b-TYPE CYTOCHROMES[1]   ◆12043

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CONTENTS

INTRODUCTION, SUMMARY, AND PERSPECTIVES

The b-type cytochromes are a class of electron-transfer proteins, which, by definition, contain a noncovalently bound heme (protoporphyrin IX) as

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prosthetic group (1, 2). These cytochromes exhibit a high structural diversity, and they function in a variety of redox reactions and electron transfer chains in aerobic, anaerobic (3, 4), and photosynthetic (5) bacteria, as well as in eukaryotic animal and plant cells (5). In some of these systems, the oxidation energy is conserved for the synthesis of ATP.

This review concentrates on three examples of membrane-bound b cytochromes: (a) cytochrome b_5 of liver microsomes, (b) the b cytochromes involved in the reduction of nitrate and fumarate in the cytoplasmic membrane of anaerobic bacteria, and (c) the b cytochromes of the respiratory chain of the mitochondrial inner membrane. These cytochromes vary in structural complexity from the molecularly dispersed microsomal cytochrome b_5, to the b cytochromes that are subunits of likewise simple complexes of two to four different subunits, and finally to the dimeric cytochrome b of the highly complicated mitochondrial unbiquinol:cytochrome c reductase which contains at least eight additional subunit polypeptides.

In recent years considerable progress has been achieved in the isolation, structural and functional analysis, and even the reconstitution of these intrinsic membrane proteins. Former soluble preparations of some of these b-cytochromes have originated from the action of proteases, and the complete proteins could only be solubilized and purified in the presence of mild detergents. Multisubunit complexes containing b cytochromes as QH_2: nitrate reductase, MQH_2:fumarate reductase, succinate:Q reductase, and QH_2:cytochrome c reductase could be isolated in a functional state. Controlled dissociation of such complexes lead to the isolation of partial complexes devoid of certain subunits and functional properties. The most intricate cytochrome b, that of the mitochondrial QH_2:cytochrome c reductase, could be obtained as a dimer that retained at least some of its functional properties.

Amino acid sequence analysis of hydrophobic polypeptides is still problematic. The primary structure of the membrane-integrated segment of microsomal cytochrome b_5 has now been elucidated for several species. Partial sequences of the extremely hydrophobic mitochondrial cytochrome b have been determined, and the further sequence analysis of this 30,000-dalton polypeptide probably will profit from studies of the nucleotide sequence of the structural gene. In general, the few available complete primary structures indicate that evolutionarily unrelated b-cytochromes exist. Three-dimensional structures show different types of cytochrome fold as well as histidine-histidine and histidine-methionine as heme-liganding residues.

An essential question concerns the topology (6) of the membrane-bound b-cytochromes. The heme-carrying segment of cytochrome b_5 appears to

be in contact with the water-phase of the cytoplasm, while the hydrophobic segment only anchors the protein in the membrane. The multisubunit complexes, however, span the membrane assymetrically with different polypeptides being accessible from the two faces of the membrane. The bacterial cytochrome *b* of the QH_2: nitrate reductase is found solely at the periplasmic side of the membrane, while the mitochondrial cytochrome *b* itself possibly traverses the membrane as a dimer.

The identification of the immediate physiological electron donors and acceptors still poses a major problem. The situation appears to be clear with microsomal cytochrome b_5 in so far as the constituents of a stearyl-CoA desaturase system have been isolated and reconstituted. The system requires, besides cytochrome b_5, a NADH-linked cytochrome b_5 reductase and the desaturase. Both the reductase and the desaturase are probably amphipathic proteins, which difuse freely in the membrane like the cytochrome. The redox centres of the three proteins are all oriented toward the same side of the membrane, and the redox reactions occur after diffusion-controlled collision of the proteins. In situ, cytochrome b_5 may have additional redox partners that are not yet unequivocally identified. Furthermore, in the case of the cytochrome b_5 of the erythrocyte methemoglobin reductase system it was postulated that an alteration of structure and biological function of the cytochrome occurs during maturation of the red blood cell. In the case of the *b* cytochromes of the multisubunit complexes, the pathway of the electron is more difficult to elucidate. In all these systems a quinone appears to be one of the redox partners (7). The *Escherichia coli* QH_2: nitrate reductase constitutes a transmembrane electron pathway. Its cytochrome *b* reacts with ubiquinol at the periplasmic surface. The nitrate reducase moiety contains a further two redox centres whose sequence is still unknown. The electron flow from formate to fumarate in *Vibrio succinogenes* is also organized transmembranously. Two distinct *b* cytochromes are involved, most likely as reductase and oxidase of menaquinone. The topology of these *b* cytochromes, however, is unknown. Two different additional redox centres detected in isolated partial complexes are possibly the other protein redox partners of the cytochrome. An especially sophisticated electron-transfer protein is represented by the dimeric *b* cytochrome of the mitochondrial QH_2: cytochrome *c* reductase. The isolated cytochrome shows, as the protein in situ, two different half-reduction potentials, even though both subunits are very similar or even identical. It is unclear whether both *b* cytochromes are arranged in parallel, in series, or otherwise. The flow of the electron is highly controversial. We depict two alternatives schematically: (*a*) the Q cycle involving bound QH· radicals and (*b*) a more classical sequence involving QH_2 as reductant and an iron-sulfur protein as oxidant of the *b* cytochromes.

Some of the cytochrome b–containing electron-transfer chains conserve the free energy of oxidation as a transmembranous proton potential that can be used for the synthesis of ATP (8). At the present state of knowledge it appears likely that a proton gradient can be produced by different mechanisms. 1. The $E. coli$ QH_2:nitrate reductase system which transfers electrons across the membrane from a proton-producing half-reaction (QH_2 oxidation) at the periplasmic face to a proton-consuming half-reaction (nitrate reduction) at the cytoplasmic face. A corresponding mechanism appears to function in the vectorial formate:fumarate reductase system of $V.$ $succinogenes.$ 2. In the case of the mitochondrial QH_2:cytochrome c reductase (site II of oxidative phosphorylation) the proton movements associated with the overall redox reactions cannot account for the proton/e ratios that have been observed in situ. An attractive hypothesis postulates that a protein constituent of the complex exhibits true proton-pumping abilities. The cytochrome b dimer appears to be a likely candidate for such a proton pump since its redox centres show a pH dependence. An alternative and at this time already classical hypothesis postulates a "Q" cycle. Here the protons are translocated by the quinone, which is reduced and oxidized at multiple sites of the complex located at different faces of the membrane.

A section on the biogenesis and genetics of mitochondrial cytochrome b is included below. Cytochrome b of the QH_2:cytochrome c reducatase has been shown to be a mitochondrial gene and translation product. A library of mutants with defective or modified cytochrome b is now available which should be extremely useful for a further elucidation of the structural and functional properties of this intricate b-type cytochrome.

CYTOCHROME b_5

Microsomal cytochrome b_5 is a membrane-bound protein functioning as a component of the NADH-dependent $\Delta 9$ stearyl CoA desaturase system (9, 10). It has also been implicated in NADPH-linked microsomal hydroxylation reactions (11, 12). Additionally, in the supernatant fraction of erythrocytes a soluble cytochrome b_5 is present which participates in the reduction of methemoglobin by transferring electrons from erythrocyte cytochrome b_5 reductase to methemoglobin (13, 14).

The microsomal cytochrome b_5 in its native state is an amphipathic protein with an M_r of 16,000 (15). It contains two domains: a hydrophilic, catalytic segment consisting of about 80 amino acid residues, and a hydrophobic segment that is required for binding the cytochrome to the microsomal membrane (15, 16). Controlled proteolytic digestion of the native

protein yields a water-soluble cytochrome b_5 with a molecular weight of about 11,000. The amino acid sequence of the soluble cytochrome b_5 has been reported for several species (17, 18). From crystals of the bovine protein a three-dimensional structure was obtained at 2 Å resolution (19). The structural and functional properties of the soluble cytochrome b_5 have been extensively reviewed (2). In the following, recent information on the native cytochrome b_5 (structure, functional properties, reconstitution, and biosynthesis) is presented.

The isolation of complete cytochrome b_5 from liver endoplasmatic reticulum requires solubilization and fractionation in the presence of detergents (15, 17, 20). In a recently improved purification procedure 2% Triton X-100 was applied (21). The final protein, exhibiting a ratio of OD_{280}/OD_{413} lower than 0.36, is soluble in the absence of detergent. Aggregates of cytochrome b_5, apparently octamers, form at neutral or slightly alkaline pH values and 0.02–0.4 ionic strength (21).

Amino acid sequences of the hydrophobic segment of cytochrome b_5 from porcine (22), equine (23), bovine (24), and rabbit (25) have been published recently. This nonpolar peptide is present at the COOH-terminus of the complete cytochrome b_5, which (in equine) now consists of a total of 133 residues (see Figure 1). The four established sequences from porcine, horse, bovine, and rabbit show extensive homology. The sequence of residues 104–126 contains only hydrophobic or uncharged hydrophilic side chains. The basic and acidic amino acid residues occur at the COOH-terminus and in the peptide linking the membranous segment to the globular heme-carrying segment. Such residues may be expected to be present

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                    90                            100                           110
Porcine          -Ile-Ala-Lys-Pro-Ser-Glu-Thr-Leu-Ile-Thr-Thr-Val-Glu-Ser-Asn-Ser-Ser-Trp-Trp-Thr-Asn-Trp

Equine      -Ser-Lys-Ile-Ala-Lys-Pro-Val-Glu-Thr-Leu-Ile-Thr-Thr-Val-Asp-Ser-Asn-Ser-Ser-Trp-Trp-Thr-Asn-Trp

Bovine           -Ile-Thr-Lys-Pro-Ser-Glu-Ser-Ile-Ile-Thr-Thr-Ile-Asp-Ser-Asn-Pro-Ser-Trp-Trp-Thr-Asn-Trp

Rabbit             -Met-Gln-Thr-Leu-Ile-Thr-Thr-Val-Asp-Ser-Asn-Ser-Ser-Trp-Trp-Thr-Asn-Trp
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                            120                           130
Porcine          Val-Ile-Pro-Ala-Ile-Ser-Ala-Leu-Val-Val-Ser-Leu-Met-Tyr-His-Phe-Tyr-Thr-Ser-Glu-Asn

Equine           Val-Ile-Pro-Ala-Ile-Ser-Ala-Val-Val-Val-Ala-Leu-Met-Tyr-Arg-Ile-Tyr-Thr-Ala-Glu-Asp

Bovine           Leu-Ile-Pro-Ala-Ile-Ser-Ala-Leu-Phe-Val-Ala-Leu-Ile-Tyr-His-Leu-Tyr-Thr-Ser-Glu-Asn

Rabbit           Val-Ile-Pro-Ala-Ile-Ser-Ala-Leu-Ile-Val-Ala-Leu-Met-Tyr-Arg-Leu-Tyr-Met-Ala-Asp-Asp
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Figure 1 Amino acid sequences of the hydrophobic segment of apocytochrome b_5 from the liver of porcine (22), equine (23), bovine (24), and rabbit (25). For the sequence of the heme-carrying segment see (2, 17, 18).

outside the hydrophobic milieu of the lipid membrane, thereby suggesting that the hydrophobic segment either penetrates the membrane or folds back on itself so that the COOH-terminus is near the cytoplasmic surface of the membrane. The results of a predictive analysis for conformational features (22, 24) according to the rules of Chou & Fasman (26, 27), are similar for the amino acid sequence of the membranous segment from horse, equine, and bovine. The sequence 103–112, which contains a cluster of three trypto-phanyl residues, seems to consist of 3–4 overlapping β-turn structures. The prediction must be viewed with caution, however, since the conformational parameters employed were derived from studies on globular hydrophilic proteins and may not be necessarily extendable to membranous peptides.

From the structure of complete cytochrome b_5, it is clear that the soluble cytochrome b_5 originated by tryptic cleavage at peptide bond 90–91 or 88–89. In addition, a short peptide (1–6 in porcine) is removed from the NH_2-terminus. Cathepsin-produced soluble cytochrome b_5 shows serine 97 as COOH-terminus (28).

The complete cytochrome b_5, including the membranous segment, is necessary for functional reconstitution of the stearyl-CoA desaturase system, although the soluble cytochrome b_5 accepts electrons from cytochrome b_5 reductase (12, 29). The spectral properties of complete cytochrome b_5 are essentially the same as those of the soluble form (heme peptide segment) (21).

The natural electron donor for cytochrome b_5 is the NADH-cytochrome b_5 reductase (30), a flavoprotein of molecular weight 33,000 (31) which is tightly associated with microsomal membranes. It is interesting that the native reductase is isolated only after detergent solubilization (31, 32), whereas a smaller soluble form is produced by lysosomal proteases contaminating the microsomal fraction (33). Thus the reductase represents an amphipathic protein, like cytochrome b_5. A physiological electron acceptor of cytochrome b_5 is the stearyl-CoA desaturase, which has been purified from rat microsomes (10, 34). It is a single polypeptide species of an apparent molecular weight of 53,000, which contains one iron atom. The desaturase is extracted only by detergents, and, accordingly, its amino acid composition shows an abundance of nonpolar amino acid residues.

These three microsomal electron transfer components have been incorporated into liposomes or microsomal vesicles (29, 35–37), in order to reconstitute a partial or complete electron transfer system from NADH to the terminal desaturase, and to examine protein-protein, protein-lipid, and diffusion-dependent interactions. All three enzymes, in any combination, bind to vesicles that retain a spherical shape with a diameter of 250–400 Å. It is found that the catalytic centers of the reductase, cytochrome b_5 and

the desaturase are all oriented toward the outer surface of the small vesicles.

Vesicles, containing the cytochrome b_5 and reductase, catalyze electron transfer (from NADH to cytochrome b_5 or to cytochrome *c*) at rates indistinguishable from those observed with microsomal preparations (35, 36). The rate of electron transfer is dependent upon the surface concentration of the components in the bilayer. At low concentrations, flavin oxidation by cytochrome b_5 becomes rate limiting, as expected for a reaction in which protein-protein interactions become diffusion-limited. This is consistent with a model involving random distribution of the nonpolar segments of these proteins in the phospholipid bilayer, and subsequent movement of the molecules through a distance of several molecular diameters to achieve productive collisions. In the complete desaturase system (34), the hydrogen abstraction from the substrate is rate limiting. Thus the overall reaction is not diffusion limited.

Recently, evidence has been presented that two forms of a soluble cytochrome b_5 isolated from the supernatant of fresh bovine erythrocytes are identical to segments of microsomal cytochrome b_5 (38). Erythrocyte cytochrome b_5 participates in the reduction of methemoglobin by transferring electrons from erythrocyte cytochrome b_5 reductase to methemoglobin (13, 14, 39). Apart from being water soluble, erythrocyte cytochrome b_5 is very similar to the microsomal cytochrome. The visible and electron paramagnetic resonance spectra of the two proteins are similar. Both forms of cytochrome b_5 can be reduced by the microsomal cytochrome b_5 reductase. Proteolysis by trypsin results in a hemoprotein indistinguishable from the tryptic products of the microsomal protein. These results agree with the hypothesis (39, 40) that soluble erythrocyte cytochrome b_5 is derived during erythropoiesis by proteolytic degradation of the membrane-bound cytochrome b_5 present in the endoplasmatic reticulum of the proerythroblasts. Such a conversion would imply that during differentation of a cell, different biological functions can be performed by a membrane-bound protein and the water-soluble catalytic segment of that protein.

BACTERIAL *b*-CYTOCHROMES

Escherichia Coli

In *Escherichia coli* at least five *b*-type cytochromes have been identified which have been designated, according to their spectral properties, *b*-556, *b*-558, *b*-562, *b*-556 (NO$^-_3$), and cytochrome *O* (4, 41, 42). In principle, the *E. coli* system would appear to be especially attractive, as defined growth conditions can be used to induce or repress specific cytochrome *b* –requiring pathways. Furthermore, specific mutants may be obtained with

lesions in defined structural genes. Despite these potentialities of the *E. coli* system, definite information is, up to now, available only for *b*-562 and the *b*-556 of the nitrate reductase.

CYTOCHROME *b*-562   a protein of about 12,000 molecular weight, is isolated from a soluble extract of disrupted cells (43, 44). Significant amounts, however, have been detected in membrane particles (45). Its function and intracellular location are still unknown. The three dimensional structure has been determined to 2.5 Å resolution (46). This *b*-type cytochrome has a methionyl and a histidyl residue as heme ligands, like cytochrome *c*. Its half-reduction potential of $E_{m7} = +110$ mV is rather positive, but it is lower than that of a *c*-type cytochrome ($E_{m7} \simeq +230$ –$+270$ mV). This may be attributed to the observation that in the cytochrome *b*-562 the heme is more exposed to the solvent than in cytochrome *c* (46). The structure of *E. coli* cytochrome *b*-562 represents a new type of cytochrome fold, and shows a different topology from that of cytochrome b_5, cytochrome *c*, or the globins.

CYTOCHROME *b*-556 OF NITRATE REDUCTASE   The ubiquinol : nitrate reductase has been isolated from *E. coli* (47–51). The multisubunit complex contains cytochrome *b*-556 (NO_3^-) as a subunit. As yet, the *b*-type cytochrome itself has not been purified. The nitrate reductase constitutes the terminal part of an anaerobic electron pathway localized in the bacterial plasma membrane (see Figure 2). This enzyme system has received special attention during the past years, since the electron transfer from QH_2 to nitrate is coupled to the formation of a transmembrane proton gradient.

The QH_2 : nitrate reductase was isolated by several laboratories after solubilization with detergent (47–51), and was found to contain three different polypeptide subunits of apparent molecular weights of 155,000, 63,000, and 19,000. Reductase preparations solubilized from the membrane by alkaline heat treatment lack spectral cytochrome *b* and also the 19,000-dalton subunit (51–54). Thus it was concluded, that the 19,000-dalton subunit represents the apoprotein of cytochrome *b*-556 (NO_3^-). Further
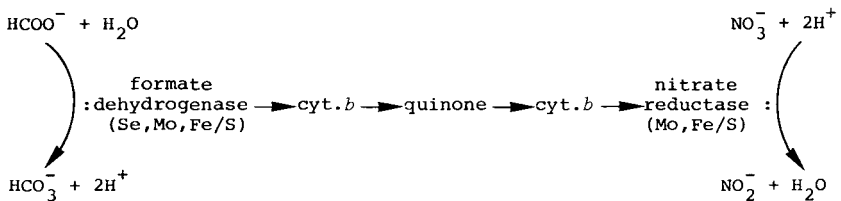

Figure 2   Scheme of the electron pathway from formate to nitrate in *Escherichia coli* (47, 64).
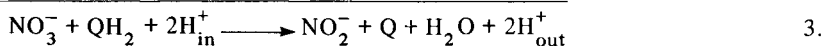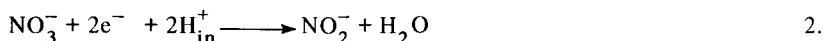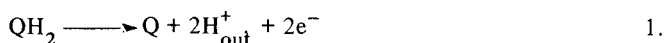
redox centres such as molybdenum (52, 54, 55) and nonheme iron (54, 56) were found to be present in the two larger subunits. The spectrum of cytochrome *b*-556 (NO_3^-) shows absorbance maxima at 559 nm, 531 nm, and 428 nm when it is integrated in the isolated nitrate reductase complex (47, 48). The molar ratio of the cytochrome in the complex is not clear. Heme contents of 6.7 (47, 48) and 15 (50) nmol/mg nitrate reductase have been reported. From the amount of the 19,000-dalton apoprotein present in the whole complex, ratios of 1 : 1 : 2 for the subunits of molecular weights 155,000, 63,000, and 19,000 have been calculated (49, 57).

The nitrate reductase is induced only in anaerobically grown cells in the presence of nitrate and molybdenum [see e.g. (42, 58, 59)]. The fully induced enzyme protein constitutes up to 15% of the cytoplasmic membrane (49, 50). Concomitant with the reductase activity the *b*-type cytochrome is formed. A heme-deficient mutant unable to synthesize δ-aminolevulinic acid, produces reduced amounts of membrane-bound reductase which contain no cytochrome *b* subunit. A large amount of a soluble form of the two-subunit reductase accumulates in the cytoplasm of this mutant (57, 60). When δ-aminolevulinic acid is added to the mutant culture, the cytoplasmic form of the enzyme is incorporated into the membrane in a stable form coincident with the formation of functional cytochrome *b* (60). These results indicate that a complex of the two large subunits binds to the membrane upon interaction with the third subunit, cytochrome *b*.

The cytochrome *b* appears to be functionally involved in the QH_2 : nitrate reductase reaction. The complete reaction is obtained with the cytochrome *b*–containing preparation, whereas in a cytochrome-free reductase, nitrate can only be reduced with artificial electron donors (57). The cytochrome *b* can be reduced by formate in the presence of *E. coli* formate dehydrogenase (47). The reduced cytochrome of the reductase is immediately reoxidized upon addition of nitrate. The whole formate : nitrate reductase reaction was reconstituted using the soluble purified formate dehydrogenase and the nitrate reductase. The rate of the reaction was stimulated 33-fold by the addition of ubiquinone-6. A cytochrome *b*–free preparation of the reductase was completely inactive in the reconstituted system. The *b*-type cytochrome of the formate dehydrogenase appears to be different from that of the reductase (48), although the molecular weights are similar and the spectral properties are indistinguishable.

Surface-labelling experiments with membrane-nonpermeating probes indicate that the cytochrome *b* apoprotein is accessible from the periplasmic face of the cytoplasmic membrane, whereas the 155,000-dalton subunit is accessible from the cytoplasmic side (61, 62). This transmembrane location of the QH_2 : nitrate reductase system of *E. coli* is further indicated by studies with membrane-permeating and nonpermeating substrates (63, 64). It has

been emphasized that the proton translocation produced during the electron flow from QH_2 to nitrate is chemiosmotic. The electron-translocating oxidoreduction connects a proton-consuming and a proton-producing half reaction which occur on opposite sides of the membrane according to following reactions:

$$QH_2 \longrightarrow Q + 2H_{out}^+ + 2e^- \qquad\qquad 1.$$

$$NO_3^- + 2e^- + 2H_{in}^+ \longrightarrow NO_2^- + H_2O \qquad\qquad 2.$$

$$NO_3^- + QH_2 + 2H_{in}^+ \longrightarrow NO_2^- + Q + H_2O + 2H_{out}^+ \qquad 3.$$

QH_2 most likely reacts directly with cytochrome b located at the periplasmic side of the membrane (Reaction 1), since the nonpermeant Diquat radical (DQ^+) is an excellent reductant for nitrate reductase in spheroplasts provided that the cytochrome b of the complex is present (63). Absence of ubiquinone or menaquinone, as in double quinone mutant, does not alter the ability of DQ^+ to reduce nitrate reductase. The immediate source of the protons and electrons for nitrate reduction (Reaction 2) is on the cytoplasmic side of the membrane. The access of nitrate to the active site, however, is still unclear. The permeation of nitrate across the membrane is too slow to be kinetically competent for the nitrate reduction rate. It has been suggested that nitrate approaches the electron- and proton-donating redox centre via a cleft extending inwards from the periplasmic surface (64).

Vibrio Succinogenes

Vibrio succinogenes, an anaerobic bacterium, contains two b-type cytochromes in the cytoplasmic membrane, which participate in the electron transfer from formate to fumarate (Figure 3). They have a common absorbance spectrum with an absorbance maximum at 562 nm in the dithionite-reduced state, but they possess quite different half-reduction potentials. The electron transport from formate to fumarate is associated with the generation of an electrochemical proton gradient across the cytoplasmic membrane of the bacterium, and with the phosphorylation of ADP.

The fumarate reductase complex, the terminal part of the electron-transferring chain from formate to fumarate, has been extracted from the cytoplasmic membrane with Triton X-100 and purified by means of chromatography oh hydroxyapatite and DEAE Sephadex (65). It consists of three polypeptide subunits of apparent molecular weights of 79,000, 31,000, and 25,000. A complex lacking the heme b absorbance signal has been isolated (65). It did not contain the 25,000-dalton subunit. Therefore cytochrome b has been attributed to the 25,000-dalton polypeptide. The

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HCOO⁻ + H₂O                                                    fumarate + 2H⁺

    \           formate                                fumarate              /
     \   :dehydrogenase ──► cyt.b ──►menaquinone ──► cyt.b ──►reductase :
      )        (Mo,Fe/S)      (-200mV)                 (-20mV)    (FAD,Fe/S)
     /
HCO₃⁻ + 2H⁺                                                         succinate
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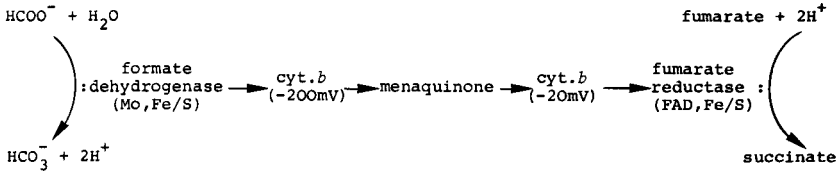
Figure 3   Scheme of the electron pathway from formate to fumarate in *Vibrio succinogenes* (68).

FAD has been found exclusively in the 79,000-molecular weight peptide. Nonheme iron and acid-labile sulfide were found in the 79,000 and 31,000-dalton polypeptides. A molar ratio of 1:1:2 in the complete complex for the subunits of molecular weight 79,000:31,000:25,000 has been calculated.

The enzyme catalyzed three types of reactions: (*a*) Fumarate reduction, by viologen radicals or anthrahydroquinone sulfonate, which was independent of the presence of cytochrome *b* and insensitive to p-chloromercuriphenyl sulfonate (p-CMS); (*b*) succinate oxidation by ferricyanide, Methylene Blue, or phenazine methosulfate, which also was independent of cytochrome *b*, but sensitive to p-CMS; and (*c*) fumarate reduction by certain naphthohydroquinones, and a succinate oxidation by certain quinones. Both of the latter activities were dependent on the presence of cytochrome *b* and sensitive to p-CMS. It has been concluded that, in the membrane of the bacterium, cytochrome *b* ($E_{m7} = -20$ mV) is the primary acceptor of the electrons of menahydroquinone, and the donor for the iron-sulfur centres of the fumarate reductase (see Figure 3).

The formate dehydrogenase complex, the upper part of the electron-transferring chain from formate to fumarate, has been extracted from the cytoplasmic membrane with Triton X-100 and enriched by means of hydroxyapatite and DEAE Sephadex chromatography, and sucrose-density-gradient centrifugation (66). It consists of two polypeptide subunits of apparent molecular weights 110,000 and 25,000. The large subunit contains molybdenum, nonheme iron, and acid-labile sulfide; the smaller subunit carries the heme IX. The complex consists of two moles of the large subunit and varying amount of the small subunit, i.e. cytochrome *b*. The authors are not yet certain whether this cytochrome *b* belongs to the formate dehydrogenase complex. They speculate that either the extent of dissociation or co-isolation may vary with different preparations. The cytochrome *b* has a half-reduction potential of $E_{m7} = -200$ mV. It can be reduced by formate. The electron transfer from formate to menaquinone has been inhibited by 2-(n-nonyl)-4-hydroxyquinoline-N-oxide (NOHOQnO). The

half-reduction potential of cytochrome b shifted from -200 mV to -240 mV under these conditions. It has been concluded that in the membrane of the bacterium this cytochrome b is the direct electron donor for menaquinone, and that the inhibition of electron transfer from formate to menaquinone by NOHOQnO is caused by the binding of the inhibitor directly to this cytochrome b (see Figure 3).

The position of the two b-type cytochromes has been confirmed by quinone-extraction experiments (67). The oxidation of cytochrome b (-200 mV) by fumarate is inhibited by the extraction of menaquinone, whereas the reduction by formate is unaffected. In contrast, the reduction of cytochrome b (-200mV) by formate, but not the oxidation by fumarate, is dependent on the presence of menaquinone.

From accessibility studies with membrane-permeating and nonpermeating dyes it has been concluded that the active site of formate dehydrogenase faces the periplasmatic space, while that of fumarate reductase is in contact with the cytoplasm of the bacterium (68). Thus it has been speculated that, possibly, in $V.$ $succinogenes$ only electrons, and not protons, may be translocated across the membrane, since the two protolytic half-reactions on opposite sides of the membrane are capable of creating a proton gradient. Protons are liberated on the outside by the reaction of formate dehydrogenase with formate, and are taken up on the inside by the reaction of fumarate reductase with fumarate. As this mechanism accounts for the observed H^+/e ratio, there would be no need for proton translocation by menaquinone.

The operation of menaquinone as H^+ translocator, however, has not been excluded. In preliminary experiments H^+ liberation during fumarate oxidation has been measured with cell suspensions of $V.$ $succinogenes$ with a ratio of $H^+/2e = 1.1–1.3$ (69). However, proton gradient measurements with these bacteria are still technically complicated. With current techniques, the data are not reliable enough to give exact $H^+/2e$ ratios, which would help to prove or disprove the existence of an ubiquinone-linked proton transport. With spheroplasts grown on formate and fumarate, P/2e ratios of 0.4–1 have been measured (69). These experiments give, however, no information about the mechanism of the proton gradient creation.

MITOCHONDRIAL b-CYTOCHROMES

Introduction

b-type cytochromes are constituents of two different multiprotein complexes of the mitochondrial respiratory chain. About 80% of the total cytochrome b is a component polypeptide of the ubiquinol:cytochrome c reductase (also named complex III), and about 20% is an integral part of

the succinate:ubiquinone reductase, the so-called complex II. Both complexes seem to form discrete structural and functional units of the mitochondrial inner membrane (70). The complexes have been isolated from the membrane by quite different methods; independent of the applied isolation procedure they contained the same number of polypeptide subunits, possessing distinct molecular weights and functional characteristics (71). Complex III consists of 8 subunits (72), complex II of only 4 (73). Cytochrome *b* of complex III has deserved special interest, since it is intimately involved in the energy conversion process at the second phosphorylation site (74). The function of the cytochrome *b* of complex II is still dubious (75). It remains to be established whether it is involved in the main path of electron flow from succinate to ubiquinone, or whether it represents a redox component of a specific metabolic pathway.

Complex III spans the mitochondrial membrane. Some of its polypeptides are placed nearer the surface of the membrane, for instance cytochrome c_1, or the iron-sulfur protein; some polypeptides (e.g. cytochrome *b*) seem to be buried more in the core of the protein architecture (76).

The discussion of cytochrome *b* function is complicated because, quite apart from other unsolved questions, even its arrangement in the sequence of the electron-transferring components is under discussion (7, 77). The complexity of the problems increases as one goes from studies on the resolved cytochrome *b* to studies of cytochrome *b* integrated in membranes. Therefore, the properties and function of cytochrome *b* is discussed in the following order:(*a*) isolated cytochrome *b*, (*b*) cytochrome *b* in complexes, and finally (*c*) cytochrome *b* in situ.

Cytochrome b of the Ubiquinol:Cytochrome c Reductase

ISOLATED CYTOCHROME b   The various preparations of cytochrome *b* are described with the aid of Table 1. The preparations from beef heart mitochondria are compiled in chronological order. Already in 1961 a highly purified cytochrome *b* was obtained (78). As in all subsequent procedures, a detergent in the presence of high salt concentrations had to be used in order to solubilize the membranous structure, and to hold the hydrophobic polypeptides in a solubilized state. With the exception of preparation (*d*) [see Table 1 (79)] bile salts were used as detergent during solubilization and isolation; for the subsequent separation of the cytochrome *b* a series of ammonium sulfate precipitations were applied. In preparation (*d*) and (*e*) (80) precipitation reactions were avoided. In case (*d*) the beef heart cytochrome *b* was prepared by a series of hydroxyapatite chromatographic treatments in the presence of Triton X-100. The starting material was a pure antimycin-loaded bc_1 complex, which was dissociated by guanidine plus Triton X-100. The *Neurospora* cytochrome *b*(*e*) was prepared by chroma-

Table 1 Isolation methods and properties of resolved cytochrome b of complex III

Source	Isolation Solubilization	Isolation Fractionation	Starting material	Heme content (μmol/g protein)	Molecular weight based on: Heme content	Molecular weight based on: Dodecyl-sulfate electro-phoresis	Molecular weight based on: Ultracentrifugation	Absorbance maxima	E_{m7} (mV)	Comments
(a) Beef heart (78)	Deoxycholate KCl	Cholate/ammonium sulfate, dodecylsulfate	Mitochondria	36	28,000	—	$S_{20,w}$ = 2.6 in cetyldimethylammonium bromide	562.5, 532.5, 429	−340	Denaturation by SDS
(b) Beef heart (82)	Cholate/ammonium sulfate	Cholate/ammonium sulfate, protease, Emasol, DEAE cellulose	"Green Brei" of mitochondria	47	21,000	—	Aggregation	561.5, 531, 428.5	−21	Cleavage of apoprotein by protease
(c) Beef heart (83)	Cholate/ammonium sulfate	Dodecylsulfate, ammonium sulfate, Sephadex G150	Succinate: cytochrome c reductase	—	38,000	38,000	—	560, 530, 427	—	
(d) Beef heart (79)	Triton X-100, P_i, NaCl	Triton X-100, guanidinium chloride hydroxyapatite chromatography	Cytochrome bc_1 complex loaded with antimycin	30	33,000	31,000	62,000 from sedimentation equilibrium in Triton X-100	560, 525, 427	−5 −85	Dimeric structure
(e) Neurospora crassa (80, 81)	Cholate, deoxycholate	Deoxycholate, Lubrol, oleyl-polymethacrylic acid resin chromatography	Submito-chondrial particles	35	30,000	32,000	51,000 from sedimentation equilibrium in bile salts	561, 530, 429	−70	Dimeric structure
(f) Yeast (84)	Deoxycholate, KCl	Cholate, deoxycholate, ammonium sulfate, protease, Tween 80	Submito-chondrial particles	2.8	—	28,000	Aggregation	562, 532, 430	—	Loss of heme b

tography on oleyl-polymethacrylic acid resin, with subsequent chromatography on DEAE cellulose and a recycling gel filtration on Sephadex G-75.

Sources (*d*) and (*e*) have provided good evidence for a 30,000 molecular weight of the monomeric cytochrome *b*, based on pyridine hemochromogen contents, and on gel electrophoresis and gel chromatography in the presence of dodecylsulfate (79, 81). The same molecular weight was already found for preparation (*a*). The molecular weight of 21,000 of preparation (*b*) (82) may be due to the cleavage of a 9.000-dalton polypeptide from cytochrome *b* due to application of a protease during isolation. The high molecular weight of preparation (*c*) (83) may be caused by special conditions during dodecylsulfate electrophoresis. The yeast preparation lost a major part of its heme *b* during isolation (84).

The absorbance spectra of the various preparations are very similar. The a-absorbance maxima of the absolute spectra of ferro-cytochrome *b* lie at 560–562 nm, those of the β-bands at 525–532.5 nm, and those of the Soret bands at 427–430 nm. The redox potentials of the heme *b* centers seem to be a more sensitive parameter for the functional state of the isolated cytochrome *b* than the absorbance spectra. Preparation (*a*) in which high concentrations of the denaturing detergent sodium dodecylsulfate were used during isolation, reveals the most negative half-reduction potential (–340 mV). The value has to be taken cautiously because only redox equilibrium studies with xanthine : hypoxanthine as redox couple, and benzyl viologen as redox mediator were undertaken over a small potential range (85). The same holds for titration of preparation (*b*) in which redox equilibrium was achieved with the ferri : ferro oxalate couple (86). The first potentiometric redox titrations were performed on the *Neurospora* preparation (G. von Jagow, unpublished results). The heme centers showed a unique potential of –70mV. The two different redox potentials and the more complex redox behavior of the heme centers of preparation (*d*) are discussed at the end of the section.

The amino acid composition of the beef heart preparation (*c*) and (*d*) are comparable. Both preparations have a low content of lysine and arginine, and a high content of nonpolar amino acids, especially leucine and isoleucine. From these results a low polarity (88) of 0.32 was calculated, characteristic for an integral membrane protein. It is noteworthy that the amino acid composition and the polarity (0.37) (88) of the *Neurospora* preparation are similar to that of beef heart preparations (81).

The isolated cytochrome *b* has been found to be a dimer. This correlates with the occurrence of two cytochromes *b* per complex III. The first indication for the existence of a dimeric protein came from gel chromatographic, electrophoretic, and ultracentrifugal studies carried out with *Neurospora* cytochrome *b* (80, 89). It exhibited a molecular weight of 55,000 in a

Sephadex G-75 chromatography performed in a bile salt plus KCl medium, while, in contrast, a molecular weight of 30,000 was found using dodecylsulfate gel electrophoresis. In hydrodynamic studies carried out in the presence of deoxycholate, values of $s_{20,w} = 4.2$, and $D_{20,w} = 6.3$ were obtained, from which a molecular weight of 63,000 was calculated (90). Sedimentation equilibrium studies led to the calculation of a molecular weight of 51,000 after correction for the bound detergent (0.3 g deoxycholate per gram of protein). A dimeric-isolated cytochrome b has also been demonstrated for beef heart preparation (d) (79). In contrast to the *Neurospora* preparation, the protein was kept in the resolved state during ultracentrifugation by Triton X-100 instead of deoxycholate. In equilibrium studies the preparation equilibrated with a molecular weight of 150,000. After correction for the bound detergent a molecular weight of 62,000 was calculated for the protein moiety. From experiments with ^{3}H-Triton X-100 a specific binding of 1.5 g Triton X-100 per gram of protein was calculated. This value corresponds to the binding of one Triton micelle per mole of dimeric cytochrome b, assuming a molecular weight of 90,000 for the Triton micelle (91).

It soon became an intriguing question, whether the dimeric cytochrome b represents a homo- or a hetero-dimer, especially since the existence of two functionally different cytochromes b in complex III has generally been postulated on the basis of many spectral, kinetic, and potentiometric data, obtained from studies on cytochrome b integrated in the mitochondrial membrane, or in the isolated complex III (see below) (77, 92). In early experiments the *Neurospora* cytochrome b was eluted in two separate fractions after hydroxyapatite chromatography in the presence of dodecylsulfate (93). It could not, however, be convincingly shown that the two fractions represented two different cytochrome polypeptides. Partial amino acid sequences of the proteins from *Neurospora* and beef heart, including the C- and N-terminal ends, showed only one unique sequence (79, 93). Genetic experiments described below indicate the existence of only one structural gene for the cytochrome b apoprotein. Thus the experimental data available at present strongly suggest the existence of two structurally identical subunits in the cytochrome b dimer. The possibility can not be excluded, however, that minor differences between the two subunits may exist, which may originate from a post-translational modification.

A further question concerns the functional state of the isolated cytochrome b dimer, i.e. the extent of preservation of the native conformation. The spectral properties of cytochrome b change during isolation. The absorbance maximum of the α-band of the dithionite-reduced cytochrome b is shifted to shorter wavelength. The splitting of the α-bands of the *Neurospora* cytochrome b, which can be obtained at $-196°C$ with the complex-

bound species, is lost with the isolated cytochrome (90). Unlike cytochrome *b* in situ, all isolated preparations so far react with the unphysiological ligands carbon monoxide and oxygen. It could well be that structural alterations are caused by the detergents, and/or the chaotripic agents applied during isolation, but it could also be visualized that the native conformation of the *b*-cytochrome depends on the association of other subunits of complex III. It cannot be decided whether the preparations of isolated cytochrome *b* are still able to carry out a physiological electron transfer reaction, since an assay for this partial reaction is not yet available.

In the case of cytochrome *b* of beef heart [Table 1, (*d*)] some functional conformation seems to be preserved, as indicated by the existence of two different half-reduction potentials (79, 94). They amount to $E_{m7} = -5$ mV, and $E_{m7}' = -85$ mV. The values are shifted by only 40 mV to the negative as compared to cytochrome *b* in situ (95). Moreover, both potentials show a pH dependence. As soon as the pH is raised from 7.0 to 8.0, both centers change their redox potential by about 40 mV to the negative, i.e. the redox change involves a protonation reaction according to the following reaction:

$$cyt.b^{3+}R + e^- + H^+ \rightarrow cyt.b^{2+}RH$$
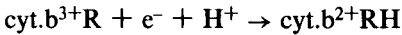
The abbreviation R in the equation represents an amino acid residue, which undergoes a protonation:deprotonation reaction. Combining this reaction with the Nernst equation, a complete protonation:deprotonation should result in a pH-dependence of 60 mV/unit pH (96). A value of only 40 mV may indicate a change of pkR due to some conformational changes of the isolated dimer. But the pH-dependence may justify the hypothesis that cytochrome *b* is a likely candidate for a proton-pumping device at the second phosphorylation site, as is discussed below (94).

CYTOCHROME *b* OF THE RESOLVED UBIQUINOL:CYTOCHROME *c* REDUCTASE   The cytochrome *b* is a building stone of complex III, and its function is intimately interconnected with the function of the other subunits. Thus the properties of cytochrome *b* have to be described in the context of the electron transfer and the energy-transducing reactions performed by the whole complex. Pure complex III preparations have been obtained from several organisms, which allows an analysis of the specific properties of this enzyme system without interference of the other complexes of the respiratory chain.

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. Of these, in Table 2, only methods (*a*) (97) and (*b*) (98) for beef heart, and (*e*) (99) and (*f*) (100) for yeast mitochondria are cited. Apparently, all these

preparations are obtained in a highly aggregated state. The heme b content of the complexes varies between 8 and 9 μmol per gram of protein. Consequently, the minimum molecular weights of the multienzyme complexes were calculated as being within the range of 200,000–250,000. Preparations (c) (101) and (d) (102, 103) were isolated by chromatography with Triton X-100, the beef heart complex by a hydroxyapatite chromatography, and the *Neurospora* complex by affinity chromatography on immobilized cytochrome c.

The aggregation states of the complexes (c) and (d) (Table 2) were investigated by ultracentrifugal studies (103, 104). From sedimentation velocity and equilibrium runs performed with the beef heart preparation, it was concluded that the isolated complex is present exclusively in a dimeric aggregation state. A molecular weight of 400,000 was calculated after correction for the bound detergent. This was in contrast to a calculated minimum molecular weight of 200,000 based on the heme b and c_1 contents (pyridine hemochromogens), and on summing up the molecular weights of the single subunits. One dimeric complex was held in solution by the specific binding of 0.2 g Triton X-100 per gram of protein. This amount is equivalent to the molecular weight of one Triton micelle (91). A dimeric complex and the same amount of specifically bound Triton were found for the *Neurospora* preparation (103). Two-dimensional crystals could be formed with this dimeric complex (105). The diffraction patterns and the optically filtered image of the electron micrographs were interpreted to show that alternate protein molecules are arranged in an up and down manner. The dimensions of one of these dimeric cytochrome c reductase molecules in projection are about 90 Å × 70 Å, which is consistent with the estimated Stokes radius and molecular weight of the dimeric enzyme protein, as well as the strict vectorial orientation of the complex in the membrane (106).

All preparations summarized in Table 2 contain, according to gel electrophoretic analysis, the same number of polypeptides, with the exception of preparation (c), which is devoid of the iron-sulfur protein due to application of antimycin during isolation (107). The mean values of the molecular weights of the subunits are in general agreement (72), and amount to about 48,000, 45,000, 30,000, 29,000, 25,000, 12,000, 10,000, and 5,000 (108). A specific adaption of the dodecylsulfate gel electrophoresis leads to a further resolution of the low-molecular-weight fractions. Three additional small polypeptides can be detected (99). The two largest polypeptides—named core protein 1 and 2—possess no redox centers (109). The 30,000–29,000-dalton polypeptide band encloses the apoprotein of cytochrome b and the cytochrome c_1 molecule, which are not regularly separated (80, 110). The 25.000-dalton subunit represents the apoprotein of the iron-sulfur protein, which is a ferredoxin-type enzyme containing, in the native state, 2 non-

Table 2 Isolation methods and properties of complex III

| Source | Isolation | | Heme b (μmol/g protein) | Mulecular weight based on: | | Number of poly-peptides | Cytochrome b absorbance maxima | E_{m7} (mV) | Enzymatic activity (units/mg) | Comments |
	Solubilization	Fractionation		Heme content	Ultra-centrifugation					
(a) Beef heart (97)	Cholate/ ammonium sulfate	Cholate, deoxy-cholate/ammo-nium sulfate	8	250,000	$S_{20,w} = 10.2$ in tauro-cholate	8	562, 565	+90 mV, −34 mV	1,000	Highest enzy-matic activity
(b) Beef heart (98)	Cholate/ ammonium sulfate	Deoxycholate, ammonium sulfate, NaOH	6.5	307,000	—	7	562 for both together	—	28	
(c) Beef heart (101)	Triton X-100, ammonium sulfate	Triton X-100, hydroxyapatite chromatography	9	200,000	400,000 from sedimentation equilibrium	6	559, 561	+80 mV, −10 mV	none	Devoid of nonheme iron protein; anti-mycin bound; isolated as dimer
(d) *Neurospora crassa* (102, 103)	Triton X-100, P_i	Triton X-100, affinity chromatography	—	—	550,000 from sedimentation equilibrium	8				Isolated as dimer
(e) Yeast (99)	Deoxycholate, KCl	Cholate/ammo-nium sulfate	8	250,000	—	8	561 for both together	—	20	
(f) Yeast (100)	Cholate/ ammonium sulfate	Deoxycholate/ ammonium sulfate, Triton X-100	9	200,000	—	7–8	562 for both together	—	108	

heme iron atoms, and 2 acid labile sulfide atoms (77, 111). Nothing is known about the function of the smaller polypeptides. The 10.000-dalton polypeptide subunit was suggested to carry an antimycin-binding site, but this assignment is still under discussion (77, 112). All preparations except (c) contain two heme b, one heme c (cytochrome c_1), and one Fe_2S_2 center per mole of monomeric complex. If a one to one stoichiometry of the single polypeptides is assumed, those polypeptides that carry no redox center amount to half of the molecular weight of the whole complex. Their existence was explained by a kind of "structural pillow function," but they could be involved also in reactions enabling an electrogenic proton transfer.

Some preliminary results concerning the architecture of the complex have been obtained with the *Neurospora* complex. The multiprotein unit could be cleaved into definite subcomplexes using increasing salt and detergent concentrations (102). The *Neurospora* complex is especially suited for such experiments, since the protein-protein interactions are weaker than in the beef heart complex. The first dissociated polypeptide, which was cleaved off already during certain preparation conditions, was the iron-sulfur protein (102). Subsequently, the two core proteins were dissociated under relatively mild conditions leading to a second subcomplex. A further dissociation yielded the dimeric cytochrome b and cytochrome c_1 associated with a smaller subunit. From these experiments it has been concluded, in contrast to the cross-linking and surface labelling studies described below, that the core proteins are arranged on the matrix surface of the mitochondrial inner membrane (113).

Surface labelling studies with isolated complex III, and complex III incorporated into phospholipid vesicles from beef heart, gave a uniform incorporation of radioactivity in all subunits except the smallest one (114). In contrast, cytochrome b from *Neurospora* integrated into the mitochondrial membrane was almost inaccessible to such a probe (93). Recent cross-linking and surface labelling studies gave additional information on the orientation of the polypeptides in the beef heart complex (106, 115). In these experiments all nine different component polypeptide subunits were labelled when the complex was present in isolated form; but in mitochondria the two core proteins, cytochrome b, cytochrome c_1, the nonheme iron protein, and only two of the smaller polypeptides (VI and VII) were tagged from the cytosolic side. In submitochondrial particles, cytochrome b and core protein 2 were exclusively accessible from the matrix side. The experiments suggest a model for the arrangement of the polypeptides (106) as depicted in Figure 4. Cytochrome b and core protein 2 span the mitochrondrial membrane. Cytochrome c_1 is placed on the cytosolic side less peripherally than the small subunit VI. The two small polypeptides VIII and IX seem to be buried in the membrane, since they were not labelled from either side of the membrane. They are not shown in the scheme of Figure 4.
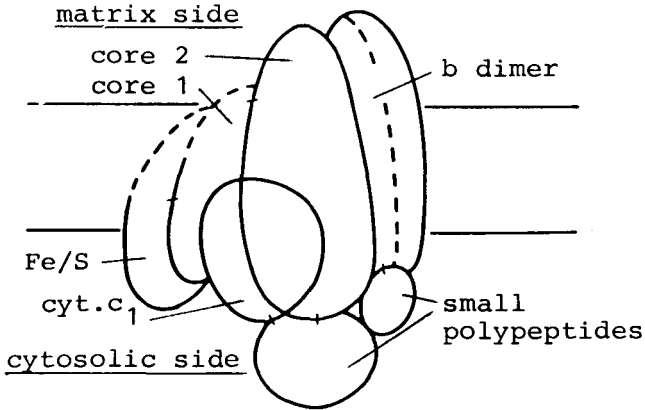
Figure 4   Model for the arrangement of the subunits of complex III based on surface labeling and cross-linking experiments [after (106)]. The cross-linking products are indicated by "ties" between the respective subunits.

The orientation of the chromophores of the complex could be determined in hydrated oriented multilayers of reconstituted proteoliposomes using optical and EPR spectroscopy (116). The hemes of cytochromes *b* and cytochrome c_1 are oriented parallel to each other, and perpendicular to the plane of the membrane. The iron-sulfur centre of the nonheme iron protein is oriented with the z-axis of the g-tensor parallel to the plane of the membrane, i.e. this center is also oriented parallel to the planes of the heme centers. Obviously the asymmetry of the complex led to an oriented incorporation of the complex in the lipid bilayer.

The two heme *b* centers of the complex possess distinct redox potentials as in situ and in the isolated cytochrome *b* dimer. Moreover, the two heme *b* centers of the resolved complex have preserved two different absorption spectra, as in situ. This contrasts to those of the resolved *b* dimer where this property has been lost. In the beef heart complex about one half of *b* can be reduced by ubiquinol or ascorbate plus TMPD, the further half only by dithionite. In preparation (*a*) the first part has an absorbance maximum at 562 nm, the second one at 565 nm (117). In preparation (*c*) the absorbance maxima are shifted 3 nm to the blue (101). This preparation differs from preparation (*a*) in having lost the iron-sulfur protein (as described above), and having a lower phospholipid content. The heme *b* centers of the complex do not react with carbon monoxide or oxygen as they did in the isolated dimer, probably because they are better protected in the core of the protein complex.

The task of the complex within the respiratory chain is to accept electrons from the ubiquinone-10 pool, and to transfer them eventually to cyto-

chrome c. Simultaneously the combustion energy has to be transferred into an energy form suitable for ATP synthesis. A present-day description of the function of cytochrome b in these processes can only be vague. In the literature, numerous hypothetical schemes are given for the pathway of the electrons (118). The following preliminary scheme (Figure 5) is offered for the arrangement of the electron carriers in complex III. The b cytochromes have been arranged in series, in parallel, and in other ways. The actual arrangement is yet unknown. The presumptive direct electron donor of the b cytochromes is ubiquinone-10 (119, 120). One hypothesis suggests that the b cytochromes transfer the electrons to a second ubiquinone-10 pool, as is discussed below (121). According to another version of the electron transport chain, cytochromes b transfer the electrons directly to the iron-sulfur center of the nonheme iron protein. The latter component seems to be inserted between cytochromes b and c_1. Recent experiments suggest that the intimate electron acceptor of cytochromes b may be the iron-sulfur protein. The so-called oxidation factor was identified as the iron-sulfur protein of complex III (122). It could be shown that this redox component is obligatory for electron flow from succinate to cytochrome c in a preparation containing complex III and II. Electron flow from succinate to cytochrome c only occurred when the iron-sulfur protein was present in the preparation. In the absence of the iron-sulfur protein only cytochrome b was reduced by succinate, but not cytochrome c_1 (123).

The electron acceptor of cytochrome c_1 is cytochrome c. It binds exclusively to the cytochrome c_1 subunit of the complex. In recent experiments it could be demonstrated, that the same surface area of cytochrome c was shielded during its binding, independent of whether it was bound to the whole complex or to isolated cytochrome c_1 (124). It did not bind to isolated cytochrome b.

In mitochondria, ubiquinone-10 is the electron donor for the complex. However, it cannot be used in the cuvette due to its high insolubility. Usually the ubiquinone analogues Q-2, or Q-3, are used, or analogues with an alkyl side chain instead of an isoprenyl side chain (125). As summarized in Table 2, the specific activities of Q-oxidation of the various complexes vary distinctly. This may be caused, among other things, by the partition coefficients of different Q analogues, as has been discussed in detail elsewhere (126).

$$\ldots \; Q \underset{\longleftarrow}{\overset{\longrightarrow}{}} \begin{array}{c} \text{cyt.}b \\ \\ \text{cyt.}b \end{array} \underset{\longleftarrow}{\overset{\longrightarrow}{}} (Q) \underset{\longleftarrow}{\overset{\longrightarrow}{}} \text{Fe/S} \underset{\longleftarrow}{\overset{\longrightarrow}{}} \text{cyt.}c_1 \underset{\longleftarrow}{\overset{\longrightarrow}{}} \text{cyt.}c \; \ldots$$
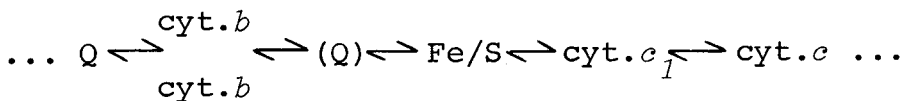
Figure 5 Preliminary scheme for the arrangement of the electron carriers in complex III.

The most potent inhibitor of the electron transferring capability of complex III is the antibiotic antimycin (127). It inhibits the electron transfer from cytochrome *b* to cytochrome c_1, and prevents the cleavage of the complex into its subunits by cholate plus salt (128). The cytochrome *c* reductase activity is blocked with a linear dependence on inhibitor concentration (129). When antimycin is bound to the complex its fluorescense is quenched (130). Studies of fluorescence quenching revealed one antimycin binding site per mole of complex (131). The same result was obtained from binding studies with [^{3}H]antimycin (132). The oxidized complex binds antimycin more firmly ($k_d = 1 \times 10^{-13}$ M) than the succinate reduced one ($k_d = 6 \times 10^{-11}$ M) (127). The decrease in binding affinity was interpreted as occurring due to a conformational change of cytochrome *b* induced by the reduction of the heme *b*-562 center by succinate.

A remarkable effect of antimycin on the redox centers of cytochromes *b* of the complex is the so-called antimycin-oxidant induced extra reduction (133). This steady-state phenomenon occurs during an antimycin-inhibited state when the electron transfer from cytochrome *b* to cytochrome c_1 is not completely blocked. Ubiquinol serving as electron donor reduces cytochrome *b* to 30%, and cytochrome c_1 completely. An increase of cytochrome *b* reduction to 90% takes place when cytochrome c_1 is oxidized, for instance by aid of ferricyanide. The whole cycle of reactions is reversible, i.e. when ferricyanide becomes reduced, cytochrome c_1 becomes reduced, and consequently the degree of reduction of cytochrome *b* declines to the original value of 30%. The effect was interpreted by three mechanisms that differ in principal: 1. a transient increase of the half-reduction potential of cytochrome *b* induced cooperatively by a second unknown redox component X, which is in redox equilibrium with cytochrome c_1 (133); 2. a transient increase of the half-reduction potential of cytochrome *b* due to its transition in an energized configuration, as long as electron flow occurs (134, 135); and 3. no increase of the half-reduction potential (E_{m7}) of cytochrome *b*, but a decrease of the overall redox potential (E^h) due to a change of the QH· radical concentration during electron flow (136). In the authors opinion the described phenomenon reflects conformational interaction between the redox components, but an exact interpretation of the reactions is not possible at the present state of knowledge. For instance, circular dichroism studies of the isolated complex from *Saccharomyces cerevisiae* revealed complex conformational interactions between cytochromes *b* and c_1 (137). In recent studies specific conformational alterations of cytochromes *b* of this complex by uncouplers have been detected that seem to promise new insights for the further understanding of uncoupler action (138).

In order to study electrogenic proton transfer, the complex was incorporated into phospholipid vesicles by the cholate dialysis method (139). An

electron flow from ubiquinol 2 to cytochrome c served as source of energy. Both the reductant and the oxidant were added to the vesicles from outside. In the presence of valinomycin plus potassium the redox reaction was coupled to an outward translocation and outside liberation of protons. A stoichiometry of one H^+/e was measured. The H^+ translocation could no longer be measured when the uncoupler FCCP or valinomycin plus nigericin were present. The electrogenic potassium ion uptake could also be measured. Its stoichiometry was one K^+/e, as expected. Discussing the mechanism of the transfer reactions, the H^+ transfer was attributed to a hypothetical component Z (139), which in our opinion may be ubiquinone, or the cytochrome b dimer.

The rate of electron flow from duroquinol to cytochrome c appears to be controlled by the membrane potential $\Delta\psi$ (140). This was concluded from experiments with a complex III reinserted into phospholipid vesicles, where a membrane potential was created by means of a valinomycin-induced potassium efflux (141). The membrane potential was estimated from the inside:outside distribution of safranine. The effect of a pH gradient ΔpH can not yet be determined due to the lack of a reliable indicator for the pH inside the vesicles, and due to the strong pH-dependence of the duroquinol:cytochrome c reductase itself.

CYTOCHOME b IN SITU   The literature on cytochrome b in situ is abundant and highly controversial. For detailed information the reader is referred to recent reviews (7, 71, 77, 90, 118, 126, 142). In the following only some classical experiments indicating the existence of two cytochromes b in complex III, and the two main proposed mechanisms of electrogenic proton transport governing at this region of the electron transfer chain are discussed. A mobile carrier–mediated hydrogen ion transport (121) is compared with a fixed protein–mediated mechanism (118), i.e. the proton-motive ubiquinone cycle is opposed to a protein-linked proton pump.

In the early fifties two types of cytochrome b were distinguished by spectrophotometry (143). In mitochondria and submitochondrial particles about one half of cytochrome b was reducible by succinate. This part showed an absorbance maximum at 562 nm, and was named cytochrome b_K in honor of D. Keilin (144). The second half of cytochrome b could be reduced by succinate in the presence of antimycin, or by dithionite. Its absorbance maximum occurs at 566 nm. It was called cytochrome b_T. It was supposed to be a high energy intermediate (74, 145). The simplest explanation for the antimycin-dependent reduction of cytochrome b-566, which was found in redox titrations with substrate couples (136, 146), is an increase of its half-reduction potential. In contrast to these studies, in potentiometric redox titrations an increase of the half-reduction potentials

of cytochromes *b* imposed by antimycin could not be detected (147). This disagreement could be due to reaction of the artificial redox mediators at different points from that of the physiological electron donors (148).

It is now generally accepted that the mitochondrial electron transfer complexes convert the free energy of oxidation into an electrochemical proton gradient $\Delta\mu_{H^+}$, which can be used for the synthesis of ATP and other energy-requiring reactions (8). In principle, a proton gradient is necessarily produced if, in the first place, the substrate is oxidized at one side and the product is reduced at the other side of the membrane; and if, second, the oxidation of the substrate involves the production of protons, and the reduction of the product involves the consumption of protons. Different or additional mechanisms for the formation of $\Delta\mu_{H^+}$ have to be postulated, however, if 1. the chemical redox reactions are not associated with the binding or dissociation of protons; 2. the redox reactions and/or the movement of protons occur at the same side of the membrane, or 3. too few protons are produced or consumed to account for the energy required for ATP synthesis.

The protonmotive ubiquinone cycle has been formulated to establish a $2H^+/e$ stoichiometry at site II (121), obtained when ubiquinone is arranged at two different positions in the electron transfer chain (121, 149). It was placed between cytochrome *b* and c_1, according to the hypothetical scheme shown in Figure 6, which represents a slight modification of the original one. The proposed mechanism will be explained going through one turn of the cycle. To start the cycle, it is proposed that the NADH-dehydrogenase transfers one electron on Q_{in}. This reaction involves the uptake of one proton from matrix side. The formed $QH_{in}^{\cdot}$ radical binds to cytochrome b_{in}, which may be identical with cytochrome *b*-566. Subsequently, this cytochrome accepts an e from b_{out} (maybe identical with cytochrome *b*-562), and re-
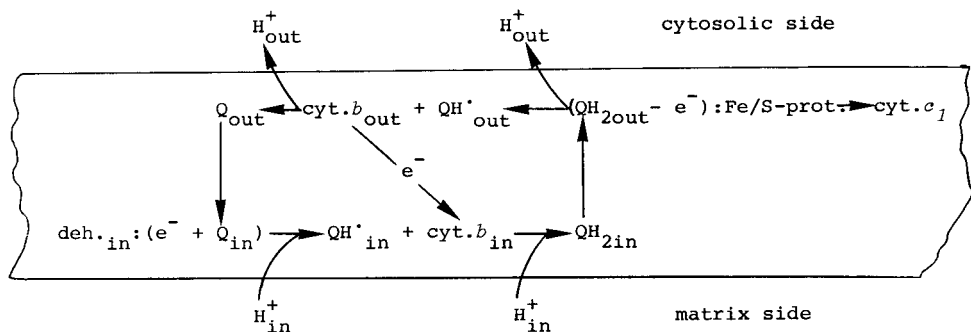

Figure 6   Hypothetical scheme of a protonmotive ubiquinone cycle.

duces therewith $QH_{in}^{\cdot}$ radical to QH_{2in}. This reaction involves a second H^+ uptake from matrix side. QH_{2in} is then released from the reaction center of b_{in} and transverses the lipid bilayer of the mitochondrial inner membrane by an unknown mechanism. It is there bound to the reaction center of the iron-sulfur protein, which accepts one e from QH_{2out}. This reaction involves a proton release to cytosolic side. $QH^{\cdot}_{out}$ radical is released from the iron-sulfur center and transferred to b_{out}. The heme center of b_{out} accepts an e that is fed subsequently into the heme center of b_{in}. The reaction leading to the formation of Q_{out} involves a second H^+ release to the cytosolic side. Retransfer of Q_{out} back across the membrane to the inner side completes the cycle. In contrast to (150), the half-life time of the $QH^{\cdot}$ radical seems not to be the most critical part of the proposed mechanism, since it can be visualized as a $QH^{\cdot}$ radical species bound to the respective reaction centers.

The ubiquinone cycle represents for the most part "paper chemistry." Essential aspects, such as the existence of $QH^{\cdot}$ radical, the occurrence of ubiquinone as reductant and oxidant of b cytochromes, and the sidedness of the redox centers, are at present not supported by experimental evidence. As an alternative mechanism to the double-arrangement of ubiquinone, a proton transport by a protein-linked pump mechanism has been visualized. Such a mechanism has been detected for bacteriorhodopsin (151, 152), and is under discussion for complex IV (153, 154). The following description is an extension of a published minimum model (94). It postulates that cytochrome b is a primary energy transformer at site II, i.e. that cytochrome b constitutes a proton pump, or is at least an essential part of it.

The proposed transfer mechanism consists of a sequence of six reactions (Figure 7). The model is based on the phenomenon that a redox change of the heme center of cytochrome b is linked with a pK change of an amino acid residue R of the protein moiety. The pK of R changes from a lower to a higher value when ferricytochrome b is converted to ferrocytochrome b (135, 155). The protonation reaction precedes the reduction reaction. In reaction 1 group R accepts a proton from the matrix face according to its pK value of 7 and the prevailing pH in the matrix space. In reaction 2 an e is accepted from the presumptive donor ubiquinol. The pK of R shifts from 7.0 to a value higher than 8.5 in the reduced state. As a consequence of the electron reception a conformational change of the molecule is induced involving a movement of RH from matrix to cytosolic position (reaction 3). The migration of group R may occur over only a short distance of some angstroms as has been discussed in models for a conformational coupling of $\Delta\mu_{H^+}$ to ATP synthesis (156), and of ΔE to creation of a membrane potential (157). In each final position group R may be linked to distinct proton channels as has been proposed for bacteriorhodopsin (151, 152). Group R can be considered as the gate of the proton pump.
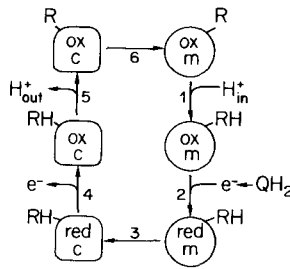
Figure 7 Hypothetical model of the reactions of an electron- and proton-transferring cytochrome *b*.

Reactions *4,5* and *6* are the symmetric back reactions of *1, 2* and *3*. The restriction of the cycle is no migration of group *R* in deprotonated state from *m* to *c* position, and vice versa (no migration of *R* in protonated state from *c* to *m* position). This constraint has to be introduced to exclude the possibility of a futile cycle. The pump yields an electrogenic proton translocation with a fixed $1H^+/e$ stoichiometry in contrast to a described variable stoichiometry (140). The equilibria of the protonation : deprotonation reactions *1* and *5* are influenced by the $\Delta\mu_{H^+}$. A high phosphorylation potential increases the proton gradient ΔH^+ with an increase of the H^+ concentration at the inner face of the mitochondrial membrane. Thus the equilibria of reactions *5* and *1* are shifted in reverse direction. Electrons will be driven in reverse direction and transferred onto ubiquinone and various redox centers of complex I. Eventually an accumulation of b^{2+} will occur, known as the energy-linked reduction of cytochrome *b*.

So far it can only be speculated whether both monomers of the cytochrome *b* dimer, or only cytochrome *b*-566 are involved in proton translocation. But, independent of this, and of whether the *b* cytochromes are arranged in series or in parallel in the electron transfer chain, the model furnishes the principal mechanism of an electrogenic proton translocation brought about by a polypeptide carrying a redox center.

BIOGENESIS AND GENETICS OF CYTOCHROME *b*   As a result of studies on the genetics and biogenesis of the mitochondria in eukaryotic microorganisms (for review see 158–163) it has been possible to establish that both the structural gene and the site of translation of apocytochrome *b* are located inside the organelle.

As shown first with *Neurospora crassa*, the highly purified cytochrome *b* of complex III is found to be labelled after incorporation of radioactive leucine in the presence of cycloheximide, a specific inhibitor of cytosolic ribosomes (80, 90). On the other hand, chloramphenicol, which inhibits

selectively the mitochondrial ribosomes, prevents the synthesis and labeling of the protein. This shows convincingly that cytochrome b of molecular weight 30,000 is translated inside the mitochondria. Analysis of complex III established that, apart from the 30,000 dalton cytochrome b, all other polypeptides, including cytochrome c_1, are provided by the cytoplasmic protein synthesis (164). Corresponding experiments with yeast *Saccharomyces cerevisiae* (165) showed that in complex III only a 32,000-dalton subunit is synthesized inside the mitochondria, whereas the other polypeptides are extramitochondrial translation products. Accordingly, the heme b-carrying protein belongs, together with three subunits of cytochrome oxidase and two to three subunits of ATPase complex, to the small number of hydrophobic proteins synthesized by the intramitochondrial protein-synthesizing system.

The isolation and genetic analysis of different types of yeast mutants (166) that had an altered function, or properties, of complex III allowed the identification of the structural gene of apocytochrome b on mitochondrial DNA (167, 168–171). Fine-structure analysis provided evidence for the existence of a single structural gene for apocytochrome b which is mosaic, i.e. the coding regions (exons) of the gene are interrupted by noncoding regions (introns) (Figure 8) (167, 170, 171).

The structural gene containing about 900 base pairs is organized in three coding regions which are interrupted by two non-coding regions that are estimated to contain 2000–4000 base pairs. The coding regions are identified by three clusters of drug-resistant mutations that confer increased resistance in vivo and in vitro to inhibitors of electron transport from the b-type

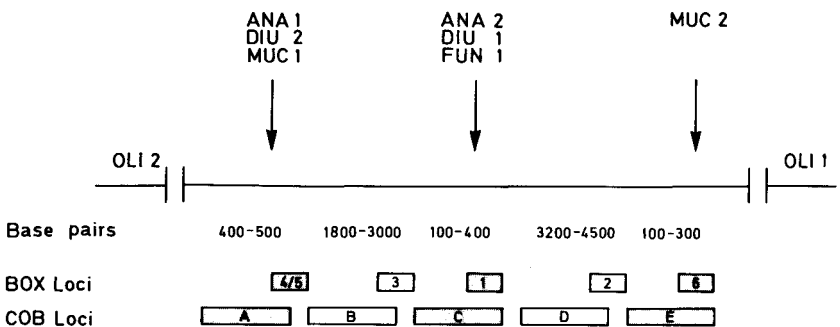*Figure 8*   The spliced structural gene of apocytochrome b (of complex III) located on mitochondrial DNA. ANA, DIU, MUC, FUN, OLI designate genetic loci which confer resistance to inhibitors of complex III and ATPase complex. BOX (COB) designates clusters of mutations leading to a nonfunctional complex III. The dotted clusters indicate the presumed coding regions. Further details are described in the text.

cytochromes to cytochrome c_1, as antimycin (172, 173), diuron (174), mucidin (175), and funiculosin (176). Remarkably, resistance to each anti-mycin, mucidin, and diuron is produced by mutations localized in two different clusters (177, 178). Additionally, so-called mit⁻ mutants (179, 180) with lesions in the ubiquinone:cytochrome *c* reductase have been isolated. These mutations are all localized in the segment of mitochondrial DNA between loci *OLI 1* and *OLI 2* where the drug-resistant mutations are also found (see Figure 8). Initially two (181), and later five (167, 171) clusters of such mutations (COB or BOX according to different laboratories) were identified. Mutations in clusters BOX 4/5 (COB A), BOX 1 (COB C), and BOX 6 (COB E) affect, specifically, complex III and they are closely linked to the drug-resistance loci (177). The mutations in clusters BOX 3 (COB B) and BOX 2 (COB D) cause pleiotropic defects in both complex III and the cytochrome oxidase. They appear to be localized in the noncoding regions, which exhibit a still unknown and possibly regulatory function (167, 169, 182).

Cytochrome *b* of the Resolved Succinate: Ubiquinone Reductase

Complex II is the multiprotein complex of the respiratory chain that trans-fers electrons from succinate to the mitochondrial ubiquinone-10 pool. It consists of four polypeptides of which at least three contain redox centers. Their molecular weights are about 70,000, 27,000, 15,000, and 13,000 (183). The two largest polypeptides constitute the so-called succinate dehydroge-nase, which can be cleaved off from the two smaller polypeptides by per-chlorate. The 70,000-dalton polypeptide has covalently linked one mole of FAD (184). It contains additionally two iron-sulfur centers of the Fe_2S_2 type, named center S-1 and S-2 according to EPR studies (185). The 27,000-dalton polypeptide contains the nonheme iron center S-3 which is an Fe_4 S_4 centre (186). After perchlorate cleavage, the two small polypeptides of complex II become insoluble, and form a precipitate. The cytochrome *b* is present exclusively in the precipitate. Therefore it has to be attributed to one or both of these polypeptide subunits.

Cytochrome *b* is present in the complex with a concentration equimolar to FAD. Its α-absorbance maximum lies at 560 nm in the dithionite-reduced state. It cannot be distinguished from the cytochrome *b* dimer of complex III by such a spectrum. But fourth derivative spectral studies performed earlier had already detected this entity (75). It differs from the resolved cytochrome *b* of complex III in that it does not react with CO. It has an unusual negative reduction potential of −200 mV when it is titrated potentiometrically in the resolved complex II (187). It is hardly reduced by succinate, as mentioned above, but it can be oxidized by fumarate or ubiqui-

none after a prereduction by dithionite. As described above, its functional characterization is still outstanding. Probably this cytochrome had already been isolated in 1977, but at that time it was not identified as cytochrome b of complex II (83). In these studies starting from a succinate: cytochrome c reductase preparation, a 17,000-dalton cytochrome b was obtained with a defective heme b content of only 10 μmol per gram of protein. The absorbance spectrum was altered, probably due to application of dodecyl-sulfate, but typical pyridine hemochromogen spectra were found.

The existence of a b-type cytochrome different to those of complex III was queried for a long time, especially because complex II isolated by ammonium sulfate fractionation is usually contaminated by 10–20% of complex III. Recent studies performed with *Neurospora crassa* have now definitely furnished evidence for the existence of a constituent b-type cytochrome in complex II (103). The fungus was grown in the presence of high concentrations of chloramphenicol. These are conditions under which the mitochondrial protein synthesis is largely inhibited. As expected, the cytochrome b of complex III was no longer synthesized. From these mitochondria the succinate: ubiquinone reductase was isolated by means of an affinity chromatography with immobilized cytochrome c. It was eluted from the column in the first fractions, whereas the incomplete complex III, devoid of the cytochrome b dimer, remained bound to the column material. Complex II had a cytochrome b content like that of beef heart, showing the same absorbance spectrum. The authors are yet uncertain whether to ascribe the heme center to the isolated 28,000-dalton or the 14,000-dalton polypeptide. When beef heart complex III is isolated by hydroxyapatite chromatography, complex II comes from the column in a similar way in the first fractions (187).

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