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Reducibility of Cytochrome *b* in Mitochondrial Inner Membrane

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Abstract

When mitochondrial inner membrane was disintegrated into Complex I–III, IV, and oligomycin-sensitive ATPase, about 50% of cytochrome *b* in Complex I–III was readily reduced with NADH, as judged by the appearance of a peak at 562 nm, while in whole mitochondria less than 25% of cytochrome *b* was reduced by succinate. On addition of antimycin to the substrate-reduced Complex I– III, cytochrome *b* was further reduced to 71% of the total, and the peak at 562 nm was red-shifted to 564 nm as in the case of dithionite reduction. These results indicate that the 562 nm and 564 nm peaks at 29°C correspond, respectively, to b_{560} and $b_{562.5}$ at 77°K of Davis et al. [7] and to "b_K" and "b_T" of Chance et al. [2]. When Complex I–III and oligomycin-sensitive ATPase were reconstituted to form a membrane, about 60% of cytochrome *b* in Complex I–III was readily reduced with NADH. In this case the 562 nm peak was not red-shifted. However, the difference spectrum of NADH-reduced membrane *minus* that in the presence of deoxycholate showed a peak at 565 nm. A mirror image of the difference spectrum was obtained on addition of an uncoupler, *m*-chlorocarbonyl cyanide phenylhydrazone. This is characteristic for "b_T". These results support the idea that the occurrence of spectral peaks of "b_T" and "b_K" is not due to two species but to single species

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Abbreviations: OS-ATPase = oligomycin sensitive ATPase; CCCP = *m*-chlorocarbonyl cyanide phenylhydrazone; F_1 = coupling factor one; OSCP = oligomycin-sensitivity-conferring protein.

of cytochrome component, and that the changes in reducibility and the spectral anomaly are merely due to modulation in molecular assembly of the membrane components. The spectral feature of reduced cytochrome *b* in the reconstituted membrane in the presence of 0.5% deoxycholate is similar to that of the original mitochondrial membrane, showing that the mitochondrial membrane is possibly fluid rather than rigid in configuration.

Introduction

As an approach to the mechanism of oxidative phosphorylation, nonphosphorylated high-energy intermediates or states in the biological energy transduction sequence should be elucidated. Cytochrome b_T has been considered to participate in energy transduction in mitochondria at the second coupling site [1, 2]. This idea was supported by the finding of Wilson and Dutton [3] that the apparent midpoint potential (E_m) of cytochrome b_T in rat-liver mitochondria increases upon addition of ATP. It has been demonstrated that the component designated as b_T also exists in submitochondrial particles [4] and in Complex III [5–7] of mitochondria. Azzi and Santoto [8] reported that the addition of ATP to coupled rat-liver mitochondria or mitochondrial fragment, or a pH increase from 7.5 to 8.7 in uncoupled membrane induces the appearance of a spectrum of b_T type, and they postulated that the pH increase may represent one of the earliest events in energy conservation. On the other hand, the Wilson–Dutton interpretation of the E_m-shift has been strongly criticized by several investigators [9–11]. Caswell [9] and Devault [10] pointed out that an artifact of the methodology to measure E_m's using a redox mediator results in an erroneously high E_m for b_T. Caswell [9] reported that the presence of ATP or of uncoupler has no material effect on the E_m of cytochrome *b*. In support of this interpretation, it has been found that antimycin abolishes the large E_m-shift caused by ATP [11, 12]. The antimycin effect is accommodated to the Wilson–Dutton hypothesis only by making the additional postulation that antimycin blocks energy transfer specifically between ATP and the coupling site II [12]. Another disturbing point regarding the Wilson–Dutton hypothesis is that in plant mitochondria none of cytochrome *b* shows an ATP-induced E_m-shift [13]. Although Dutton and Storey [13] attribute this to a fundamental difference between plant and animal mitochondria in the mechanism of energy transduction at the site II, Lambowitz et al. [14] concluded that the E_m-shift in animal mitochondria is merely due to reversed electron transport. In addition, ATP-induced reduction of both b_K and b_T has been shown [15], and the uniqueness of the function of b_T has been questioned [16].

It is with this background that we have taken a look into

cytochrome *b* in Complex I–III of beef-heart mitochondria in the absence and presence of OS-ATPase and/or CCCP. The data reported here suggest that the apparent modulation of the reducibility (redox equilibration with substrate) of cytochrome *b* producing spectral anomaly is due to changes in molecular assembly of the membrane components.

Materials and Methods

Intact beef-heart mitochondria were prepared by using Nagarse as reported previously [17]. Complex I–III was prepared by the method of Hatefi et al. [18]. OS-ATPase was prepared by the method of Tzagoloff et al. [19]. Reconstitution of membrane from Complex I–III and OS-ATPase was performed as described by Kopaczyk et al. [20]: the preparation of Complex I–III was diluted to a protein concentration of 10 mg/ml with 10 mM Tris-HCl, pH 8.0. The preparation was mixed with OS-ATPase at 20° C in the ratio of 1:1.5 on a protein basis. The mixture was diluted to a protein concentration of 0.15 mg/ml with a solution of 0.25 M sucrose, 0.1 M Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, and 3 mM $MgCl_2$. The diluted mixture was centrifuged at 50,000 rpm for 60 min. The sediment was suspended in a solution of 20 mM potassium phosphate, pH 7.4, and 2 mM sodium azide. Deoxycholic acid used in the experiments was recrystallized three times from 50% ethanol. Spectrophotometry was carried out using a Shimadzu Multipurpose Spectrophotometer, type MSP-501. The specimens for electron microscopy were prepared by negative staining with phosphotungstic acid [21]. All specimens were examined in a Hitachi type HU-11B electron microscope operated at 75 kv.

Results

The difference spectrum of intact mitochondria reduced by succinate and that of Complex I–III reduced by NADH are shown in Fig. 1, curves I and II, respectively. In curve II, the peak at 562 nm is that of cytochrome *b*, and 554 nm is that of cytochrome c_1, as reported by Hatefi et al. [22]. A shoulder at 532 nm and a peak at 525 nm are characteristic of β-bands of *b* and c_1, respectively. Addition of antimycin (final concentration 0.1 mM) to the substrate-reduced Complex I–III (curve II) caused characteristic spectral changes as shown in curve III: further reduction of cytochrome *b* with a red-shift of 2 nm in the α-band of cytochrome *b* and the shift of the negative peak from 540 nm to 545 nm by the enhanced reduction of flavin. Further addition of dithionite resulted in the complete reduction of the redox components,

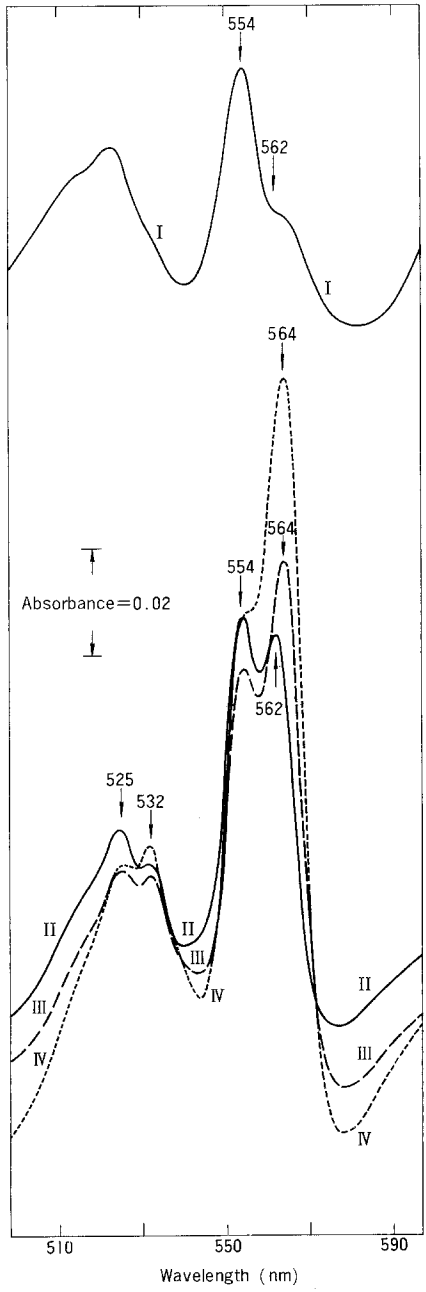


Figure 1. The reduced *minus* oxidized spectra of intact mitochondria and Complex I–III. Curve I: The heavy beef-heart mitochondria (3.8 mg protein) were suspended in a medium (1 ml) containing 0.25 M sucrose and 10 mM Tris-HCl, pH 7.4. The reduction was carried out at 29° C by adding 5 μmole succinate. Curve II: The solution (1 ml) consisted of 3.2 mg protein of Complex I–III, 20 mM potassium phosphate, pH 7.4, 2 mM sodium azide, 1 mg bovine serum albumin, and 0.15 mg mitochondrial phospholipid mixture. NADH (250 nmole) was added to the sample cuvette at 29° C. Curve III: Antimycin, 100 nmole, was added to the NADH-reduced enzyme. Curve IV: A crystal of dithionite was further added to the sample cuvette.

as shown in curve IV. Estabrook [23] observed a shift of about 3 nm in the α-band of cytochrome *b* when the substrate-reduced enzyme was further reduced with dithionite.

Judging from the 554 *minus* 540 nm absorption in curve II, i.e. extent of cytochrome c_1 reduction, it was calculated that 86% of the total (the 554 *minus* 545 nm absorption in curve IV) was reduced by the substrate, whereas extent of cytochrome *b* reduction (the 562 *minus* 577 nm absorption in curve II) by the substrate is 51% of the total (the 564 *minus* 577 nm absorption in curve IV). Since it was estimated [5] that Complex III contains approximately 4 nmole cytochrome c_1 and 8 nmole cytochrome *b* per mg protein, it could be concluded that approximately equimolar amounts of cytochrome c_1 and *b* are reduced by the substrate in curve II, and another half of cytochrome *b* remained in oxidized form. Addition of antimycin to the substrate-reduced enzyme caused a slight oxidation of cytochrome c_1 (86–80% reduction of the total) and enhanced reduction of cytochrome *b* (51–71% reduction of the total), as shown in curve III in Fig. 1.

Figure 2 shows the relative reduction of cytochrome *b* to c_1 in the membrane reconstituted from Complex I–III and OS-ATPase. Curve I represents the difference spectrum recorded by employing NADH as reductant, as in the case of Fig. 1, curve II. Cytochrome *b* (the 562 *minus* 577 nm absorption) was more reducible while cytochrome c_1 (the 554 *minus* 540 nm absorption) less reducible than Complex I–III before reconstitution with OS-ATPase (curve II in Fig. 1). The relative increase in the magnitude of the peak at 532 nm in the reconstituted membrane indicates the enhanced reduction of cytochrome *b*. Since the dithionite-reduced spectrum of the reconstituted membrane was essentially the same as in curve IV in Fig. 1, the extents of reduction of cytochrome *b* and c_1 were calculated to be 59% and 71% of the total, respectively. The overall trend is similar to the effect of antimycin on the substrate-reduced enzyme (curve III in Fig. 1), except that the red-shift of the 562 nm peak in the case of antimycin was not observed in the NADH-reduced reconstituted membrane. From these results, it is reasonably assumed that the reconstitution of Complex I–III and OS-ATPase to form a membrane causes the conformational changes occurring in the redox components, and we tried to modulate the conformation of the reconstituted membrane by deoxycholate. Spectra in Fig. 2, curves II–IV, demonstrate the change in absorbance of cytochrome bands when titrated with various concentrations of deoxycholate. The increase in the c_1 peak was observed with increasing deoxycholate concentration. At 0.5% deoxycholate, it is cytochrome c_1 that attained almost maximal reduction (96% of the total) while cytochrome *b* remained in comparatively oxidized state, as depicted by the shoulder at 562 nm; the extent of the reduction of *b* is 45% of the total. The relative reduction of cytochrome *b* to c_1 in the reconstituted

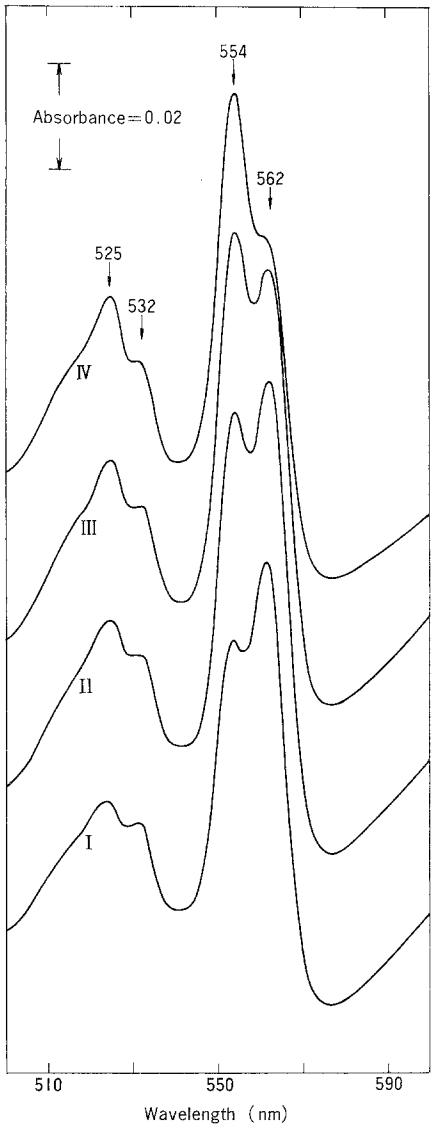


Figure 2. The reduced *minus* oxidized spectra of Complex I–III recombined with OS-ATPase with graded amounts of deoxycholate. The conditions were the same as in Fig. 1, Curve II, except that Complex I–III was recombined with OS-ATPase [20]. Deoxycholate was added to the solution in the following order: Curve I, none; curve II, 1.25 mg; curve III, 2.5 mg; curve IV, 5 mg.

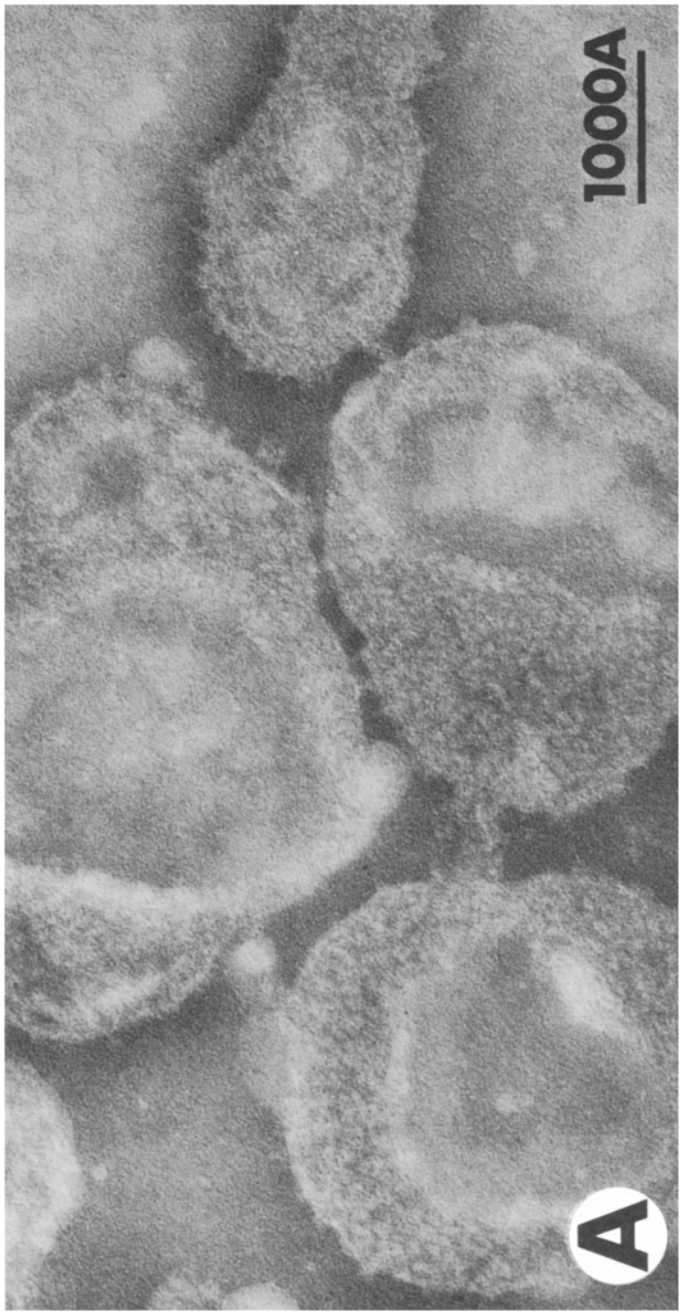
membrane in 0.5% deoxycholate is similar to that in intact mitochondrial inner membrane shown in Fig. 1, curve I. It was noted that with linear increase of deoxycholate from 0% to 0.125%, 0.25%, and 0.5%, the extent of reduction of cytochrome c_1 was linearly increased from 71% to 87%, 96%, and 96% of the total, respectively. On the other hand, in the case of cytochrome *b*, the linear relation was not observed. The extents of reduction, *viz.* 59%, 62%, 57%, and 45%, indicate the occurrence of reduction followed by oxidation of cytochrome *b* upon increasing deoxycholate concentration. The extents of reduction of cytochrome *b* and c_1 suggest the complicated interaction of the redox components with the modulation in the conformation of the reconstituted membrane. Electron micrographs of the reconstituted membranes corresponding to curves I and IV in Fig. 2 are shown in Figs. 3A and B, respectively. Tight vesicular structures with projections (F_1 and OSCP) are observable in Fig. 3A, as reported by Kapaczyk et al. [20], while more loosely arranged structures are seen in Fig. 3B, in which 0.5% deoxycholate was present.

The addition of ATP had no effect on the 562 nm peak in the reconstituted membrane as well as in Complex I–III after the reduction with NADH.

Figure 4 shows the difference spectra of the reconstituted membrane reduced with NADH *minus* that in the presence of 0.5% deoxycholate. By this procedure, it became possible to separate the spectrum of cytochrome *b*, which is sensitive to the modulation of membrane structure by deoxycholate. It was noted that the difference spectrum (curve I in Fig. 4) has a positive peak at 565 nm and a negative peak at 558 nm. When various concentrations of CCCP were added to the sample cuvette, the difference spectrum was changed as shown by curves II–VI. With 8 μM CCCP (curve IV), there was marked decrease in the height of the 565 nm peak and when CCCP concentration was raised to 16 μM (curve VI) cytochrome *b* in the reference cuvette was in a more reduced state. It was noted that the peak at 565 nm was clearly observable in a mirror image (curves V and VI). The negative peak at 558 nm in curve I remained in curves V and VI. The addition of dithionite completely reduced the remaining cytochrome *b* even in the presence of 16 μM CCCP. The similar results were obtained with Complex I–III reduced with NADH in the absence and presence of deoxycholate. Although the height of the 565 nm peak per mg protein was lower than that of the reconstituted membrane, the 565 nm peak in Complex I–III was also sensitive to CCCP.

Discussion

Evidence [24–27] has been accumulated to suggest that ATP formation in oxidative or photosynthetic phosphorylation is not the reversal of the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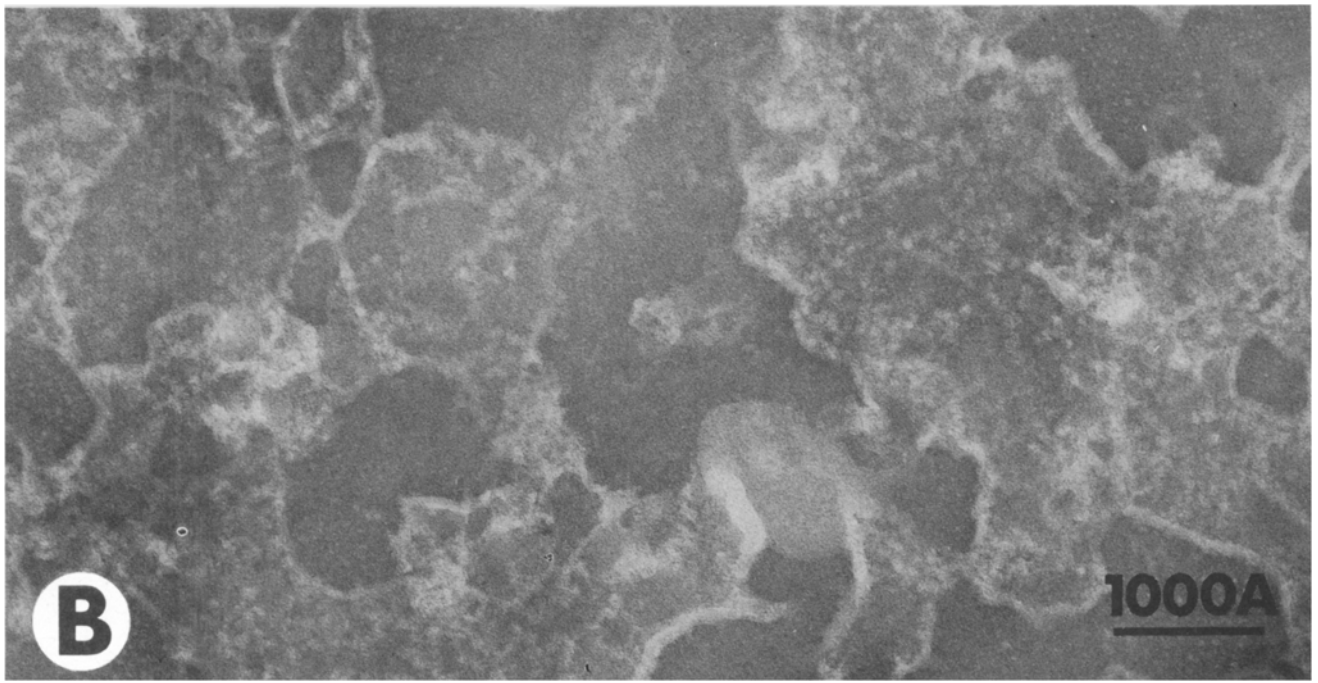

Figure 3. Electron micrographs of Complex I–III recombined with OS-ATPase in the absence and presence of deoxycholate. (A) Specimen made from Fig. 2, curve I "sample." (B) Specimen made from Fig. 2, curve IV "sample."

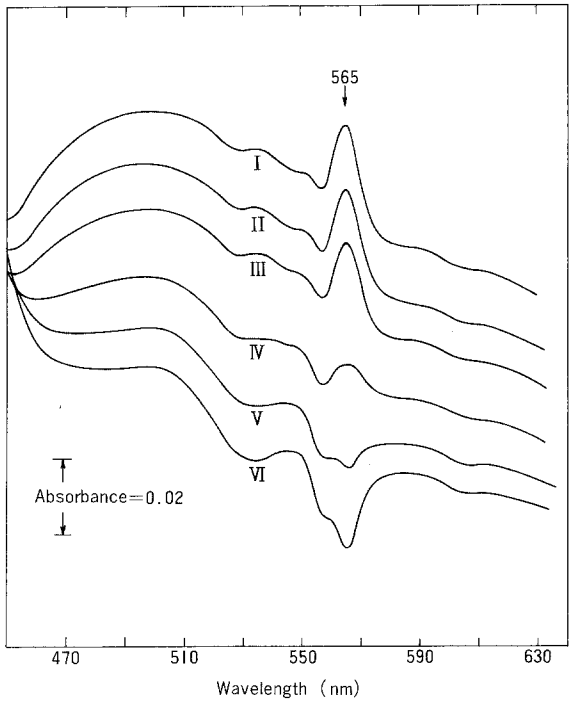

Figure 4. Difference spectra of Complex I–III recombined with OS-ATPase in the absence and presence of deoxycholate. Difference spectra were recorded by taking the "sample" in Fig. 2, curve I as sample and the "sample" in Fig. 2, curve IV as reference. The uncoupler, CCCP, was added to the sample in the following order: curve I, none; curve II, 2 nmole; curve III, 4 nmole; curve IV, 8 nmole; curve V, 12 nmole; curve VI, 16 nmole.

hydrolysis of ATP to ADP and inorganic phosphate. Therefore, the elucidation of the proposed nonphosphorylated intermediates or states, such as cytochrome b_T, is an important problem. It was noticed by Hatefi et al. [22] that NADH reduction of NADH-cytochrome *c* reductase (Complex I–III) resulted in almost complete reduction of cytochrome c_1 (554 nm) and a partial reduction of cytochrome *b* (562 nm). Complete reduction of cytochrome *b* was only attained by the addition of dithionite. They claimed that antimycin inhibits the reoxidation of cytochrome *b* in Complex I–III but not that of cytochrome c_1. We have confirmed their results and further noticed that 2 nm red-shift of cytochrome *b* peak at 562 nm was brought about by the addition of antimycin as in the case of dithionite-reduction of the

enzyme. A 3 nm red-shift of the 562 nm peak of cytochrome *b* by dithionite reduction has been noticed by Estabrook [23], but this red-shift was not observed by Hatefi et al. (Fig. 2 [22]). The discrepancy may be due to incomplete reduction of cytochrome *b* in the case of Hatefi et al., as judged from the height of the 554 nm and 562 nm peaks in their dithionite-reduced enzyme on the basis of the molar ratio (2.0) of cytochrome *b* to cytochrome c_1 in Complex III [5, 28]. Reconstitution of the membrane from Complex I–III and OS-ATPase resulted in a modulation of reducibility of cytochromes; *b* becomes more reducible and c_1 less reducible by the substrate (cf Fig. 1, curve II with Fig. 2, curve I). It was also found that the addition of deoxycholate to the reconstituted membrane with components reduced with NADH caused a linear reduction of cytochrome c_1 and an oxidoreduction of cytochrome *b*. These results indicate that the reducibility of cytochrome *b* in the reconstituted membrane is modulated in a complicated way by the conformational changes induced by deoxycholate (Fig. 2, curves II–IV, also see Fig. 3). In the presence of 0.5% deoxycholate, the relative degree of reduction of cytochrome *b* to c_1 is similar to that in the original mitochondrial membrane reduced by the substrate (cf Fig. 2, curve IV with Fig. 1, curve I).

The difference spectrum of the reduced form of the reconstituted membrane in the absence and presence of deoxycholate (Fig. 4, curve I) showed the peak at 565 nm, and a mirror image of the spectrum (Fig. 4, curve VI) is obtained by the addition of the uncoupler, CCCP. It was noticed that the shapes of these spectra are similar to those of cytochrome b_T in mitochondria in the presence of ATP or an uncoupler as reported by Sato et al. [29], Fig. 2. In Complex I–III, it has been observed by Davis et al. [7] that cytochrome b_{560} was readily reduced with NADH at 77° K, but $b_{562.5}$ was not reduced with NADH unless the preparation was treated with antimycin. From these results, Davis et al. concluded that b_{560} and $b_{562.5}$ in the binary complex (Complex I–III) correspond, respectively, to b_K and b_T described by Chance et al [2]. Therefore, the 562 nm and 564 nm peaks at room temperature in the present study correspond to b_K and b_T, respectively.

The Wilson–Dutton hypothesis was proposed by assuming that two E_m values, high and low, correspond to two different cytochrome *b*'s, "b_T" and "b_K," respectively. Sato et al. [29] observed the red-shift of the α-band of cytochrome *b* in mitochondria similar to that in Complex I–III observed in the present study (Fig. 1, curves III and IV). They interpreted this phenomenon to mean that besides "b_K," another species of cytochrome *b*, namely "b_T" is newly reduced by antimycin which lowers the E_m of cytochrome *b*, and, therefore, the red-shift of the peak is apparent.

However, as mentioned in the *Introduction,* Caswell [9] concluded from both theoretical and practical analyses that the reported

high-potential form of cytochrome *b* associated with energized mitochondria has not been demonstrated. The lack of the ATP-induced E_m-shift in plant mitochondria [13] also seems to refute the Wilson–Dutton hypothesis. Moreover, the existence of two materially different species of cytochrome *b* has never been demonstrated aside from this kinetic and phenomenologic evidence. On the other hand, it was revealed in the present study that Complex I–III recombined with OS-ATPase showed no red-shift of the 562 nm peak when it was reduced with NADH (Fig. 2), but the reducibility of cytochrome *b* in the reconstituted membrane is sensitive to deoxycholate. It was further confirmed that the difference spectrum, shown by curve I in Fig. 4, of the substrate-reduced reconstituted membrane *minus* that in the presence of deoxycholate clearly reveals a 565 nm peak, which is sensitive to an uncoupler. This is characteristic for "b_T." These findings indicate a possibility that the occurrence of the spectral peaks of "b_T" and "b_K" is not due to two species but a single species of cytochrome whose absorption spectrum splits under certain conditions.

In accord with the above view, Zaugg and Rieske [28] succeeded in the essentially complete recovery (95%) of cytochrome *b* from Complex III as well as from the electron transfer particles after cleaving them by using bile acid and ammonium sulfate. The cytochrome *b* recovered has sharp and symmetrical α-band, having a peak at 563 nm and a trough at 577 nm [28]. From the spectrum, they established the molar absorbancy index for the quantitative estimation of cytochrome *b* in Complex III as well as in submitochondrial particles. Their results imply that only one species of cytochrome *b* is present in Complex III. Their results and the evidence presented in this paper support the idea that the reducibility and spectral anomaly of cytochrome *b* is merely due to modulation in the molecular assembly of the membrane components.

From the above considerations, we prefer to adopt an interpretation to explain our results different from the "b_K" and "b_T" hypothesis: cytochrome *b* in the electron-transfer chain has an intimate and complicated interaction with other membrane components and the shape of its reduced-form spectrum is modulated by the interaction. Azzi and Santoto [8] postulated an equilibrium of protonated and unprotonated forms of cytochrome *b* to explain the appearance of a b_T type spectrum by increasing pH's in the suspending medium of submitochondrial particles. Assuming a spectral difference in the reduced form of protonated and unprotonated cytochrome *b* as they postulated [8], the phenomena reported in this paper might also be explained. A conformation favourable to maintaining the unprotonated form of *b* is changed by the addition of reagents to one favouring the maintenance of the protonated form of *b*, or the reagents directly change the unprotonated form of *b* to the protonated form; a lipid-soluble weak acid, CCCP, may convert the unprotonated form of *b* to the protonated form.

In this paper, it was clearly demonstrated that the reducibility of cytochrome *b* can be modulated by the addition of reagents such as antimycin, CCCP, and dithionite or by the conformational changes interacting with other membrane components such as OS-ATPase or deoxycholate. Since the presence of deoxycholate disintegrates the vesicular structure of the reconstituted membrane (Fig. 3), it seems that the molecular assembly of the membrane components regulates the reactivity of the redox components. A similar phenomenon was observed in our previous study on the reactivity of cytochrome oxidase with ascorbate [30]. It is noticed that the reducibility of cytochrome *b* in the reconstituted membrane is easily modulated nonlinearly to the same level as that in an intact mitochondrial membrane. The fact implies that the intact mitochondrial membrane is rather more fluid than rigid in configuration, as postulated by Singer and Nicolson [31], and that cytochrome *b* in the intact mitochondrial membrane might act as a regulatory enzyme by interacting with the membrane components or by influencing with the local environment.

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