electrophoresis experiment (Fig.17 d). However, a partial reaction between purified protein I and antiserum against protein II was shown by the broken precipitin line observed between them (Fig.17 b).

Counterimmunoelectrophoresis was also performed with the crude cytochrome b preparation to test the possibility that the antisera was strong enough to immunoprecipitate apocytochrome b. The spectrally pure cytochrome b preparation was obtained as described previously and dissolved in tris-glycine buffer, pH 8.6, containing 5% Triton and 1% sodium dodecyl sulfate. Antiserum against protein I collected on the 64th day gave an obvious round precipitin line, whereas antisera against protein II collected on the 40th and 64th days gave a straight precipitin line. No precipitin line was observed with control serum (Fig.18). The reason that the precipitin lines are formed so close to the well containing antiserum could result from any of the following factors: (1) a higher mobility of antigen in the cytochrome b extract than that of antibody; (2) much more antigen was used than antibody, i.e. the ratio of antigen to antibody was not balanced; (3) comparably weak antigenic activity of the antiserum. These results suggested that cytochrome b even in a crude preparation can react with both antisera against proteins I and II.

A further test was necessary to learn if

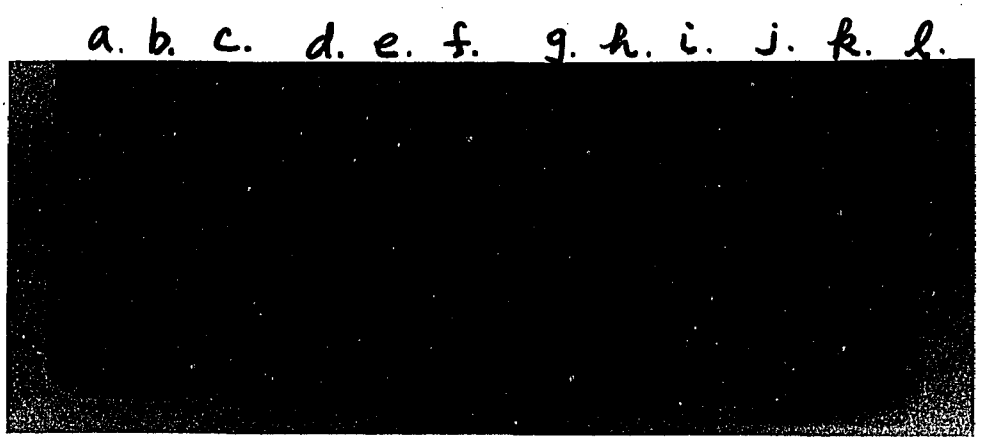

Fig. 18. Immunological detection of apocytochrome b-I and b-II in a spectrally pure, but crude cytochrome b preparation. An aliquot of 10 µl of spectrally pure cytochrome b preparation, 8.5 µg protein/µl, containing 5% Triton X-100 and 1% sodium dodecyl sulfate was loaded in each bottom well. Aliquots of 10 µl of various sera were placed in the top wells in the following order: a, antiserum against protein I collected on the 40th day after first injection of the antigen; b,g, antiserum against protein I collected on the 47th day; c,j, antiserum against protein I collected on the 64th day; d, antiserum against protein II collected on the 40th day; e,h, antiserum against protein II collected on the 47th day; f,k, antiserum against protein II collected on the 64th day; i,l, control serum. Counter immunoelectrophoresis was performed as described in the legend to Fig. 17.

cytochrome b present in the mitochondrial membrane could be immunoprecipitated. To test the ability of the antisera against apocytochrome b to immunoprecipitate cytochrome b, mitochondria isolated from yeast cells grown in [^{3}H]leucine were titrated with both antisera (Fig.19). The optimum ratio of antiserum to mitochondrial extract was found to be 9 for antiserum I and 12 for antiserum II when the mitochondrial protein concentration was maintained at 10 mg per ml. At the optimum ratio, antiserum I precipitated 3.5% of the radioactive material from labeled mitochondria while antiserum II precipitated 5%. It is noteworthy that both antisera gave an arch-like titration curve, i.e. increasing the amount of antisera used above the optimum ratio resulted in reducing the amount of radioactive material precipitated from mitochondria. Addition of control serum resulted in a rather flat titration curve and brought down less than 2% of the radioactive material even when the ratio of serum to extract was increased to 18 again suggesting the specificity of both antisera.

It is well known that solubilization techniques play an important role in immunoassay experiments in the following two critical steps: (1) the solubilization of intact organelles or membranes prior to immunoincubation with antiserum and (2) the isolation and solubilization of immunoprecipitates, the antigen antibody complex, after immunoincubation.

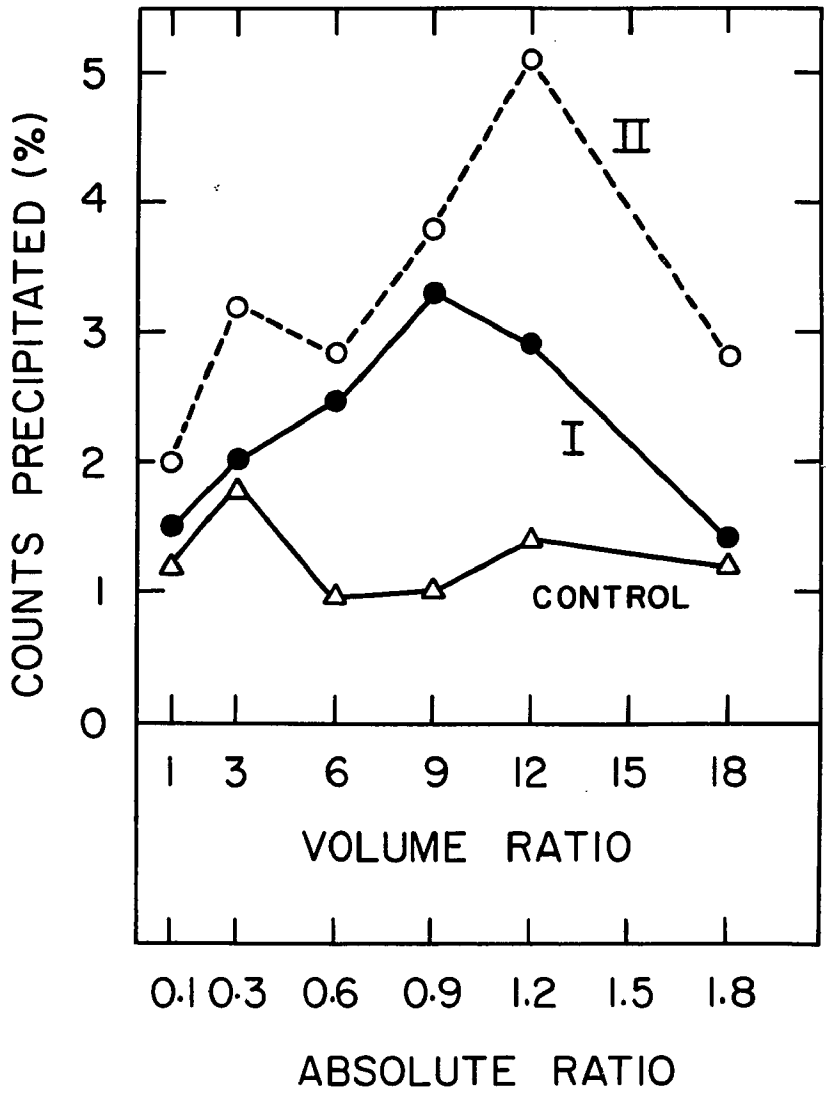

Fig. 19. Titration of mitochondrial extract with (1) antiserum against apocytochrome b-I (●——●), (2) antiserum against apocytochrome b-II (○--○), (3) control serum (△——△). Mitochondria were isolated from wild type strain grown in medium containing 0.67% yeast nitrogen base, 0.2% yeast extract, 1% glucose and 5 mCi per liter of [^{3}H]-leucine and extracted with 1% Lubrol and 1% deo-

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xycholate as described under Materials and Methods. Increasing amounts of antisera or control serum were added to 10 µl aliquots of mitochondrial extracts containing 10 mg/ml protein and 10,000,000 counts x min^{-1} x ml^{-1}.

For the first step, we also tried to lyse mitochondria with 1%, 2% or 3% sodium dodecyl sulfate, 1 mM EDTA, 1 mM EGTA, pH 7.4, boiling 3 min or sitting at room temperature overnight followed by a 10-fold dilution with 3% Triton X-100, 154 mM sodium chloride, 10 mM sodium phosphate, pH 7.4. This method, which was first described by Côte et al (1979) for the solubilization of the cytochrome b-c_1 complex of yeast mitochondria, was unsuccessful for the immunoprecipitation of cytochrome b. Boiling the samples in sodium dodecyl sulfate caused an irreversible aggregation of cytochrome b while incubating them at room temperature was insufficient to solubilize cytochrome b and other membrane proteins. Solubilization of mitochondria with 1% Triton X-100 and 1 M KCl (Bell et al 1979), led to incomplete dissociation of all membrane components such that many proteins appeared in the immunoprecipitate. For the subsequent studies on the biogenesis of cytochrome b, we found that treatment of mitochondria with 1% Lubrol and 1% deoxycholate for 1 hr at 4°C gave the best solubilization of the mitochondrial membrane.

For the second step, the isolation and solubilization of immunoprecipitates, protein A-Sepharose CL-4B (Pharmacia) or denatured Staphylococcus aureus cells coated with protein A are popularly used to isolate antigen-antibody complexes from other membrane components prior to solubilization (Werner and Machleidt, 1978; Côte et al, 1979; Maccecchini et al, 1979). Both of these methods were tried with a minimum of success. The major

difficulty was the solubilization of cytochrome b from the protein A-Sepharose or the Staphylococcus aureus cells bound to the antibody since cytochrome b is hydrophobic and heat labile. It was concluded that the best way to isolate cytochrome b immunoprecipitates is simply by centrifugation without any preincubation with protein A-Sepharose or Staphylococcus aureus cells.

In the following immunoassays, mitochondria isolated from yeast cells grown in [^{3}H]leucine were solubilized with 1% Lubrol and 1% deoxycholate for 1 hr followed by immunoincubation with either antiserum against protein I or protein II. The washed radioactive immunoprecipitates were dissociated with 5% 2-mercaptoethanol and 5% sodium dodecyl sulfate and further analyzed by polyacrylamide gel electrophoresis in a tube gel as described in Materials and Methods . The immunoprecipitate obtained with antiserum I contained a predominant peak at a molecular weight of 30,000, whereas, that from antiserum II contained a major peak of 32,000 dalton (Fig.20). Both molecular weights are approximately the same as the molecular weights of the pure antigens,29,000 and 31,000. It is well known that deviations in molecular weight determination are unavoidable during tube gel electrophoresis. Two other labeled minor peaks of higher molecular weight range were seen in both immunoprecipitates from antiserum I and antiserum II. Those might be due to either non-specific

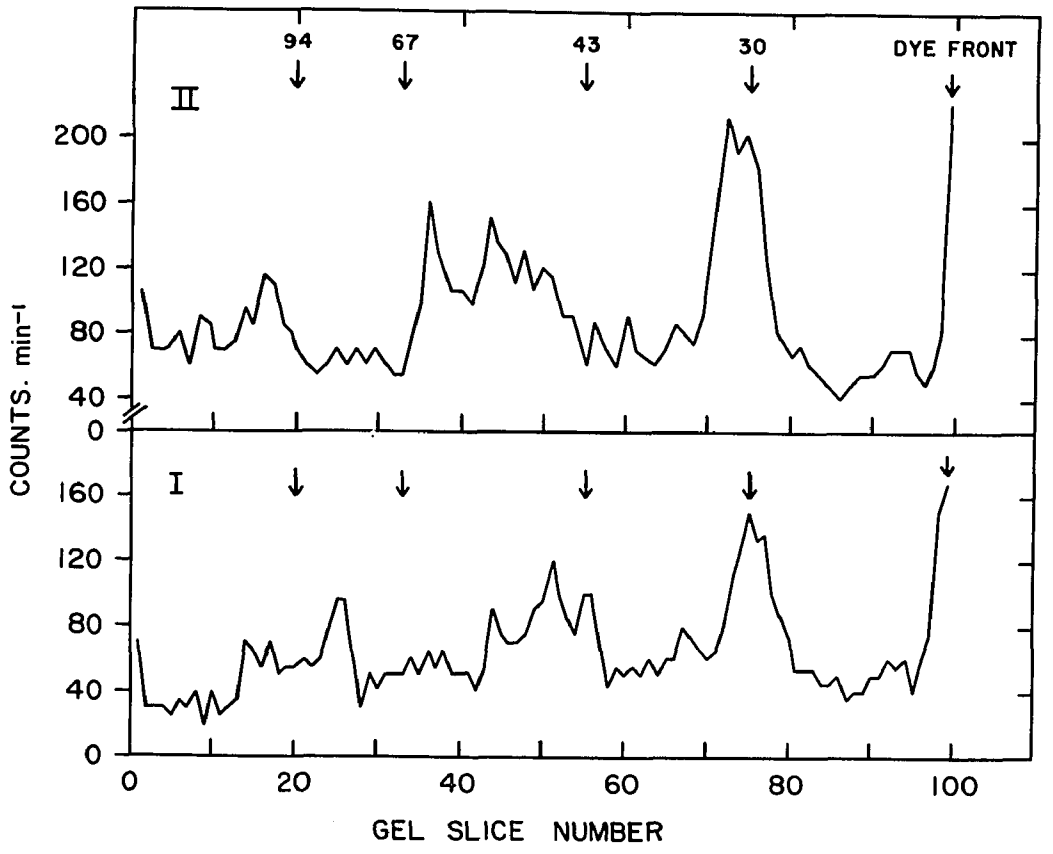

Fig. 20. Electrophoretic analysis of cytochrome b immunoprecipitated from labeled yeast mitochondria. Labeled mitochondria were prepared from yeast cells grown in 0.67% yeast nitrogen base, 0.2% yeast extract, 1% glucose and 5 mCi/liter [^{3}H]-leucine for 10 generations and then solubilized in 1% Lubrol and 1% deoxycholate. After immunoincubation with both antisera to cytochrome b, the washed immunoprecipitates were analyzed by sodium dodecyl sulfate tube gel electrophoresis and sliced every 2 mm with a Gilson gel slicer. Numbers above the arrows refer to molecular weights of the standards. (I, immunoprecipitate obtained with antiserum to apocytochrome b-I and II, immunoprecipitate obtained with antiserum to apocytochrome b-II)

immunoprecipitation or to aggregation of cytochrome b.

The results obtained suggest that both antisera against protein I and protein II were able to react during counter-immunoelectrophoresis and immunodiffusion with the purified proteins I and II, an ultrogel fraction containing proteins I and protein II and the spectrally pure but crude, cytochrome b preparation. In addition, both antisera are capable of isolating cytochrome b from mitochondria of cells grown in media containing [^{3}H]leucine. From all these observations, we have concluded that both antisera I and II were specific against apocytochrome b.

Biogenesis of Apocytochromes b-I and b-II.

The availability of specific antisera to both protein I and protein II have enabled us to investigate further the biogenesis of two apocytochromes b : apocytochrome b-I and b-II in Saccharomyces cerevisiae. Yeast cells were pulse-labeled with [^{35}S]SO_4 in the presence of cycloheximide and mitochondria were isolated and solubilized with 1% deoxycholate, 1% Lubrol, prior to immunoprecipitation with optimum amounts of either antiserum. Electrophoretic analysis of the labeled immunoprecipitates were performed to reveal if protein I and protein II are synthesized on mitochondrial ribosomes which are cycloheximide resistant. As shown in Fig. 21, antiserum I caused the precipitation of three major peaks of 44,000, 31,000 and 21,000 daltons. The 31,000 dalton peak corresponded exactly to the molecular weight of the antigen, protein I, one of the purified forms of apocytochrome b. It was clear

that this 31,000 dalton peak was cytochrome b as judged by the molecular weight, the site of synthesis-mitochondria and the specificity of antiserum I as demonstrated above. The 44,000 molecular weight peak probably is a protein called VAR-1, a protein of the mitochondrial small ribosomal subunit. The 21,000 dalton peak is most likely subunit III of cytochrome c oxidase brought down non-specifically by antiserum I. It should be noted that a protein of that molecular weight was not present in the gel profile of Fig. 20. Identical gel patterns with a predominant labeled peak of molecular weight 31,000 and two other peaks of 42,000 and 21,000 daltons were obtained in the immunoprecipitate formed between antiserum II and mitochondria pulse-labeled with sulfate in the presence of cycloheximide. In this gel pattern, cytochrome b peak of 31,000 dalton is more prominent than the other two non-specific peaks. In these experiments, it was difficult to distinguish between protein I and protein II, the two forms of apocytochrome b in the labeled immunoprecipitates. Cytochrome b seemed to be present in the "Protein I" form with a molecular weight of 31,000. The data obtained with the specific antisera against apocytochromes b-I and b-II indicate that both protein I and protein II are translated on mitochondrial ribosomes.

Because the crude antisera precipitated proteins other than cytochrome b from labeled mitochondria, we decided to purify an IgG fraction from both antisera and the control serum to see if this non-specific immunoprecipitation could be avoided.

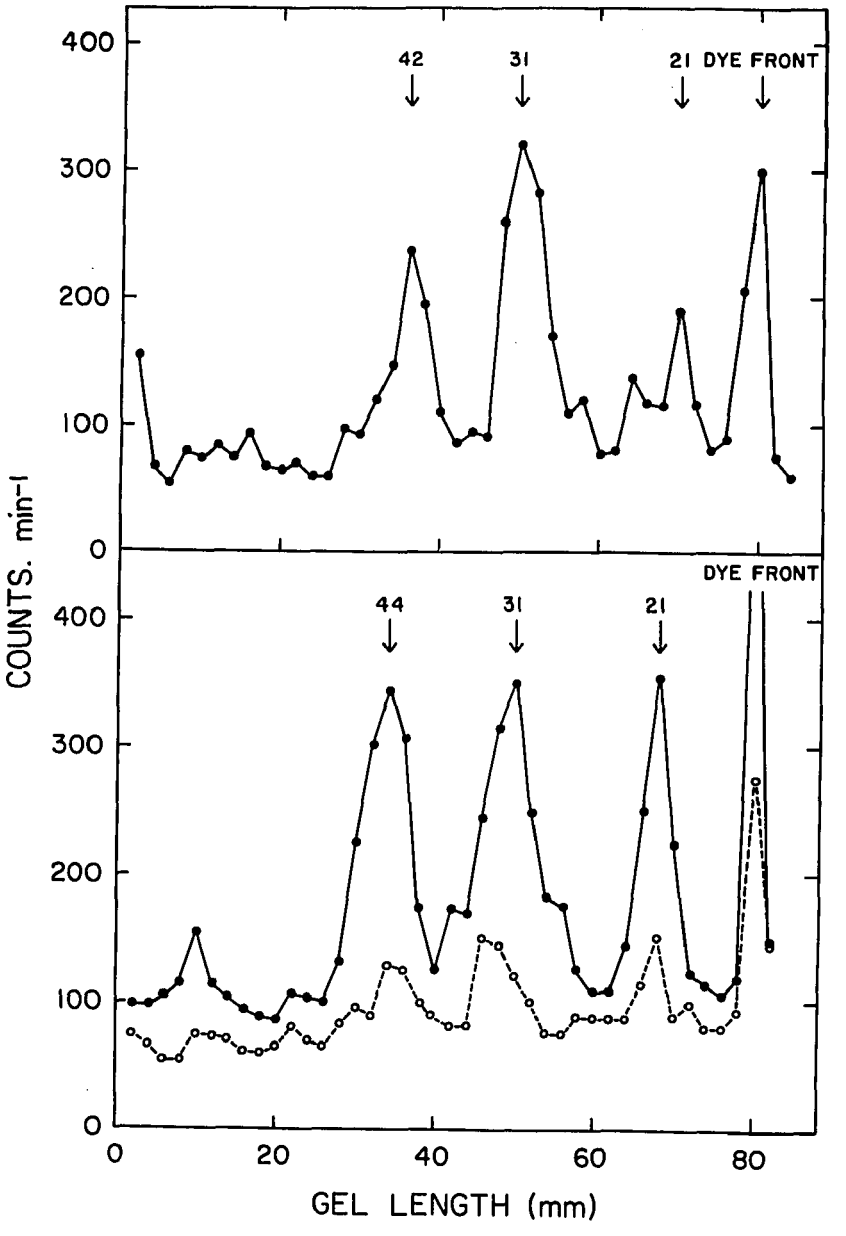

Fig. 21. Gel electrophoresis of immunoprecipitated cytochrome b. Yeast cells were pulse labeled with [^{35}S]sulphate for 15 min at 30°C in the presence of 1 mg/ml cycloheximide. Mitochondria were prepared by cell-breakage with glass beads, extracted with 1% Lubrol and 1% deoxycholate and subjected to immuno-precipitation with two types of antisera against cytochrome b and control serum separately. An

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Legend to Fig. 21 contd....

aliquot of the immunoprecipitates was analyzed by tube gel electrophoresis in 10% acrylamide/sodium dodecyl sulfate and sliced every 2 mm with a Gilson gel slicer. Lower panel is immunoprecipitate obtained with antiserum against apocytochrome b-I, while the upper panel is that obtained with that against apocytochrome b-II. Numbers above arrows represent molecular weights of the labeled peaks.

For this purpose, crude IgG fractions of the two antisera against apocytochrome b and the control serum were obtained by treating the sera with 34% saturated ammonium sulfate, and then dialyzing against phosphate buffer to remove the ammonium salts. A new immunotitration curve was performed using mitochondria prepared from yeast cells pulse-labeled with [^{35}S]methionine in the presence of cycloheximide. As seen in Fig. 22, increasing amounts of the IgG fractions obtained from antisera I and II precipitated increasing percentages of radioactive material from the labeled mitochondria, while IgG fraction from control serum precipitated less than 1% of precipitates at all ratios of serum to mitochondria extract tested.

In order to compare the titration curve in Fig. 19 with that of Fig. 22, the absolute ratio was expressed as the volume of serum or IgG fraction in µl to the protein content of extract in µg rather than plotting the ratio of serum volume to the volume of mitochondria extract.

$$\text{Absolute ratio} = \frac{\text{volume of serum or IgG fraction (µl)}}{\text{protein content of extract (µg)}}$$

$$\text{Volume ratio} = \frac{\text{volume of serum or IgG fraction (µl)}}{\text{volume of extract (µl)}}$$

It is obvious that the antigen content in an extract is proportional to the protein content of that extract rather than to the volume of that extract and, furthermore, that the

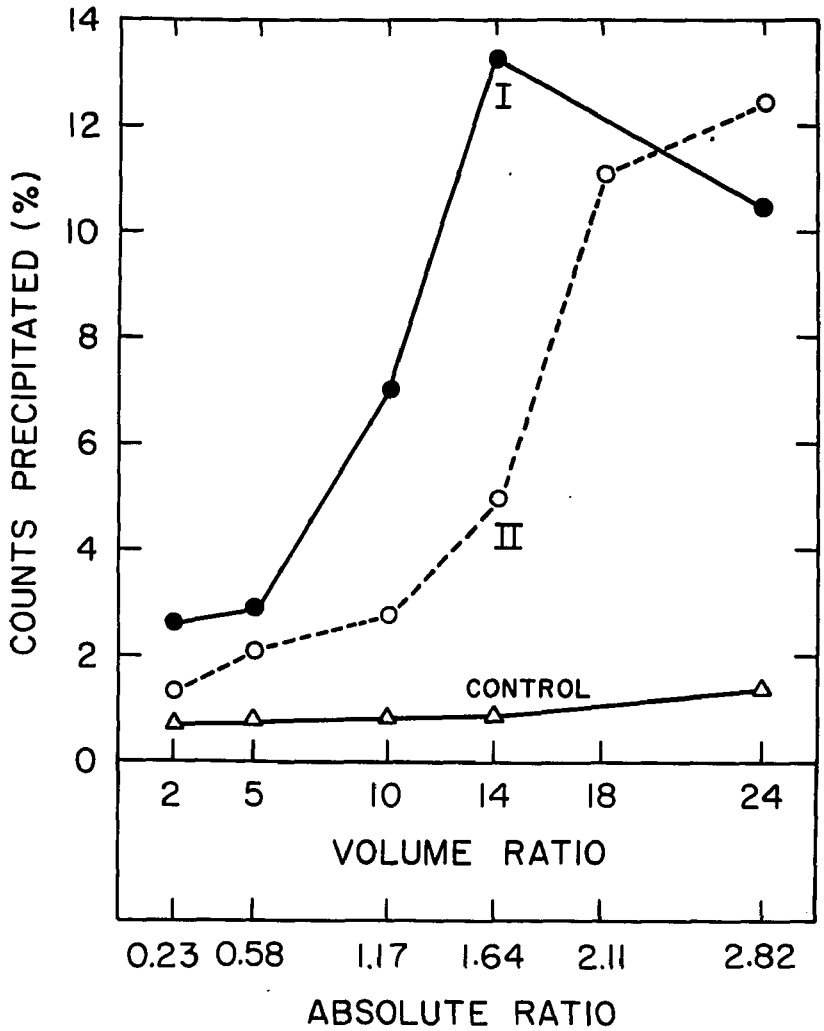

Fig. 22. Immunotitration curve of mitochondrial extract with (1) purified IgG fraction against apocytochrome b-I (●——●), (2) purified IgG fraction against apocytochrome b-II (○- - -○), and (3) purified IgG fraction from non-immunized rabbit serum (△——△). IgG fractions were purified from antisera to apocytochrome b-I and b-II and control serum by 3 precipitations with

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34% saturated ammonium sulfate at 4°C followed by a dialysis against sodium phosphate buffer to remove the ammonium salt. One gram of yeast cells were pulse labeled with 1 mCi [^{35}S]methionine for 5 min at 10°C in the presence of 1 mg/ml cycloheximide. Mitochondria were prepared and extracted as described in the legend to Fig.21. Increasing amounts of IgG fractions from antisera or control serum were incubated with 10 µl aliquots of mitochondrial extracts containing 8.5 mg/ml and 4,800,000 counts x $min^{-1}xml^{-1}$. Absolute ratio of IgG fraction to extract was calculated by dividing the volume of IgG fraction (µl) by protein concentration of mitochondrial extract (µg) in each incubation.

protein concentration of an extract usually varies from experiment to experiment. Therefore, an absolute ratio of serum to extract provides more meaningful and useful information than a regular ratio.

In the immunotitration curve of Fig. 19, the protein concentration of the mitochondria extract was 10 µg per µl while that in Fig. 22 it was 8.5 µg per µl. When the absolute ratio was less than 1, both antisera and the IgG fractions precipitated increasing amounts of radioactivity from the mitochondrial extracts; however, above a ratio of 1 the IgG fractions against both proteins continued to precipitate more radioactivity, while the crude antisera precipitated less amounts of radioactive material from labeled mitochondria. These data showed that both IgG fractions from antiserum I and antiserum II are more potent than the crude antisera.

These IgG fractions were used in subsequent experiments to immunoprecipitate proteins I and II from mitochondria isolated from yeast cells pulse-labeled with [^{35}S]methionine in the presence of cycloheximide. The washed immunoprecipitates were subjected to sodium dodecyl sulfate gel electrophoresis on a slab gel which was then autoradiographed as described in Materials and Methods. As seen in Fig. 23, the immunoprecipitate obtained with the IgG fraction from the antiserum against apocytochrome _b_-I (lane 2) contained 3 labeled bands corresponding in molecular weight to VAR-I, cytochrome _b_ and subunit III of cytochrome _c_ oxidase as compared to a

parallel electrophoresis of mitochondrial translational products (lane 1). IgG fraction II also precipitated 3 components (lane 3), and the control serum precipitated very few counts distributed in every component of the total mitochondrial translation proteins (lane 4). It is apparent that the gel patterns in the radioautogram are identical to the gel profile in Fig. 21 (obtained by crude antisera) including the presence of non-specific binding of IgG fractions to VAR-I and subunit III of cytochrome c oxidase. Unfortunately, the non-specific precipitation could not be avoided even when using a purified IgG fraction of the antiserum.

The presence of proteins precipitated non-specifically was avoided when mitoplasts were used instead of mitochondria as starting material for the immunoprecipitation. Yeast cells of strain KL14 were pulse-labeled with [^{3}H]leucine in the presence of cycloheximide, spheroplasts were prepared by Zymolyase digestion and mitochondria were isolated. Mitoplasts were obtained by treating the mitochondria with 3 mg digitonin per 10 mg protein for 5 min at 4°C and centrifuging 10 min at 10,000 g. Mitoplasts were solubilized with Lubrol and DOC[1] prior to immunoincubation with the optimum amount of IgG fraction I or II. The washed and dissociated immunoprecipitates were analyzed on a slab gel electrophoresis together with the labeled mitoplast as a control. Each lane of the slab gel was cut into thin strips which were cut into 1 mm slices manually. The gel slices were counted after

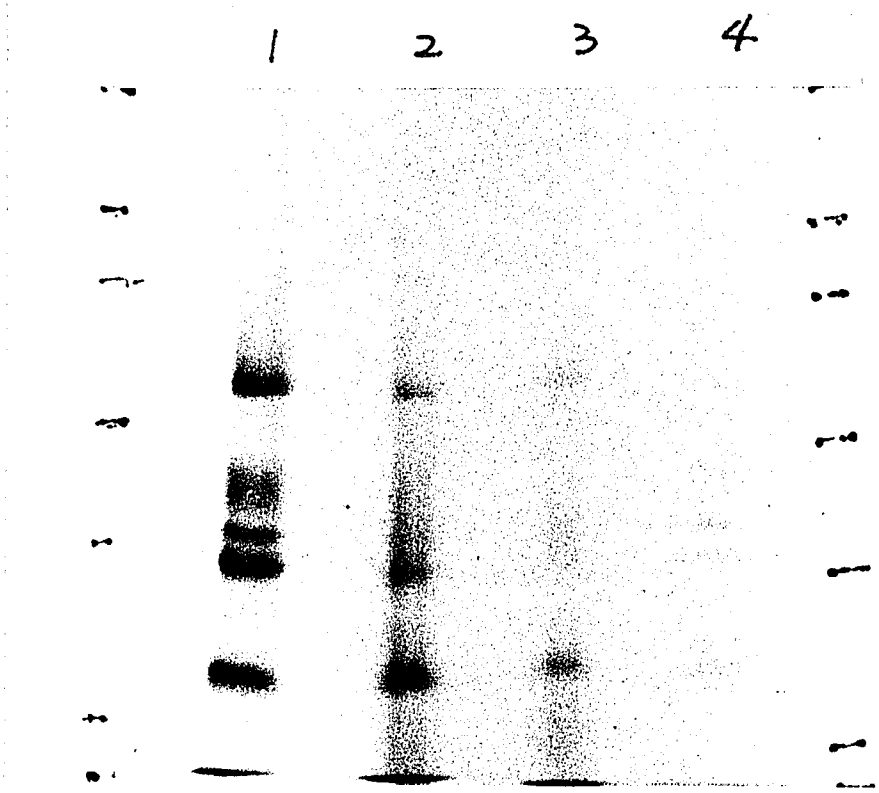

Fig. 23. Radioautogram of sodium dodecyl sulfate polyacrylamide gel analysis of immunoprecipitates obtained by incubation of IgG fractions from antisera I and II and control serum with mitochondria extracted from cells labeled with [^{35}S]methionine in the presence of cycloheximide. Lane 1, mitochondrial translational products, obtained by pulse labeling one gram of yeast cells with 1 mCi of [^{35}S]methionine in the in the presence of cycloheximide for 5 min at 10°C. Mitochondria were prepared after breakage of cells with glass beads and solubilized with 1% deoxycholate and 1% Lubrol at 4°C for 1 hr prior to immunoincubation. Lane 2, immunoprecipitate of IgG fraction against protein I. Lane 3, immunoprecipitate of IgG fraction against protein II. Lane 4, immunoprecipitate of control IgG fraction.

digestion with hydrogen peroxide as described in Materials and Methods. As shown in Fig. 24a, IgG fraction I precipitated from labeled mitoplasts one single predominent peak with a molecular weight of 31,000 dalton. The minor labeled peaks of 96,000, 63,000 and 23,000 daltons are negligible. A parallel gel profile of the labeled mitoplasts also contained a pronounced peak of 31,000 molecular weight, as well as other translation products. Fig.24b shows that IgG fraction II also precipitated one prominent peak with an apparent molecular weight of 31,000. This 31,000 dalton peak contained less label than that in the immunoprecipitate form IgG fraction I. The 58,000 dalton minor peak might be an aggregation of cytochrome b since a peak of that molecular weight does not appear in the gel profile of mitoplasts. The high molecular weight minor peak (higher than 100,000 dalton) might be an artifact or an aggregation of cytochrome b. The observation that IgG fractions I and II are able to precipitate only one protein with the apparent molecular weight of cytochrome b from a mitoplast preparation obtained from cells labeled in the presence of cycloheximide, provides striking evidence that mitochondrial ribosomes are the site of translation of the two forms of cytochrome b, apocytochrome b-I and b-II.

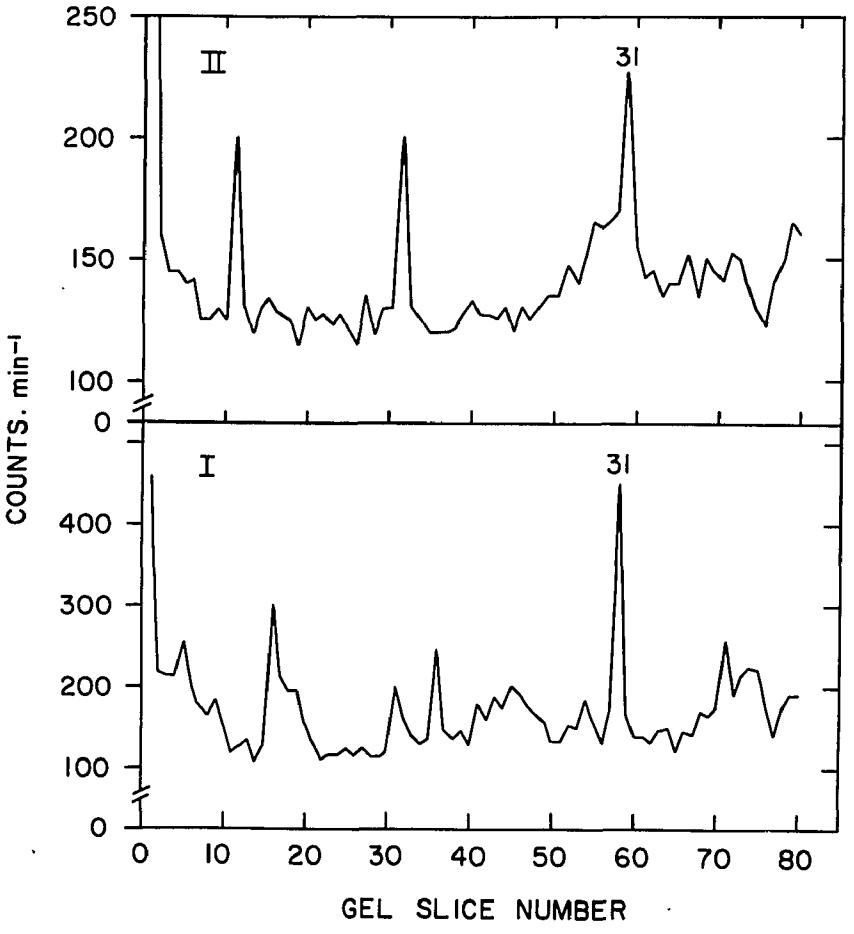

Fig. 24. Electrophoretic analysis of apocytochrome b-I and b-II immunoprecipitated from labeled yeast mitoplasts. Yeast cells of strain KL14-4A were harvested at early stationary stage and pulse labeled with 1 mCi [^{3}H]leucine per g of cells for 15 min at 30°C under non-growing conditions in the presence of cycloheximide. Labeled cells were lysed with 2 mg Zymolyase 5000 per g cells at 30°C for 1 hr and sphero-

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plasts were prepared. Mitochondria were isolated by breaking spheroplasts in a mini-Waring blendor for 25 sec. Mitoplasts were obtained by incubating mitochondria with 3 mg digitonin per 10 mg mitochondrial protein for 5 min at 4°C, and sedimented by a 10 min centrifugation at 12,000 g. Mitoplast extracts in 1% Lubrol and 1% deoxycholate were immunoincubated with the IgG fractions against apocytochrome b-I and b-II. Immunoprecipitates were subjected to slab gel electrophoresis and 10 mm wide slab gel strips were sliced manually. Panel I is immunoprecipitate formed with antiserum against apocytochrome b-I while Panel II is that formed with antiserum against apocytochrome b-II.

5. LOCALIZATION OF APOCYTOCHROMES b-I AND b-II

Inhibition of cytochrome c reductase activity by IgG fractions I and II.

Before studying the effect of IgG fractions I and II on DBH_2:cytochrome c reductase activity, it was essential to demonstrate that all membrane fractions, e.g. mitochondria, submitochondrial particles and mitoplasts, were enzymatically active. The preparation of a fraction containing mitoplasts, an inner membrane-matrix fraction, was obtained by digitonin treatment of yeast mitochondria (Velours et al 1977). The effect of various concentrations of digitonin treatment on the cytochrome c reductase activity of mitochondria was first investigated. Mitochondria prepared from spheroplasts of yeast strain KL14-4A were incubated with 1 mg, 3 mg or 10 mg of digitonin per 10 mg of mitochondrial protein for 5 min at 4°C, respectively. After centrifugation, the sedimented pellets were resuspended in sodium phosphate buffer at a concentration of 1.5 mg protein per ml and treated with 0%, 1%, 2% or 3% of cholate separately prior to enzyme assay. The assay DBH_2:cytochrome c reductase was performed as described in Materials and Methods.

As seen in Fig.25, the enzyme activities of 1 mg, 3 mg and 10 mg digitonin per 10 mg protein treated mitochondria were identical without addition of cholate. The addition of 1%

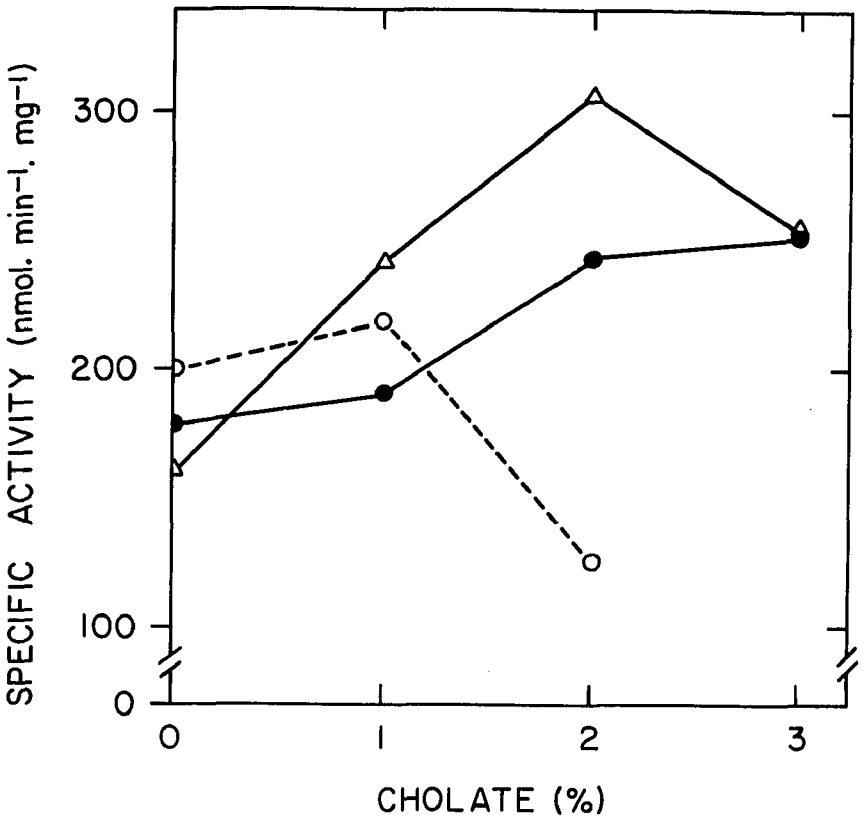

Fig. 25. Effect of cholate concentration on DBH_2-cytochrome c reductase activity of mitoplasts, 1 mg digitonin treated (●—●), 3mg digitonin treated (△—△) and 10 mg digitonin treated (○--○) mitochondria. Mitochondria, prepared from spheroplast as described under Materials and Methods, were incubated 5 min at 4°C with 1 mg, 3 mg and 10 mg digitonin/10 mg protein, respectively, and centrifuged at 12,000 g for 10 min. A stock solution of 20% cholate was added to each mitoplast suspension of 1.5 mg/ml to indicated final concentration just prior to assay. DBH_2-cytochrome c reductase activity was performed according to the methods of Brown and Beattie (1977).

cholate stimulated the enzymatic activities of mitochondria treated with 1 mg and 10 mg of digitonin to a small extent but stimulated significantly mitochondria treated with 3 mg of digitonin. The addition of 2% cholate stimulated enzyme activities of mitochondria treated with 3 mg digitonin to a greater extent than that of mitochondria treated with 1 mg digitonin, but inhibited the enzyme activity of mitochondria treated with 10 mg of digitonin. The addition of 3% cholate overdispersed the mitoplasts obtained from 3 mg digitonin-treated mitochondria and reduced their enzyme activity, although 3% cholate slightly stimulated the enzyme activity of 1 mg digitonin-treated mitochondria. These data suggested that the optimum condition to obtain an enzymatically active mitoplast preparation is to treat the mitochondrial suspension with 3 mg digitonin per 10 mg protein and to disperse the mitoplasts thus obtained with 2% cholate prior to enzyme assay.

Subsequently, the DBH_2:cytochrome c reductase activities of mitochondria, submitochondrial particles and mitoplasts from the haploid strain KL14-4A were assayed with and without addition of cholate at the indicated concentrations (Table 3). Before addition of cholate, the cytochrome c reductase activities of mitochondria, mitoplasts and submitochondrial particle preparations are all approximately the same. The DBH_2:cytochrome c reductase activity of mitoplasts increased

TABLE 3

The Effect of Cholate Treatment on DBH_2-cytochrome c reductase activity in mitochondria, mitoplasts and submitochondrial particles.

Preparations	DBH_2-cytochrome c reductase activity					
	Control		1 % Cholate		2 % Cholate	
	$nmol.min^{-1}.mg^{-1}$	%	$nmol.min^{-1}.mg^{-1}$	% increase	$nmol.min^{-1}.mg^{-1}$	% increase
Mitochondria	613.	100	594.	- 2.94	641.	4.7
Mitoplasts	537.	100	595.	10.7	745.	38.7
Submitochondrial particles	571.	100	888.	55.5	977.	71.2

10% by solubilization with 1% cholate, and 38% by 2% cholate. The greatest stimulation of cytochrome c reductase activity by cholate was observed with the preparation of submitochondrial particles. The enzyme activity was stimulated up to 55% by addition of 1% cholate, and up to 71% by 2% cholate.

To study the inhibition of enzyme activity by antibodies raised against the two forms of apocytochrome b, inside out vesicles (submitochondrial particles) and outer membrane depleted organelles (mitoplasts) were used. An aliquot of 10, 20, and 40 µl of IgG fractions against apocytochrome b-I, b-II and control IgG fraction was incubated with 10 µl of 2% cholate treated submitochondrial particles or mitoplasts at a protein concentration of 1.5 µg per µl for 45 min at room temperature prior to enzyme assay. In a parallel experiment, it was shown that the 45 min incubation without any serum or IgG fraction at room temperature did not affect the enzyme activity. The degree of inhibition was obtained by comparing the enzyme activity of IgG fraction I or II treated mitoplasts or submitochondrial particles with the enzyme activity of that preparation treated with the corresponding volume of control IgG.

As seen in Fig.26a, the inhibition effect of both IgG fractions I and II on the DBH_2:cytochrome c reductase activity of mitoplasts increased linearly with increasing volume of IgG fraction, showing that both IgG fractions exerted a significant inhibition effect on the enzyme activity of

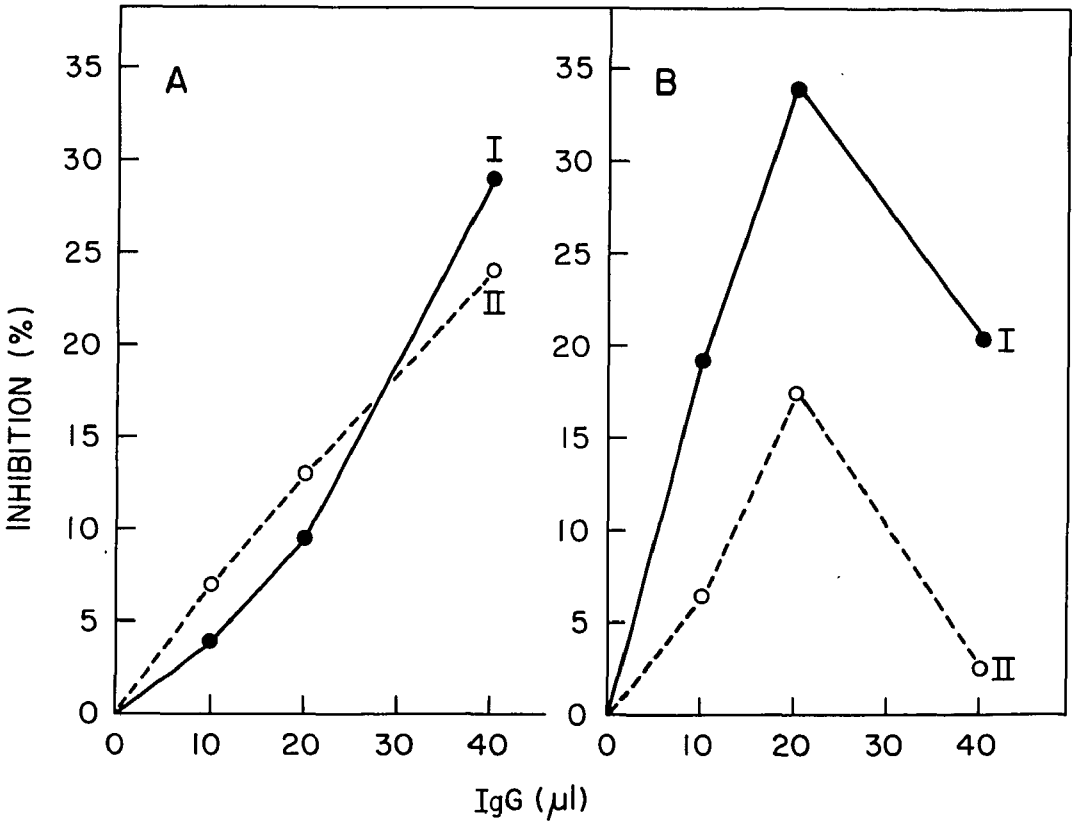

Fig. 26. Inhibitory effects of IgG fractions against apocytochrome b-I (●—●) and b-II (○--○) on DBH_2-cytochrome c reductase activity of (A) mitoplast and (B) submitochondrial particles. IgG fractions were prepared according to the legend of Fig.22. Mitoplasts were obtained by treating mitochondria with 3 mg digitonin/10 mg protein as described in the legend to Fig.25. Aliquots containing 10 μl of 2% cholate treated mitoplasts and submitochondrial particles (1.5 mg protein/ml) were incubated individually with various amounts of IgG fractions for 45 min at room temperature. Enzyme activity was compared with that obtained when a corresponding amount of control IgG fraction was incubated with the preparation. The results are expressed as percent inhibition.

mitoplasts. The inhibition of cytochrome c reductase activity of submitochondrial particles is plotted in Fig.26b. IgG fraction I inhibited the enzymatic activity of submitochondrial particles to a greater extent than did IgG fraction II. However, it is clear that both IgG fractions I and II inhibited significantly the DBH_2:cytochrome c reductase activity of submitochondrial particles.

In general, IgG fractions I and II raised against two forms of apocytochrome b, are capable of inhibiting cytochrome c reductase activity from both the matrix side (as shown in submitochondrial particles preparation) and the outer membrane side (as seen in mitoplast preparation), providing evidence that the two forms of apocytochrome b span the mitochondrial inner membrane.

Labeling of Apocytochromes b-I and b-II with [^{125}I]DABS.

To confirm that these two apocytochromes b span the mitochondrial inner membrane,the membrane non-penetrating reagent, diazotized iodosulfanilic acid([^{125}I]DABS) was used to label protein components which are exposed on the surface of the membrane from both the matrix side and the cytochrome c side. It has been shown that diazotized sulfanilic acid and iodosulfanilic acids do not enter into the interior of cells or organelles, such that only proteins extending from or exposed on the membrane are labeled (Berg, 1969; Sears et al, 1971; Tinberg et al, 1974; George et al, 1976; Prochaska et

al , 1980).

Mitochondria and submitochondrial particles, freshly prepared at the suspensions of 2 mg protein per ml sodium phosphate buffer, were incubated with 400 µCi, 40 µCi or 8 µCi of [^{125}I]diazotized iodosulfanilic acid (2200 Ci/mM) at 4^{o}C for 30 min or 60 min with frequent vortexing. After extensive washing by centrifugation, labeled mitochondria and submitochondrial particles were solubilized with either 1% Lubrol, 1% deoxycholate or 1 M potassium chloride, 1% Triton X-100, and immunoincubated with optimum amounts of IgG fractions I and II and control IgG fraction. Labeled mitochondria, submitochondrial particles and immunoprecipitates were analyzed on a 10% acrylamide gel electrophoresis and then radioautographed.

As illustrated in Fig.27, the incorporation of diazotized [^{125}I] iodosulfanilic acid into mitochondrial surface proteins and submitochondrial particles was linearly proportional to the concentration of [^{125}I]DABS in the incubation mixture, rather than to the incubation time, since no difference in the incorporated specific activity was observed between the 30 min and 60 min incubation.

As shown in the autoradiogram (Fig.28), four proteins at molecular weights of 65,000, 52,000, 31,000 and 29,000 in the mitochondria were labeled with [^{125}I]DABS (lane 1) while in submitochondrial particles (lane 8) seven labeled

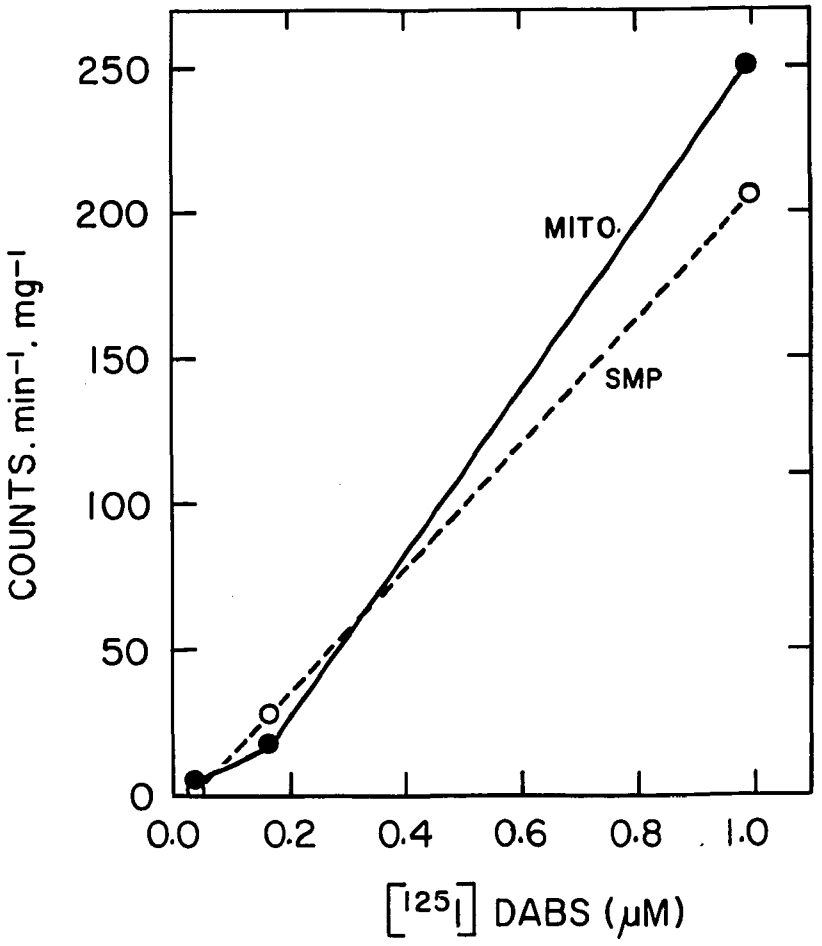

Fig. 27. Incorporation of [^{125}I]diazobenzene iodosulfanilic acid into mitochondria and submitochondrial particles. Aliquots containing 100 µl of mitochondria (●—●) and submitochondrial particles (○- - -○) 2 mg/ml) were incubated with various amounts of 2200 Ci.mmol [^{125}I]diazobenzene iodosulfonate acid for 30 min at 4°C. Incorporation was terminated by addition of 4 ml of 5 mM histidine and immediate

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centrifugation. The treated mitochondria and submitochondrial particles were extensively washed by several centrifugations to remove free [^{125}I]diazobenzene iodosulfonate.

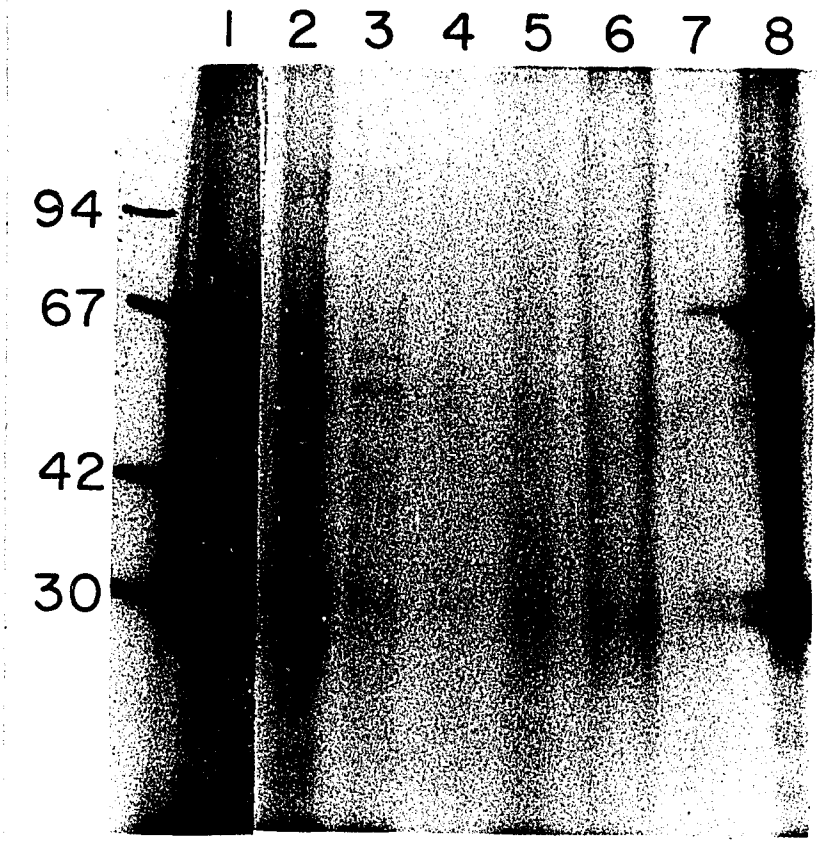

Fig. 28. Autoradiogram of sodium dodecyl sulfate polyacrylamide gel pattern of immunoprecipitates formed with antibodies against apocytochrome b-I and b-II and mitochondria and submitochondrial particles labeled with [^{125}I]DABS. Aliquots of 100µl of mitochondria and submitochondrial particles at 2 µg protein/ 1 were labeled with 400 Ci of [^{125}I]diazobenzene iodosulfonate for 30 min at 4°C. Labeled mitochondria were solubilized with 1% deoxycholate and 1% Lubrol, whereas submitochondrial particles were solubilized with 1M KCl and 1% Triton X-100 prior to immunoincubation. Lane 1, mitochondria. Lane 2, immunoprecipitate of IgG fraction against apocytochrome b-I with [^{125}I]DABS labeled mitochondria. Lane 3, immunoprecipitate of IgG fraction against apocytochrome b-II with [^{125}I]DABS incorporated mitochondria. Lane 4, immunoprecipitate of control IgG fraction with [^{125}I]DABS labeled mitochondria. Lane 5, immunoprecipitate of IgG fraction against apocytochrome b-I with [^{125}I]DABS labeled submitochondrial particles. Lane 6, immunoprecipitate of IgG fraction against apocytochrome b-II with [^{125}I]-DABS labeled submitochondrial particles. Lane 7, immunoprecipitate of control IgG fraction with [^{125}I]-DABS labeled submitochondrial particles. Lane 8, [^{125}I]DABS incorporated submitochondrial particles.

bands were observed at molecular weights of 96,000, 68,000, 62,000, 55,000, 50,000, 31,000 and 29,000. The different gel patterns of mitochondria and submitochondrial particles indicated the asymmetrical arrangement of the protein components in the mitochondrial inner membrane.

Immunoprecipitates from IgG fractions I, II and control IgG fraction incubated with mitochondria are shown in lanes 2, 3, and 4 (Fig.28). Two labeled bands of 56,000 and 31,000 were observed with IgG fraction I, and three labeled bands of 60,000, 55,000 and 30,000 were in the immunoprecipitate of IgG fraction II, while no protein was precipitated by the control IgG fraction, showing again the specificity of IgG fractions I and II. The 31,000 dalton protein in the immunoprecipitate of IgG fraction I and the 30,000 dalton protein in the precipitate of IgG fraction II are apocytochromes *b* with their antigenic active site(s) exposed to the outside of the mitochondrial inner membrane. The two labeled bands of molecular weight between 55,000 and 60,000 in the immunoprecipitates might be aggregates of cytochrome *b* or proteins which non-specifically precipitate with the IgG fractions.

IgG fractions I, II and control IgG fraction treated immunoprecipitates from submitochondrial particles are shown in Fig.28, lanes 5,6 and 7,respectively. Only one single band was found labeled in both IgG fractions I and II precipitated submitochondrial particles with molecular weights of 30,000

and 29,000, respectively. The 30,000 dalton protein may be apocytochrome b-I while the 29,000 dalton protein may be apocytochrome b-II; however, it is clear that both proteins are cytochrome b. Both proteins are less highly labeled in the immunoprecipitates from the submitochondrial particles than those from mitochondria, suggesting that apocytochrome b may be more exposed to the cytoplasmic side of the inner membrane than to the matrix side.

The results of the [^{125}I]DABS labeling experiments provide further evidence that the two forms of apocytochrome b span the inner membrane of mitochondria.

CHAPTER IV: DISCUSSION

Several different values for the molecular weight of cytochrome b from the respiratory chain of yeast as well as mammalian mitochondria have been reported (Weiss, 1976). These varying estimates may have occured because of species differences in the molecular weight while others may have resulted because of the anomalous behavior of cytochrome b during gel electrophoresis (Marres and Slater, 1977; Groot, et al, 1978). While most proteins migrate according to the logarithm of their molecular weight in sodium dodecylsulfate, some proteins behave abnormally whether due to the conformation of the molecule, to the inability of the protein to bind sodium dodecylsulfate or to the presence of unusual charged groups on the surface. When the migration of these proteins in different concentrations of acrylamide is measured, the curve obtained in the plot of R_f values versus acrylamide extrapolates back to a different value than that of standard proteins. The slope of this line, defined as the retardation coefficient, K_r, can be used to estimate the molecular weights of peptides which have an anomolous behavior in dodecylsulfate electrophoresis (Frank and Rodbard, 1975). For cytochrome b, the K_r value corresponds to a molecular weight of 55,000. Groot et al (1978) have suggested that this value may be the true molecular weight of the protein, in contrast to the suggestion of Weiss and Ziganke (1974) that cytochrome b from Neurospora exists as a dimer with a molecular weight of 56,000. In all of these

studies, where an anomalous migration of cytochrome b was observed, this protein was present either in the intact mitochondrial membrane or associated with other proteins in the cytochrome b-c_1 complex.

Hence we decided to reinvestigate the behavior of cytochrome b during sodium dodecylsulfate gel electrophoresis using a purified preparation. For these studies it was impossible to use our previously purified cytochrome b since proteolytic digestion was a necessary step during the purification (Lin and Beattie, 1978). By solubilizing sub-mitochondrial particles in low concentrations of cholate followed by ammonium sulfate precipitation, we have been able to obtain a spectrally pure cytochrome b preparation which contains only 4 major polypeptides. The band in this preparation corresponding to cytochrome b, identified by comparison with total mitochondrial translation products, displayed normal migration behavior during sodium dodecyl-sulfate electrophoresis in different concentrations of acrylamide with an apparent molecular weight of 31,000. It thus appears that considerable changes occur when cyto-chrome b is separated from cytochrome c_1 and other associated proteins in the b-c_1 complex. Perhaps cytochrome b may change upon cleavage of the cytochrome b-c_1 complex. This suggestion is supported by the observations that two specifi-

cally and kinetically different species of cytochrome b are present in mitochondria and an isolated cytochrome b-c_1 complex but cannot be observed when the complex has been split (Weiss, 1976). We have concluded from these studies that the actual molecular weight of cytochrome b is 31,000. In addition, this polypeptide migrates with the same molecular weight in the sodium dodecylsulfate-urea gels of Swank and Munkres (1971). This value for the molecular weight also correlates well with the molecular weight of cytochrome b isolated from beef heart (von Jagow, 1978b) and from Neurospora crassa (Weiss,1978).

The major difficulty encountered in the isolation of cytochrome b is the contamination by cytochrome c_1 in the preparation due to the close relationship of these two proteins in the mitochondrial inner membrane.For instance, the cytochrome b preparation from yeast reported by Katan et al (1976) is contaminated by cytochrome c_1 as judged by its absorption maximum at 555 nm. In the present purification, cytochrome c_1, together with cytochromes a-a_3, is removed at step 2 - an ammonium sulfate fraction which contains spectrally pure cytochrome b as shown by difference absorption spectra (Fig.5).

Upon separation by ultrogel column chromatography, heme b content was estimated by the absorbance at 418 nm, the Soret peak of oxidized cytochrome b, and the polypeptide composition of each fraction was revealed by preliminary sodium dodecyl-

sulfate gel electrophoresis. In order to reduce any disulfide bonds, to dissociate cytochrome b from other membrane proteins and to solubilize the ammonium sulfate precipitate, 5% sodium dodecylsulfate and 5% β-mercaptoethanol was added to the ammonium sulfate precipitate prior to ultrogel filtration. Despite these precautions, the majority of cytochrome b when dissociated from other proteins has a tendency to aggregate and forms oligomers with molecular weights above 90,000 dalton which appear in the first peak off the ultrogel column. The heme of cytochrome b is also present in some small polypeptide or as free heme residues probably resulting from partial or complete degradation of cytochrome b.

An ultrogel fraction containing two major polypeptides at molecular weights around 30,000 dalton was further separated by super gel electrophoresis. Two major proteins were eluted individually from super gel slices with a modified funnel-shaped acrylamide gel electrophoresis (cf Fig. 1) and Coomassie brilliant blue was removed by acetone extraction (cf Fig. 3). The funnel-shaped acrylamide gel for protein elution from gel slices is an original design, while the preparative gel electrophoretic separation and acetone extraction are modifications of Chua and Blomberg's (1979) methods for the separation of chloroplast membrane proteins.

Protein I of 31,000 daltons and protein II of 29,000 daltons are thus purified by this purification procedure.

Both proteins show only one single band upon sodium dodecylsulfate polyacrylamide gel electrophoresis (cf Fig. 13). Furthermore, protein I and protein II are identified as apocytochrome *b*-I and *b*-II by their similiar antigenic response to antibody against cytochrome *b* prepared by Lin *et al* (1978), by their normal migration behavior and their identical fingerprints obtained by limited proteolytic digestion (cf Fig. 15). Despite the fact that the yield for protein I is merely 0.09% of the starting mitochondrial protein and that for protein II is 0.05% (Table 2), these yields were satisfactory to serve the second goal of this dissertation, the purification of two homogenous cytochromes *b* with distinct molecular weights.

The existence of two functionally different cytochromes *b* in complex III has been postulated on the basis of spectrophotometric, redox kinetic and potentiometric studies performed in intact mitochondria or in an isolated *b*-c_1 complex since the 1950's (Chance, 1958; Wainio, 1977; von Jagow and Sebald, 1980). The present study is the first report of the purification to apparent homogeneity of two species of cytochrome *b* from one source - yeast *Saccharomyces cerevisiae*. Although Weiss and Ziganke (1976) have separated, by means of hydroxylapatite column chromatography in the presence of sodium dodecylsulfate, two subunits of cytochrome *b* from *Neurospora crassa* with similiar amino acid composition

and identical C and N terminal residues, no further information about either the homogeneity or the molecular weights of these subunits have been published.

Purified apocytochrome b-I migrates with a molecular weight (31,000) identical to that of native cytochrome b which was immunoprecipitated by antiserum against cytochrome b from mitochondria pulse labeled in the presence of cycloheximide or from cells grown in [^{3}H]leucine (Lin et al, 1978). Purified apocytochrome b-II possesses the same electrophoretic mobility (29,000) as the cytochrome b band of the total mitochondrial translational products in a 10% acrylamide gel. In addition, both cytochromes b-I and b-II gave similar fingerprint patterns after limited proteolytic digestion. It is concluded from all these data that cytochromes b-I and b-II are two forms of cytochrome b, sharing most of the peptide sequence including antigenical active site(s), and that cytochrome b-II is a part of cytochrome b-I.

It was not feasible to distinguish these two purified cytochromes b spectrally or functionally. Firstly, throughout the purification procedures the preparation of cytochrome b is in sodium dodecylsulfate for at least 2 weeks, from step 2 to step 5 under Materials and Methods. During this time, heme b is lost. Secondly, less than 1 mg of protein is obtained in each preparation. The quantities of purified

protein obtained do not allow a spectral analysis. Moreover, no enzymatic assay is appropriate just for cytochrome b.

In order to explore the relationship between apocytochrome b-I and b-II and to investigate the biogenesis of the two purified cytochrome b, antisera against apocytochrome b-I and b-II were raised and the specificity of both antisera was established by Ouchterlony double diffusion test and counter-immunoelectrophoresis experiments. It was not surprising that antiserum against apocytochrome b-I (antiserum I) fully reacts with cytochrome b-II while antiserum to apocytochrome b-II (antiserum II) only partially reacts with cytochrome b-I. As discussed above, apocytochrome b-I and b-II share some peptide sequences probably including some antigenic active sites; however, b-II contains fewer amino acids than b-I and thus may have lost some antigenically active sites. Therefore, the binding sites which are made complementary to active sites of an antigen are fewer in antiserum II than in antiserum I. As a consequence, antiserum I may be stronger in precipitating both cytochrome b-I and b-II. When cytochrome b-II reacts with antiserum I, all the active sites in cytochrome b-II are occupied and a complete precipitin line is formed. However, owing to the lack of binding sites in antiserum II, when it reacts with b-I, some of the active sites in b-I are still free, which resulted in a partial

reaction (cf Fig. 17b). These observations provided further evidence that both cytochrome b-I and b-II share certain common antigenic sites.

The data obtained from the counterimmunoelectrophoresis and the immunodiffusion test indicate that both antisera against apocytochrome b-I and b-II are capable of reacting with purified cytochrome b-I and b-II as well as the spectrally pure but crude cytochrome b preparation. Furthermore, cytochrome b can be immunoprecipitated from small quantities of labeled extracts of yeast mitochondria by immunoincubation with either antiserum I or II (cf Fig. 20). We have concluded from all these data that both antisera I and II are specific against apocytochrome b.

To avoid non-specific binding in the immunoprecipitation experiment, IgG fractions were further purified from both antisera and control serum basically according to Chan and Schatz (1978). Immunotitration curves obtained using crude sera and that obtained from purified IgG fractions are comparable in terms of the absolute ratio which is defined as the ratio of the volume of serum or IgG fraction in µl to the protein content of the extract in µg in an immunoincubation. Generally, a volume ratio or regular ratio of serum to extract is expressed in immunoincubation or immunotitration assays (Ebner et al, 1973; Line et al, 1978; Poyton and McKemmie, 1979).

Since the antigen content in an extract is proportional to the protein content rather than to the volume of that extract, we found that the absolute ratio provides more meaningful and useful information than a volume ratio. For these particular antisera and IgG fractions, absolute ratio 1 gives the best precipitation.

The synthesis of apocytochrome b-I and b-II on mitochondrial ribosomes has been shown by sodium dodecylsulfate gel analysis of radioactively labeled immunoprecipitates. Incorporation of [^{3}H]leucine or [^{35}S]methionine into apocytochrome b-I and b-II in yeast cells is resistant to cycloheximide, an inhibitor of cytoplasmic protein synthesis. The presence of only one protein with the molecular weight of cytochrome b in the immunoprecipitate obtained with IgG fraction I or II from a mitoplast preparation obtained from cells labeled in the presence of cycloheximide (cf Fig.24), provides a striking evidence that apocytochrome b-I and b-II are translated on mitochondrial ribosomes. These results are in good agreement with previous studies on Neurospora crassa cytochrome b (Weiss and Ziganke, 1974; Weiss, 1976) and studies on yeast cytochrome b (Katan et al., 1976; Lin et al., 1978).

Recent biochemical and genetic analyses have demonstrated the existence of only one single mosaic structural gene

coding for apocytochrome b of molecular weight around 30,000 dalton on the mitochondrial DNA (Alexander et al, 1979; Haid et al, 1979; Alexander et al, 1980). In the present study, we have purified two forms of cytochrome b: apocytochrome b-I of 31,000 dalton, and apocytochrome b-II of 29,000 dalton; both are structurally and antigenically similar and are translated on mitochondrial ribosomes. It is most likely that these two forms of cytochrome b are coded on the COB-BOX region of mitochondrial DNA and translated as one single homologous protein which is then modified posttranslationally. It has been suggested by Weiss and Ziganke (1974) and mentioned by von Jagow and Engel (1980) that the difference between the two functionally distinct cytochromes b is possibly due to posttranslational modification. The appearance of two forms of cytochrome b in Saccharomyces cerevisiae does not confirm previous suggestions of a cytochrome b homo-dimer. Von Jagow and Engel (1980) have recently proposed that one cytochrome b monomer of the cytochrome b dimer translocates protons by undergoing a conformational change. This hypothesis is based on the observation that cytochrome b has a pH dependence on the half-reduction potential.

A preparation of mitoplasts was obtained from yeast mitochondria by treatment with 0.4 mg of digitonin per mg of protein by Velours et al (1977). The mitoplast preparation thus

obtained was shown by these workers to consist of an inner membrane-matrix fraction by electron microscopy respiratory control and the release of the outer membrane marker enzyme, kynurenine hydroxylase. Poyton and McKemmie (1979) also showed, using marker enzymes, that treatment of mitochondria with 0.35 mg of digitonin per mg of mitochondrial protein for 1 min produces an inner mitochondrial membrane fraction. In accordance with the above findings, our observations on the cytochrome c reductase activity indicated that a mitoplast fraction obtained by treating mitochondria with 3 mg digitonin per 10 mg protein for 5 min is active and sensitive to cholate stimulation (cf Fig. 25).

The table of cytochrome c reductase activities (Table 3) indicates that our mitochondria, mitoplasts and submitochondrial particles are good closed membrane systems. DBH_2, an anologue of ubiquinone, was used to replace ubiquinone in the electron transport chain because of its hydrophilicity and solubility. In the submitochondrial particles and mitoplasts, the electron transfer components including cytochromes b and c_1 are exposed after cholate treatment to facilitate electron transfer from DBH_2 to cytochrome c. In the mitochondria, owing to the presence of the outer membrane, which interferes with the contact of electron carriers with substrate DBH_2, even 2% cholate did not stimulate enzyme activity significantly.

The localization of apocytochrome b-I and b-II was explored by two different approaches: (1) inhibition of cytochrome c reductase activities of mitoplasts and submitochondrial particles by IgG fractions I and II, (2) iodination of apocytochrome b-I and b-II by [^{125}I]diazobenzene iodo sulfonate, the membrane non-penetrating reagent from the matrix and cytosolic sides. Both IgG fractions I and II inhibited cytochrome c reductase activity from both the cytoplasmic and matrix side of the membrane. In addition, sodium dodecyl sulfate gel analysis of immunoprecipitates revealed that both apocytochrome b-I and b-II are labeled by [^{125}I]DABS in mitochondria and submitochondrial particles. These data give direct proof for the localization of two cytochromes b in the model proposed by Bell et al.(1979) for the arrangement of polypeptides in complex III. It is concluded that both apocytochrome b-I and b-II span the mitochondrial inner membrane and protrude from both the matrix and cytoplasmic sides.

LIST OF ABBREVIATIONS

ATP:	adenosine triphosphate
DABS:	diazobenzene sulfonate
DB, DBH_2:	2,3-dimethoxy-5-methyl-6-n-decyl-1,4-benzoquinone and its reduced form
DCCD:	dicyclohexylcarbodiimide
DOC:	deoxycholate
EDTA:	ethylenediaminetetraacetic acid
EGTA:	ethyleneglycol-bis-(β-amino-ethyl ether) N,N'-tetraacetic acid
FAD:	flavin adenine dinucleotide
[^{125}I]DABS:	[^{125}I]diazobenzene iodosulfonate
K_r:	retardation coefficient
mtDNA:	mitochondrial DNA
NADH:	reduced nicotinamide adenine dinucleotide
PMSF:	phenylmethylsulfonyl flouride
Q:	coenzyme Q or ubiquinone
STE buffer:	0.25 M sucrose, 0.05 M Tris-HCl, 1 mM EDTA, pH 7.5
VAR-1:	a protein of the mitochondrial small ribosomal subunit.

REFERENCES

1. Alexander, N.J., Perlman, P.S., Hanson, D.K. and Mahler, H.R. (1980) Cell 20, 199.

2. Alexander, N.J., Vincent, R.D., Perlman, P.S., Miller,D.H., Hanson, D.K. and Mahler, H.R. (1977) J.Biol.Chem. 254, 2471.

3. Ball, M.B., Bell, R.L. and Capaldi, R.A. (1977) FEBS Lett. 83, 99.

4. Beattie, D.S., Battie, C. and Weiss, R. (1980) submitted for publication.

5. Beattie, D.S. Chen, Y.S., Clejan, L. and Lin, L.F.H. (1979) Biochemistry 18, 2400.

6. Bell, R.L., Sweetland, J., Ludwig, B. and Capaldi, R.A. (1979) Proc.Nat'l.Acad.Sci. USA 76, 741.

7. Bell, R.H. and Capaldi, R.A. (1976) Biochemistry 15, 996.

8. Berg, H. (1969) Biochim.Biophys.Acta. 183, 69.

9. Berk, A.J. and Clayton, D.A. (1974) J.Mol.Biol. 86, 801.

10. Borst, P. (1972) Ann.Rev.Biochem. 41, 333.

11. Borst, P. and Grivell, L.A. (1978) Cell. 15, 705.

12. Bossard, M.J., Vik, T.A. and Schuster, S.M. (1980) J.Biol.Chem. 255, 5342.

13. Boxer, D.H. (1975) FEBS Lett. 59, 149.

14. Boyer, P.D., Chance, B., Ernster, L., Mitchell, P., Racker, E. and Slater, E.C. (1977) Ann.Rev.Biochem. 46, 955.

15. Brdiczka, D. and Schumacher, D. (1976) Biochem.Biophys.Res. Commun. 73, 823.

16. Brown, G.G., and Beattie , D.S. (1977) Biochemistry 16, 4449.

17. Cabral, F., Lolioz, M., Rudin, Y., Schatz, G., Clavilier,L. and Slonimski, P.P. (1978) J.Biol.Chem. 253, 297.

18. Case, G.D., Ohnishi, T. and Leigh, J.S. (1976) Biochem.J. 160, 785.

19. Chan, S.H. and Schatz, G. (1979) in: Methods in Enzymology. Vol. 56, pp 223.

20. Chance, B. (1958) J.Biol.Chem. 233, 1223.

21. Chance, B., Williams, E.R. Adv.Enzym. (1956) 17, 65.

22. Chance, B., Wilson, D.F., Dutton, P.L. and Erecinska, M. (1970) Proc.Nat'l. Acad.Sci. USA 66, 1175.

23. Chua, N.H. and Blomberg, F. (1979) J.Biol.Chem. 254, 215.

24. Cleveland, D.W., Fisher, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) J.Biol.Chem. 252, 1102.

25. Cote, C., Solioz, M. and Schatz, G. (1979) J.Biol.Chem. 254, 1437.

26. Criddle, R.S., Packer, L. and Shieh, P. (1977) Proc. Nat'l.Acad.Sci. USA 74, 4306.

27. Davis, K.A., Hatefi, Y., Poff, K.L. and Butler, W.L. (1973) Biochim.Biophys.Acta 325, 341.

28. Dervartanian, D.V., Albracht, S.P.L., Berden, J.A., Van Gelder, B.F. and Slater, E.C. (1973) Biochim.Biophys. Acta 292, 496.

29. Di Jeso, F., Giorgini, D. and Bartolucci, S. (1978) Comptes Rendus Soc.Biol. (Paris) 172, 67.

30. Dujon, B., (1979) Nature 282, 777.

31. Dujon, B., Colson, A.M. and Slonimski, P.P. (1977) in: Mitochondria 1977. Berlin: Walter de Gruyter pp 579-669.

32. Dutton, P.L., Erecinska, M., Sata, N., Mukai, Y., Pring, M. and Wilson, D.F. (1972) Biochim.Biophys.Acta. 267, 15.

33. Dutton, P.L., Wilson, D.F. and Lee, C.-P (1970) Biochemistry 9, 5077.

34. Ebner, E., Mason, T.L. and Schatz, G. (1979) J.Biol.Chem. 248, 5369.

35. Enns,R.K. and Criddle, R.S. (1977) Arch. Biochem. Biophys. 182, 587.

36. Eytan, G.D. and Schatz, G. (1975) J.Biol.Chem. 250, 767.

37. Eytan, G.D., Carroll, R.C., Schatz, G. and Racker, E. (1975) J.Biol.Chem. 250, 8598.

38. Frank, R.N. and Rodbard, D. (1975) Arch.Biochem.Biophys. 171, 1.

39. Freedman, G.A., Tracy, R.P. and Chan, S.H.P. (1979) J.Biol.Chem. 254, 4305.

40. Ferguson, K.A. (1964) Metab.Clin.Exp. 13, 985.

41. Gellerfors, I. and Nelson, B.D. (1977) Eur.J.Biochem. 80, 275.

42. George, J.N., Potterf, R.D., Lewis, P.C. and Sears, D.A. (1976) J.Lab.Clin.Med. 88, 232.

43. Gilbert, W. (1978) Nature 271, 501.

44. Green, D.E. and Hatefi, Y. (1961) Science 133, 13.

45. Groot, G.S.P., Van Harten-Loosbroek, N. and Kreike, J. (1978) Biochim.Biophys.Acta 517, 457.

46. Haid, A., Schweyen, R.J., Bechmann, H., Kaudewitz, F., Solioz, M. and Schatz, G. (1979) Eur.J.Biochem. 94, 451.

47. Hanson, D.K., Miller, D.H., Mahler, H.R., Alexander, N.J. and Perlman, P.A. (1979) J.Biol.Chem. 254, 2480.

48. Hatefi, Y. and Stempel, K.E. (1969) J.Biol.Chem. 244, 2350.

49. Hatefi, Y., Hanstein, W.G., Gallante, Y. and Stigall, D.I. (1975) Fed.Proc. 34, 1699.

50. Hatefi, Y. and Galante, Y.M. (1980) J.Biol.Chem. 255, 5530.

51. Katan, M.B., Van Harten-Loosbro k, N. and Groot, G.S.P. (1976) Eur.J.Biochem. 70, 409.

52. Keilin, D. (1925) Proc.Roy.Soc., Ser., B, 98, 312.

53. Keihl, R. and Hatefi, Y., (1980) Biochemistry 19, 541.

54. Kim, I.C. and Beattie, D.S. (1973) Eur. J. Biochem. 36, 509.

55. Klingenberg, M. (1968) In Biological Oxidations, ed. T.P. Singer, New York: Interscience pp 3-54.

56. Kroger, A. and Klingenberg, M. (1970) Vitam.Horm. NY. 28, 533.

57. Laemmli, V.K. (1970) Nature (London) 227, 680.

58. Lewin, A.S., Gregor, I., Mason, T.L., Nelson, N. and Schatz, G. (1980) Proc.Nat'l.Acad.Sci. USA. 77, 3998.

59. Lin, L.F.H. and Beattie, D.S. (1978) J.Biol.Chem. 253, 2412.

60. Lin, L.F.H., Clejan, L. and Beattie, D.S. (1978) Eur.J.Biochem. 87, 171.

61 Lin, L.F.H., Kim, I.C. and Beattie, D.S. (1974) Arch. Biochem. Biophys. 160, 458.

62. Locker, J. and Rabinowitz, M. (1979) in: Methods in Enzymology. Vol. 56, pp. 3.

63. Maccecchini, M.L., Rudin, Y., Blobel, G. and Schatz, G. (1979) Proc.Nat'l.Acad.Sci. USA 76, 343.

64. Marres, C.A.M. and Slater, E.C. (1977) Biochim.Biophys. Acta 462, 531.

65. Mason, T.L. and Schatz, G. (1973) J.Biol.Chem. 248, 1346.

66. Mendel-Hartvig, I. and Nelson, B.D. (1978) FEBS Lett. 92, 36.

67. Mihara, K. and Blobel, G. (1980) Proc. Nat'l.Acad.Sci. USA 77, 4160.

68. Nelson, B.D. and Mendel-Hartvig, I. (1977) Eur.J.Biochem. 80, 267.

69. Ohnishi, K. (1966) J. Biochem. 59, 1.

70. Orme-Johnson, N.R., Hansen, R.E. and Beinert, H. (1974) J.Biol.Chem. 249, 1928.

71. Ouchterlony, O. (1953) Acta Pathol.Microbiol.Scand. 32, 231.

72. Papa, S. (1976) Biochim.Biophys.Acta 456, 39.

73. Perlman, P.S. and Mahler, H.P. (1974) Arch.Biochem. Biophys. 162, 248.

74. Poyton, R.O. and McKemmie, E. (1979) J.Biol.Chem. 254, 6763.

75. Prochaska, L., Bisson, R. and Capaldi, R.A. (1980) Biochemistry 19, 3174.

76. Pumphrey, A.M. (1962) J.Biol.Chem. 237, 2384.

77. Ragan, C.I. (1976) Biochim.Biophys.Acta 456, 249.

78. Rieske, J.S. (1976) Biochim.Biophys.Acta 456, 195.

79. Rubin, M.S. and Tzagoloff, A. (1973) J.Biol.Chem. 248, 4275.

80. Saltzgaber, J. and Schatz, G. (1978) J.Biol.Chem. 253, 305.

81. Schatz, G. and Kovac, L. (1974) in: Methods in Enzymology. Vol. 31, pp 627.

82. Schneider, D.L., Kagawa, Y. and Racker, E. (1972) J.Biol.Chem. 247, 4074.

83. Sears, D.A., Reed, C.F. and Helmkamp, R.W. (1971) Biochim. Biophys. Acta 233, 716.

84. Seabald, W. and Wachter, E. (1978) in: Energy Conservation in Biological Membranes (Schafer, C. and Klingenberg, M. eds.) Springer Verlag, W. Berlin. pp. 228-236.

85. Sevarino, K.A. and Poyton, R.O. (1980) Proc. Nat'l.Acad. Sci. USA 77, 142.

86. Siedow, J.N., Power, S., DeLa Rosa, F.F. and Palmer, J. (1978) J.Biol.Chem. 253, 2392.

87. Smith, R.J. and Capaldi, R.A. (1977) Biochemistry 16, 2629.

88. Solioz, M. and Schatz, G. (1979) J.Biol.Chem. 254, 9331.

89. Stiggall, D.L., Galante, Y.M. and Hatefi, Y. (1979) in: Methods in Enzymology. Vol LV, pp 308.

90. Stiggall, D.L., Galante, Y.M. and Hatefi, Y. (1978) J.Biol.Chem. 253, 956.

91. Studier, F.W. (1973) J.Mol.Biol. 79, 237.

92. Swank, R.T. and Munkres, K.D. (1971) Anal.Biochem. 39, 462.

93. Tanaka, M., Hanin, M., Yasunobu, K.T., Yu, C.A., Yu, L., Wei, Y.H. and King, T.E. (1979) J.Biol.Chem. 254, 3879.

94. Tinberg, H.M., Melnick, R.L., Maguire, J. and Packer, L. (1974) Biochim.Biophys.Acta 345, 118.

95. Trumpower, B.L. and Edwards, C.A. (1979a) FEBS Lett. 100, 13.

96. Trumpower, B.L. and Edwards, C.A. (1979b) J.Biol.Chem. 254, 8697.

97. Tzagoloff, A., Macino, G. and Sebald, W. (1979) Ann.Rev. Biochem. 49, 419.

98. Tzagoloff, A., Rubin, M.S. and Sierra, M.F. (1973) Biochim.Biophys.Acta 301, 71.

99. Velours, J., Guerin, B. and Duvert, M. (1977) Arch. Biochem.Biophys. 182, 295.

100. von Jagow, G. and Engel, W.D. (1980) FEBS Lett. 111, 1.

101. von Jagow, G., Schagger, H., Riccio, P., Klingenberg, M. and Kolb, H.J. (1977) Biochim.Biophys.Acta 462, 549.

102. von Jagow, G., Schagger, H., Engel, W.D., Riccio, P., Kolb, H.J. and Klingenberg, M. (1978a) in: Methods in Enzymology 53, 92.

103. von Jagow, G., Schagger, H., Engel, W.D., Machleidt, W. and Machleidt, I. (1978b) FEBS Lett. 91, 121.

104. von Jagow, G. and Sebald, W. (1980) Ann.Rev.Biochem. 49, 281.

105. Wainio, W.W. (1977) J. Bioenerg.Biomem. 9, 321.

106. Weber, K. and Osborn, M. (1969) J.Biol.Chem. 244, 4406.

107. Weiss, H. (1976) Biochim.Biophys.Acta 456, 291.

108. Weiss, H. and Juchs, B. (1978) Eur.J.Biochem. 88, 17.

109. Weiss, H. and Kolb, H.J. (1979) Eur.J.Biochem. 99, 139.

110. Weiss, H. and Ziganke, B. (1974) Eur.J.Biochem. 41, 63.

111. Weiss, H. and Ziganke, B. (1976) in: Genetics and Biogenetics of Chloroplasts and Mitochondria, Th. Bucher, et al eds. Elsevier, North Holland Biomedical Press. pp 259-67.

112. Weiss, H. and Ziganke, B. (1977) in Mitochondria 1977. Berlin: Walter de Gruyter pp 463-76.

113. Weiss, H. and Ziganke, B. (1978) in: Methods in Enzymology. Vol. 58, pp 212.

114. Werner, S. and Machleidt, W. (1978) Eur. J. Biochem. 90, 99.

115. Wikstrom, M.K.F. (1971) Biochim.Biophys,Acta 253, 332.

116. Wikstrom, M.K.F. (1973) Biochim.Biophys.Acta 301, 155.

117. Wikstrom, M.K.F. and Berden, J.A. (1972) Biochim.Biophys. Acta 283, 403.

118. Yu, C., Yu, L. and King, T.E. (1975) Biochem.Biophys. Res.Commun. 66, 1194.