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REVIEW

Has Mitochondrial Cytochrome *b* Two (or More) Components or Is It a Dimer?

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Introduction

Cytochrome *b* of the mitochondrial respiratory chain is an electron-transferring, heme-containing protein which has protoheme IX as its prosthetic group. It is classified as belonging to the *b* type cytochromes by the Commission on Biochemical Nomenclature of the International Union of Pure and Applied Chemistry and International Union of Biochemistry on the basis of its containing protoheme which is not covalently bound to a protein.

For details of the older literature the reader is referred to a monograph by Wainio [1] which contains a chapter on cytochrome *b* of the mammalian mitochondrial respiratory chain. In that chapter it is suggested that the mitochondrion contains only one chemical species of cytochrome *b* which may be partitioned so that the molecules lie in two or more environments or compartments, with each compartment being more or less accessible to a number of reducing agents. In a recent review on cytochrome *b*,

Wikström [2] favors the view that two, or possibly three, main cytochrome *b*-like components can be identified, i.e., cytochrome *b*-562 with an $E_m = +40$ mV and cytochrome *b*-566 (and *b*-558) with an $E_m = -30$ mV, but that only two functionally different groups may be distinguished with each group containing both the *b*-562 and *b*-566 (and *b*-558) components.

The complexity of cytochrome *b*, i.e., its possible multiple nature and its anomalous reactivity, suggests that the components of cytochrome *b* may be close to one another in the membrane or even parts of one complex. Therefore, the same question may be asked about cytochrome *b* as has been asked about cytochrome *c* oxidase—is it constituted of two separate components or is its a dimer? With respect to cytochrome *c* oxidase the progression of thought has been from a firm belief in two separable components, namely cytochromes *a* and a_3, to an acceptance of the possibility that the two components are intimately associated as in cytochrome aa_3, or even that the two parts are similar, i.e., cytochrome *aa*, of which one part is modified by its environment.

There is only one published report to support the concept that cytochrome *b* in the respiratory chain might be a dimer. Weiss and Ziganke [3] isolated cytochrome *b* from *Neurospora crassa* and obtained a molecular weight of about 30,000 based on the heme content or by gel electrophoresis in dodecyl sulfate, whereas gel filtration on Sephadex G-75 in a bile salt and KCl medium yielded a molecular weight of 58,000. Weiss [4] has developed this concept of cytochrome *b* being a dimer at some length in a review article. He suggests that in a conglomerate, as in complex III prepared from the mitochondrial inner membrane, the two cytochrome *b* subunits might interact each with a different polypeptide leading to a different conformation and thus to the different properties of the two heme centers.

A study in our laboratory has also suggested that cytochrome *b* could be a dimer [5]. Succinylation of mammalian heart mitochondrial cytochrome *b* produced a preparation which was soluble and had a minimum molecular weight of 64,000 based on the heme content. However, thin-layer chromatography on Sephadex G-200 gave a molecular weight of 118,000 for the same preparation. It may be that the dimer of cytochrome *b* has previously escaped detection because of the marked tendency of the protein to polymerize and because depolymerizing agents such as cetyldimethylethylammonium bromide produced only a monomer. In one of several theoretical formulations which Slater and his associates [6, 7] have proposed for the mechanism of action of cytochrome *b*, it has been suggested there might be a dimer containing two species of cytochrome in equal amounts, i.e., bb_i, of which b_i might be a high-potential cytochrome *b* which participates in the synthesis of ATP.

Isolated Cytochrome b

The known characteristics of purified mammalian cytochrome *b* are those of a typical conjugated protein. The preparation of Goldberger et al. [8] was a monomer in cetyldimethyethylammonium bromide. It had a single boundary with an $S_{20,w}$ value of 2.6 in the analytical ultracentrifuge. The minimum molecular weight based on the iron or heme content was 28,000. However, in dodecyl sulfate, which was necessary for the purification process, the ultracentrifuge revealed a mixture of huge aggregates which sedimented at rates indicating molecular weights in excess of 4,000,000. Thus the aggregating properties of cytochrome *b* were revealed early and are probably due to the hydrophobic properties of the protein [9]. It should be understood that cytochrome *b* is firmly attached to the inner mitochondrial membrane and can only be released by treatment of the membrane with surface active agents, and can be separated from cytochrome c_1 only by heating in the presence of cholate and ammonium sulfate or by treatment of the complex with proteinase.

Ohnishi [9, 10] also observed aggregation when studying sedimentation in cholate or in polyoxyethylene sorbitan monolaurate, and concluded that the state of aggregation of the purified mammalian heart muscle cytochrome *b*, in contrast to housefly larval cytochrome *b*, varied greatly according to the dispersing conditions. He calculated a mean molecular weight of 21,300 for the enzyme from the heme content. The γ peak of the reduced form shifted from 428.5 to 424 nm when the preparation was treated with CO. This may have been an indication of denaturation.

Cytochrome *b* from *Neurospora crassa* [3] and *Locusta migratoria* [11] has a molecular weight of about 58,000 in deoxycholate or cholate and about 30,000 in dodecyl sulfate.

Purification of mammalian cytochrome *b* to a minimum molecular weight of 21,300 or 28,000 renders the enzyme inactive. On the other hand, a reconstitutively active preparation of cytochrome *b* having a minimum molecular weight of 78,000 (calculated from 12.8 mμ atoms heme iron/mg protein) has been reported by Yamashita and Racker [12]. A succinate-cytochrome *c* oxidoreductase of good activity was reconstituted from succinic dehydrogenase, the cytochrome *b* just mentioned, a cytochrome c_1 of molecular weight 73,000 (less than 50% pure), a commercial preparation of cytochrome *c*, and phospholipids containing ubiquinone-50. In order to achieve maximum activity, periods of incubation at 37°C of up to several hours were required.

Isolated cytochrome *b*, sometimes called classical cytochrome *b* or cytochrome b_K (after Keilin [13]) has maxima in its visible absorption spectrum at room temperature in the reduced state at 429, 532.5, and

562.5 nm [8] or at 428.5, 531.0, and 561.5 nm [10]. There is no evidence from either of these isolation studies that cytochrome *b* is other than a uniform enzyme. However, several attempts have been made to separate multiple components. Yu et al. [14] isolated two cytochrome *b* proteins from succinate-cytochrome *c* oxidoreductase and from the cytochrome b–c_1 complex of beef heart mitochondria. The one of molecular weight 37,000 had maxima in its absorption spectrum in the reduced state at 427, 530, and 560 nm, whereas the other of molecular weight 17,000 had poorly discernible peaks in the reduced state, although its reduced pyridine hemochrome was identical to that obtained from protoheme IX. It was suggested by the authors that the second protein was a denatured cytochrome *b*. The separation involved a 2-h incubation at 37°C in dodecyl sulfate. An earlier separation by Wambier et al. [15] also required heating at 40°C for 30 min at steps 3 and 5 in the procedure of Rieske and Tisdale [16]. This modification separated two fractions which were both homogeneous on polyacrylamide gel electrophoresis and which had maxima in the reduced state at 430, 528, and 560 nm (fraction II) and at 430, 535, and 568 nm (fraction I). However, their reduced pyridine hemochromes had slightly different peaks than would be expected for a protoheme IX pyridine derivative. It is likely that one or both of these fractions obtained by Wambier et al. were denatured cytochrome *b*.

Is it possible that isolated cytochrome *b* is not wholly native cytochrome *b*? Erecinska et al. [17] prepared a succinate-cytochrome *c* oxidoreductase from pigeon breast muscle mitochondria and found two *b* cytochromes whose properties were easily modified by detergents and lyotropic agents. Deoxycholate, which was used by Goldberger et al. [8] for the initial extraction of the *b*, and cholate, which was used by Ohnishi [10] for extraction and by both purification, caused the disappearance of the absorption on the long wavelength side of the cytochrome *b* spectrum, i.e., in the region of *b*-566. The spectrum of *b*-566 changed to a form indistinguishable from that of *b*-562. Nikodem and Waino [5] identified three spectral peaks in purified cytochrome b–c_1 particals, and found that incubation of the particles with cholate and ammonium sulfate at pH 6.5, which was the treatment used to cleave the complex, resulted in a single spectral species. These results suggest that at least one-half of isolated cytochrome *b* is not native. Furthermore, as mentioned earlier, isolated cytochrome *b* of molecular weight 21,300–28,000 is enzymatically inactive, i.e., it does not react with either ubiquinol, its possible electron donor, or with ferricytochrome c_1, its electron acceptor, and does react with CO. Thus cytochrome *b in situ* may consist of two components, of which one is in special positon or environment, i.e., in a special relationship to its electron donor or acceptor or the energy-transducing mechanism.

Treatment of the enzyme *in situ* with detergents could modify one component in a special environment and change its spectral properties to approximate those of classical cytochrome *b*.

Absorption Spectra of Cytochrome b in situ

The multiple nature of cytochrome *b* was suggested by Chance [18] in 1958 when he concluded from spectral data that three cytochrome *b* pigments existed in a preparation of mitochondrial fragments from bovine heart. The first was characterized as a succinate-reducible pigment with an α maximum in its absorption spectrum in the reduced state at 562 nm; the second as a pigment reducible by succinate, but only in the presence of antimycin A and exhibiting a maximum at 566 nm; and the third as a pigment reducible only by dithionite and having a maximum extending from 556 to 566 nm. Pumphrey [19] found that succinate reduced 70% of the dithionite-reducible cytochrome *b*, and that antimycin A increased the reduction to 95%. There was no evidence for a third, solely dithionite-reducible cytochrome *b*. The existence of a cytochrome *b* reducible by dithionite alone was questioned by Slater and Colpa-Boonstra [20], who felt that the increased absorption after dithionite probably represented the reduction of myoglobin contained in the preparation as a contaminant. Colpa-Boonstra and Minnaert [21] had shown that even carefully washed heart muscle preparations contained sufficient myoglobin to cause interference in the spectral measurement of cytochrome *b*. Slater and Colpa-Boonstra [20] concluded that their own data and those of Chance provided evidence for the existence of two, but not three, cytochrome *b*-like pigments.

In 1971 Wikström [22] found three maxima in mitochondrial and submitochondrial particles of rat liver. Classical cytochrome *b*, i.e., *b*-562, was identified by difference spectroscopy in an experiment in which the sample cell was made anaerobic with tetramethyl-*p*-phenylenediamine (TMPD) and ascorbate in the presence of rotenone and carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) and the aerobic reference cell contained rotenone, FCCP, ascorbate, and KCN to which TMPD was added after a 2-min incubation. It was believed that the cytochrome *b* was reduced in the sample cell due to endogenous substrates and was not reduced in the aerobic reference cell because of a leak through the cyanide-inhibited site. Classical cytochrome *b*, the only one identified in this experiment, since the α maximum was at 562 nm and was symmetrical, could have resulted from the presence of the uncoupler FCCP or from the presence of any or of all of the other agents. Cytochrome *b*-558 (a shoulder

on the difference spectrum) and *b*-566 (a peak) were identified by difference spectroscopy under anaerobic conditions in the presence of rotenone and FCCP by employing two different succinate: fumarate ratios to effect reduction. 3-Hydroxybutyrate was a better reductant, although 25 min was required to reach complete reduction. Beef heart mitochondria and Mg^{2+}-ATP submitochondrial particles prepared from beef heart mitochondria yielded similar results. The relative amounts of the three components in rat liver mitochondria were estimated to be *b*-558 : *b*-562: *b*-566 = 1 : 4 : 2. Since the 558- and 566-nm maxima appeared under the same conditions, the possibility was considered that the two maxima belonged to the same component. It was also pointed out by Wikström that it may not be correct to consider these maxima as belonging to three (or two) cytochromes *b*, but that there may be only one cytochrome *b* in three (or two) environments. It is strange that none of the 558- and 566-nm components was seen in the first experiment. If indeed endogenous substrates were responsible for the reduction, it should have been succinate that was the effective reductant, since the inhibitor rotenone was present to block the oxidation of NAD-linked substrates. The 25-min period required for complete reduction in the second experiment leaves room for the possibility that some unwanted alterations in cytochrome *b* took place.

Yu et al. [23] and Higuti et al. [24] claim that the 566- and 558-nm maxima belong to two different components, i.e., to make a total of three components together with the 562-nm component. Yu et al. studied succinate-cytochrome *c* oxidoreductase and found that the 566/558 nm ratio was not constant in several preparations. The evidence of Higuti et al. seems more conclusive, in that when they added succinate to rotenone- and KCN-inhibited and ATP-treated rat liver mitochondria under conditions in which the cytochromes *b*-562, *b*-566, $c-c_1$, and aa_3 were already reduced, the succinate caused the reduction of *b*-558.

It may be asked what happens to the cytochrome b_5 of the outer mitochondrial membrane when cytochrome *b* is prepared from intact mitochondria or from submitochondrial particles having an outer membrane. Does the cytochrome b_5 fractionate with the cytochrome *b* and become a contaminant? Is cytochrome b_5 all or any part of the *b*-558 which seems to be a second peak accompanying the cytochrome *b*-556 component? The amount of cytochrome b_5 in pig liver mitochondria has been estimated to be about one-half as much as the cytochrome *b* (564 nm) and more than the cytochrome c_1 (555 nm) [25].

Sato et al. [26] also concluded that there are three cytochromes *b* in mitochondria from yeast, but whereas two of the cytochromes *b* were found to have maxima at 561.5 nm, and 558 and 565 nm, the third

cytochrome *b* had a maximum at about 563 nm. To identify *b*-561.5, the curves were obtained as a difference spectrum between a reference cuvette treated with piericidin A, ascorbate, TMPD, dicoumarol, and KCN and a sample cuvette containing the same reagents plus glycerol-1-phosphate. The *b* with a double maximum (558 and 565 nm) was seen in a difference spectrum obtained with reference cuvette also containing piericidin A, ascorbate, TMPD, dicoumarol, and KCN and a sample cuvette containing the same reagents plus glycerol-1-phosphate and antimycin A. The 563-nm peak developed after 18 min of anaerobiosis of the cell in which the 561.5 maximum was identified.

Davis et al. [27], working with bovine heart mitochondria at 77 K found maxima at 553.5, 557, 559.5, and 562.5 nm. Three of these, with the 557 nm maximum at 77 K excepted, are presumably the 558-, 562-, and 566-nm maxima obtained by others at room temperature. The 559.5- and 562.5-nm components (562 and 566 nm at room temperature) were found to fractionate into complex III, i.e., ubiquinol-cytochorome *c* oxidoreductase, while the 557-nm component (560 nm at room temperature), not previously recognized by others, was found in complex II, i.e., succinate-ubiquinone oxidoreductase. It was concluded that there are three *b*-type cytochromes in bovine heart mitochondria, two of which have been recognized before, and that the third is *b*-557 at 77 K or *b*-560 at room temperature. The spectra presented by these investigators are the most convincing because only dithionite was used as a reductant in some instances and because the spectra of the fourth derivative helped to identify the peak unambiguously in submitochondrial particles and in cytochrome *c*-depleted mitochondria, as well as in the succinate-ubiquinone oxidoreductase complex.

The existence of a fourth *b* component has been suggested by Phelps and Crane [28] following the use of a new inhibitor, 2-ω-cyclohexylpentyl-3-hydroxyl-4-napthoquinone, and a new reductant, dithiothreitol, without electron mediators, on beef heart submitochondrial particles. However, the resulting peaks at room temperature were such that instead of being at 558, 561, 564, and 567 nm as proposed by the author's, they could be at 558, 560, 562, and 566 nm. These peaks would then correspond to those of Davis et al. and support their contention that there are three components rather than four.

Thus, if we accept the existence of maxima at 558, 560, 562, and 566 nm (room temperature) and assign 562 nm to one component and 558 nm to be the split peak of the 566-nm component, we are left with the 560-nm peak to be assigned to a third component. This component is reducible in complex II, in submitochondrial particles, and in cytochrome *c*-depleted mitochondria with dithionite, but not by the addition of substrates. The

presence of a solely dithionite-reducible component recalls the suggestion by Slater and Colpa-Boonstra that there may be contaminants in heart muscle preparations. Furthermore, before its existence as a separate component can be accepted, it must be shown that its enzymatic inactivity is the result of its having been detached from its normal electron donor and acceptor, and that it can be prepared as a nonclassical cytochrome *b* from succinate-ubiquinone oxidoreductase. However, the occurrence of a 560-nm component in complex II in a concentration equal to that of succinic dehydrogenase, makes it a component that cannot be ignored.

Effects of Antimycin A

Antimycin A is a mixture of at least four closely related antibiotics which were found originally to be inhibitory to the growth of pathogenic fungi. It was almost immediately established that antimycin A was effective as an inhibitor of the respiratory chain at extremely low concentrations. For example, Ahmad et al. [29] in 1950, demonstrated that the oxidation of succinate by rat liver homogenates was 90% inhibited by 40 ng of antimycin A/3 ml in a Warburg respirometer flask. Chance [30] estimated that the amount of antimycin A which resulted in a complete steady state reduction of cytochrome *b* and in a complete oxidation of cytochromes *c* and c_1 when added to a preparation of heart muscle submitochondrial particles was in a 1:1 ratio to the cytochrome *b*. The mechanism of inhibition by antimycin A has not yet been established.

The proximity of the antimycin-inhibitory site to cytochrome *b* has dictated that the relationship of the two be thoroughly investigated. Pumphrey [19] reported that when antimycin was added to NADH-reduced submitochondrial particles in an amount just sufficient to cause a significant increase in the level of cytochrome *b* reduction (which larger amounts will produce), a red shift of 0.7 nm was observed in the maximum of the α peak of the absorption spectrum of cytochrome *b*. The addition of a larger amount of antimycin increased the height of the α peak and shifted the maximum from 563.7 to 564.5 nm. In the presence of a high concentration of cholate, the α maximum of cytochrome *b* underwent a blue shift (from 563 to about 561 nm) and lost its property of being reduced by menadiol. Antimycin A prevented these changes. Many have subsequently investigated these actions of antimycin with the result that it is now believed that the effect may be on the 562-nm component of cytochrome *b*. Berden and Opperdoes [31] titrated the decrease in absorption at 562 nm (relative to 575 nm as a reference) that was caused by the addition of antimycin to beef heart mitochondria maintained at a potential of 100 mV with NADH and mediators. The stoichiometry was one molecule of

antimycin per molecule of cytochrome c_1. The same stoichiometry was obtained for the increase in absorption at 565 nm (relative to 558 nm as a reference). Dutton et al. [32] found that the red shift induced by antimycin when added to pigeon heart mitochondria was less than 1 nm in the maximum of the reduced 562-nm component. The stoichiometry was approximately one molecule of antimycin per molecule of cytochrome *b*-562.

The phenomenon of the increased reduction of cytochrome *b* by succinate in the presence of antimycin was first reported by Chance [18] in 1958. He concluded that antimycin revealed a nonclassical cytochrome *b* which was reduced by succinate only in the presence of antimycin and had an α maximum in the reduced state at 566 nm. Results with soluble preparations containing all cytochrome members of the respiratory chain [33, 34] supported the view that cytochrome *b* reacts with reductants and oxidants according to its accessibility to the kind and concentration of the reagents used in the experiments. NADH, or succinate with antimycin, reduced essentially 100% of the cytochrome *b* when measured at 562 nm. Reduction by dithionite was taken to equal 100%. From 65 to 70% of the cytochrome *b* was reduced by such reagents as succinate alone, NADH preceded by *O*-phenanthroline or 8-hydroxyquinoline, and by dithionite preceded by α,α-bipyridyl or *O*-phenathroline. Thirty-six per cent of the preceded by α,α-bipyridyl or *O*-phenanthroline. Thirty-six per cent of the cytochrome *b* was reduced by succinate preceded by 2,3-the presence of α,α-bipyridyl or thenoyltrifluoroacetone. The interpretation that was placed on these experiments was that the mitochondrion might contain only one chemical species of cytochrome *b* in several locations with each molecule more or less accessible to the reducing (or oxidizing) agents. There appeared to be no evidence here that there were several species of cytochrome *b*. Lee and Slater [35] concluded from their studies with beef heart mitochondria that the effect of antimycin in the presence of oxygen involved all three cytochromes *b*, i.e., *b*-562, *b*-566, and *b*-558. Wikström and Berden [36] found that the increased reduction of cytochrome *b* induced by antimycin was always dependent on the initial presence of an oxidant such as oxygen and that the increased reduction involved all three spectroscopically resolvable components (*b*-562, *b*-566, and *b*-558). The increased reducibility was considered to be the result of two effects: inhibition of oxidation of ferrocytochrome *b* by ferricytochrome c_1, i.e., the effect of antimycin, and oxidation of the semiquinone form of a two-equivalent redox couple such as ubiquinone/ubiquinol by the added oxidant, leading to a decreased redox potential of the QH_2/QH couple and reduction of cytochrome *b*. There was no evidence that the use of antimycin could resolve additional components.

Oxidation–Reduction Potentials

The oxidation–reduction potential of cytochrome *b* has been reported to be −340 mV for a purified highly aggregated preparation having a molecular weight in excess of 4,000,000 [37], −21 mV for another purified preparation having an $S_{20,w} = 33.3$ [9], +66 mV for a partially purified preparation [38], and +77 mV for cytochrome *b* in nonphosphorylating submitochondrial particles prepared from mammalian heart mitochondria [39].

Dutton et al. [40] studied the course of reduction and oxidation of cytochrome *b* at 561 nm (relative to 575 nm as a reference wavelength) in bovine heart submitochondrial particles as a function of the oxidation–reduction potential using NADH (and $Na_2S_2O_4$ below −100 mV) as a reductant and ferricyanide as an oxidant. The complex curve suggested the presence of three components. The three were estimated to have $E_{m,7}$ values of −110, +40, and +135 mV. Sato et al. [26] claimed that these three components in *Candida utilis* had $E_{m,7}$ values of −50 mV (for *b*-566—or 565), +5 mV (for *b*-563), and +65 mV (for *b*-562—or 561). The 560-nm component identified by Davis et al. [27] in complex II from bovine heart mitochondria was reported by them to have a low midpoint potential. It was reduced only by dithionite, but was oxidized by fumarate or ubiquinone.

Since the titration curves of Dutton et al. and Sato et al. are complex and require considerable manipulation in order for them to yield straight lines for the separate components, the assignments of the values are approximations at best. This may account for some of the differences in the values obtained in the two experiments: −110 versus −50 mV, +40 versus +5 mV, and +135 versus +65 mV, although it must be remembered that the preparations are from different organisms. Furthermore, the three values of Sato et al. and the value of Davis et al. for the 560-nm component depend on the correctness of the assumption that several spectrally different forms of cytochrome *b* do exist. Dutton et al. make a tentative assignment of only their +40-mV component being cytochrome *b*, i.e., the classical 562-nm component. These authors admit that accurate midpoint potentials cannot be assigned to the other two components because these lie at the extremities of the titration where the ratio, oxidized to reduced form, becomes very sensitive to small errors in absorbance measurements and to contributions from other non-cytochrome *b*-absorbing species. von Jagow [41] titrated cytochrome *b* in intact mitochondria of *Neurospora crassa* using the same technique as did Dutton et al. and revealed two components with midpoint potentials of −40 and +60 mV. Titration of purified cytochrome *b* revealed only one component with an $E_{m,7}$ of −56 mV. The criticism given previously also applies here.

Effect of Energization

In 1970 Wilson et al. [42, 43] analyzed the oxidation–reduction potential titration of cytochrome *b* with NADH in rat liver mitochondria with *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, phenazine methosulfate, and phenazine ethosulfate as redox mediators and concluded that there were two chemically different species of cytochrome *b* in the respiratory chain. The midpoint potential of one was presumably energy dependent, i.e., the two-component system in the presence of excess ATP with E_m values of +35 and +245 mV was changed to a one-component system having an E_m of 0 mV when the uncoupler, 5-chloro-3-*p*-phenyl-2,4',5'-trichlorosalicylanilide, was added.

It was pointed out almost immediately by DeVault [44] and Caswell [45] that there were shortcomings in the data of Wilson et al., notably that the redox mediators might not have been reacting directly with the cytochrome being studied. Wikström and Lambowitz [46] finally demonstrated that very high concentrations of the redox mediators, phenazine methosulfate or phenazine ethosulfate, were required to react efficiently on both sides of site 2 of oxidative phosphorylation in the respiratory chain, i.e., in the vicinity of cytochrome *b*, and that the titrations previously carried out in the presence of ATP had been at concentrations of mediator far too low to overcome the effects of reversed electron transport. The ATP-induced shift in E_m of the one component was believed to be due to a lack of equilibrium being established with the redox mediators. Therefore the shift could not be considered evidence for an energy-transducing function for part of cytochrome *b*.

The concept of a cytochrome b_T, an energy-transducing cytochrome *b*, will therefore have to be discarded. However, someone will have to explain the results of Slater et al. [6, 7] who showed that ATP caused a small shift in the absorption maximum of ferrocytochrome *b* and an oxidation of cytochrome *b*. Energization of respiring mitochondria or of mitochondria suspended in a K^+-free medium by adding valinomycin was also associated with a red shift in the cytochrome *b* maximum. Are these spectral changes simply due to the reduction of one cytochrome *b* component and the oxidation of another?

Kinetics of Cytochrome b *Oxidation*

When coupled anaerobic pigeon heart mitochondria were pulsed with oxygen by Chance et al. [47], the cytochrome *b* as measured at 560 nm (with 575 nm as a reference) showed a marked biphasic response. About 50% of the cytochrome *b* was readily oxidized with a $t_{1/2}$ of 100 msec, whereas the remainder was only slowly oxidized ($t_{1/2} \sim 1$ sec). ATP, ADP,

and P_i had negligible effects on the fast phase, but ADP slowed down the slow phase by a factor of 4, while ADP + P_i and an uncoupler enhanced it. Chance et al. felt that a portion of the cytochrome *b* was under the control of the energy-transducing mechanism and identified that part as b_T. Subsequently, Papa et al. [48] from the same laboratory studied submitochondrial particles from beef heart and placed the fast reacting *b* on the oxygen side of flavoprotein, which they also monitored. The remainder of the *b* was so slow reacting compared with the flavoprotein that it could not be placed in the main respiratory chain. It was recommended that it should be placed on a side path.

More recently Grimme [49] reported that in phase I, the fast phase, *b*-562 was rapidly oxidized, *b*-566 was reduced, and cyctochrome c_1 did not accept electrons. In phase II, up to 350 msec, the oxidation of *b*-562 continued and cytochrome c_1 started to accept electrons. The response of cytochrome *b*-566 was not mentioned. A phase III was identified as that slow phase during which a quasi equilibrium was established among the three redox components, i.e., the two cytochromes *b* and cytochrome c_1.

Other Evidence for Multiple Components

Electron paramagnetic resonance spectroscopy has been used to distinguish two cytochrome *b* components in ubiquinol-cytochrome *c* oxidoreductase (complex III) of beef heart mitochondria. Dervartanian et al. [50] identified a component with a signal at $g=3.44$ (ferricytochrome *b*-562) and another component with a signal at $g=3.8$ (ferricytochrome *b*-566). Orme-Johnson et al. [51] also assigned the $g_z=3.44$ signal to cytochrome-562, and the $g_z=3.78$ signal to cytochrome-566. Dervartanian and co-workers indicated that there were only these two major species of *b* present in complex III. The other species were present in much smaller amounts and were possibly modified forms of cytochrome *b*. The signal of *b*-562 was two to three times as intense as the signal of *b*-566 which was in reasonable agreement with spectrophotometric measurements made by others [22]. No mention was made of a signal arising from *b*-558.

Storey and Lee [52] recorded the circular dichroism spectra of oxidized and reduced submitochondrial particles from beef heart mitochondria and were unable to detect any contribution from cytochrome *b*-562 (or 561). However, under conditions in which *b*-566 was reduced, an ellipticity change was observed: a positive band at 426 nm and a negative one at about 436 nm. There were no detectable differences in this CD spectrum when reduction took place in the presence of antimycin A or 2-heptyl-4-hydroxyquinoline-*N*-oxide or when the component was reduced in the energized membrane by reversed electron transport or by an apparent shift

in the midpoint potential. Their suggestion was that the most likely explanation for the conformational change in *b*-566 was reversed electron transport, rather than a shift in the midpoint potential.

Conclusions

There is no evidence from the properties of isolated cytochrome *b* that it consists of two components. Isolated mammalian cytochrome *b* has a minimum molecular weight per heme of 21,300 or 28,000, but exists in solution in the form of huge aggregates. It has an α maximum in its spectrum in the reduced state at about 562 nm. Since it is enzymatically inactive and since its γ maximum in the reduced form is shifted from 428.5 to 424 nm when treated with CO, it must be considered that the isolated enzyme may not be native.

Absorption spectroscopy of cytochrome *b* in intact mitochondria and submitochondrial particles reveals major α maxima at 562 and 566 nm, and a lesser absorption peak at 558 nm which is probably associated with the 566-nm maximum. The relative amount of these absorbances in rat liver mitochondria has been estimated to be 558 : 562 : 566 = 1 : 4 : 2. A minor component absorbing at 563 nm has been reported in yeast mitochondria and a component absorbing at 560 nm has been found in succinate-ubiquinone oxidoreductase (complex II) prepared from beef heart mitochondria. The latter may be a *b* component which has been dissociated from its position in the respiratory chain by cleavage during isolation.

The effect of antimycin A, i.e., the red shift in the α maximum of cytochrome *b*, suggests that there may be more than one component: one cytochrome *b* component is reduced while another is oxidized. However, the increase in the absorption when antimycin A is added to succinate-reduced mitochondria or to submitochondrial particles or soluble preparations may not necessarily indicate that there are two or more separate components.

Oxidation–reduction potentials offer weak evidence for the presence of more than one component. The values are at best estimates, and because the titration curves are complex, the calculated values may be influenced by the redox state of noncytochrome *b* components.

The least reliable evidence for two components comes from kinetic experiments which measure the response of anaerobic mitochondria or submitochondrial particles to a pulse of oxygen. Measurements at one wavelength offer questionable evidence for the existence of two components, unless the measurements are made kinetically.

The concept of an energy-transducing cytochrome *b*, i.e., cytochrome

b_T should be discarded. The apparent ATP-induced shift in E_m on which the existence of b_T was based has been shown by Wikström to be due to reversed electron transport.

Electron paramagnetic resonance spectroscopy and circular dichroism spectroscopy distinguish two components: a *b*-562 and a *b*-566.

Therefore, the evidence appears inescapable that cytochrome *b* is constituted of more than one component. Whether these are two (or more) separate components having different physical and chemical properties, or two (or more) identical components in two (or more) different attachments or environments cannot be distinguished at this time. It is possible that the spectral, oxidation–reduction, and kinetic anomalies might better be explained if the assumption were made that cytochrome *b* is a dimer of closely interacting monomers.

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