Potential Induced Redox Reactions in Mitochondrial and Bacterial Cytochrome b-c₁ Complexes*

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Purified cytochrome b-c₁ complexes from beef heart mitochondria and Rhodobacter sphaeroides were reconstituted into potassium-loaded asolectin liposomes for studies of the energy-dependent electron transfer reactions within the complexes. Both complexes in a ubiquinone-sufficient state exhibit antimycin-sensitive reduction of cytochromes b (both low and high potential ones) upon induction of a diffusion potential by valinomycin in the presence of ascorbate. Addition of N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) to the ascorbate-reduced potassium-loaded asolectin proteoliposomes resulted in reduction of cytochrome b₅₆₂. Upon addition of valinomycin, the induced diffusion potential caused a partial reoxidation of cytochrome b₅₆₂ and partial reduction of cytochrome b₅₆₆ in beef heart cytochrome b-c₁ complex in the presence of antimycin and/or myxothiazol. Surprisingly, when ubiquinone-depleted beef heart cytochrome b-c₁ complex liposomes were treated under the same conditions, no cytochrome b₅₆₆ reduction was observed but only the oxidation of cytochrome b₅₆₂, and the oxidation was not oxygen-dependent. We explain this effect by b₅₆₂ iron-sulfur protein short-circuiting under these conditions, assuming that both antimycin and myxothiazol markedly affect subunit b conformation. The electrochemical midpoint potential of heme b₅₆₆ appears to be significantly higher than that of heme b₅₆₂ in the presence of myxothiazol, which cannot be accounted for only by the potential-driven electron transfer between these two hemes plus the shift in chemical midpoint potentials caused by myxothiazol. A model for energy coupling consistent with structural findings by Ohnishi et al. (Ohnishi, T., Schagger, H., Meinhardt, S. W., LoBrutto, R., Link, T. A., and von Jagow, G. (1989) J. Biol. Chem. 264, 735-744) is presented. This model is a compromise between pure "redox-loop" and pure "proton-pump" mechanisms. Redoxiation of high potential heme b is observed in an antimycin- or antimycin plus myxothiazol-inhibited, ascorbate plus TMPD-repricuated R. sphaeroides b-c₁ complex, upon membrane potential development, suggesting that a similar electron transfer mechanism is also operating in the bacterial complex.

Ubiquinol-cytochrome c oxidoreductase (b-c₁ complex or complex III) accomplishes vectorial proton translocation across the mitochondrial inner membrane coupled to electron flow from ubiquinol to cytochrome c. To date, experimental data on electron-transfer mechanisms are more or less consistent with Mitchell’s Q₀ Q cycle hypothesis (1-4). However, the details of membrane energization resulting from charge separation in the b-c₁ complex reactions remain unclear.

Electron transfer between the low and high potential cytochromes b (b₅₆₆ and b₅₆₂ respectively) contributes about 40% to the total energetic span of mitochondrial complex III (5-7). The remaining 60% of the membrane potential is believed to be associated with vectorial proton translocation (1, 6, 8, 9). This 60% portion of the total electrogenicity is closely related to the structural arrangement of b-c₁ across the membrane. On the basis of electrochemical and structural studies (5-13), different authors place the b₅₆₆ heme near the outer (P) side of the mitochondrial membrane and b₅₆₂ heme in the middle (5, 7, 11), or place b₅₆₆ in the middle of the membrane with b₅₆₂ on the inner (N) side (6, 8, 9, 12), or place the hemes at approximately equal distance from the outer and inner sides (13), respectively.

Important data on electron pathways and energy transduction has been obtained with the help of the specific inhibitors myxothiazol and antimycin (2-7). Although it is generally believed that the binding sites of these two inhibitors have essentially different locations (antimycin blocks electron transfer between high potential b and quinone and myxothiazol inhibits quinol oxidation by ISP and low potential b (2-4)), the specific action of the inhibitors remains somewhat ambiguous. To reconcile contradictory data on the transmembrane arrangement of the b-c₁ complex, Konstantinov (9) proposed that antimycin also arrests redox-linked proton uptake and release in center o by affecting low potential b and changing its Eₚ/pH dependence (where Eₚ indicates midpoint redox potential) (14). Furthermore, some spectroscopic and potentiometric studies suggest the proximity of myxothiazol to both center o and i (15-17).

Most of the electrochemical data for the cytochrome b-c₁ complex were obtained from submitochondrial particles, mitochondria or chromatophores. For studying electron transfer within the complex, several advantages can be gained by the use of the purified cytochrome b-c₁ complex, reconstituted in asolectin liposomes. The influence of other components of the electron transfer chain is eliminated, and the effect of quinone can be studied using quinone-depleted and -replenished b-c₁ complexes. Recently Miki et al. (13) performed an elegant study of reverse electron transport in purified cytochrome b-c₁ complexes reconstituted into potassium-loaded vesicles. Potential induced reduction of b₅₆₂, without apparent b₅₆₆ reduction, was

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1 The abbreviations used are: Q, ubiquinone; QH₂, ubiquinol; Q₁, Q₁₀, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; Q₂, Q₂₀, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; MOPS, 3-(N-morpholino)propanesulfonic acid; MCLA, 2-methyl-6-(p-methoxyphenyl)-3,7-dihydropyrano[1,2-alpyrazin-3-one hydrochloride; ISP, iron-sulfur protein; SMP, submitochondrial particles; EPR, electron paramagnetic resonance; AA, antimycin A; myx, myxothiazol.
observed in the presence of ascorbate. When high potential $b_{562}$ is preceded by an ascorbate and TMPD mixture, an induced membrane potential causes low potential $b_{562}$ heme reduction at the expense of high potential $b_{557}$ heme. The important role of quinone in $b_{562}$ reduction was shown, as was the inhibitory effect of antimycin and myxothiazol on reverse electron transport (13). However, a detailed study of the effects of inhibitors on energy-linked electron transfer in the mitochondrial cytochrome $b_{562}$ complex, in the presence and absence of quinone, was not performed (13). We have examined potential induced reduction of cytochromes $b$ in intact and Q-depleted preparations of beef heart $b_{562}$ complex at saturating concentrations of antimycin and myxothiazol. Additionally, we describe the energy-linked cytochrome $b$ reduction in the Rhodobacter sphaeroides cytochrome $b_{562}$ complex reconstituted into liposomes.

**EXPERIMENTAL PROCEDURES**

Asadectin (crude soybean phospholipids) was obtained from Associate Concentrates (Woodside, Long Island, NY) and partially purified according to Sone et al. (18). Horse heart cytochrome $c$, valinomycin, MOPS, and sodium ascorbate were purchased from Sigma. N,N,N-trimethyl-p-phenylendiamine (TMPD) and sodium cholate were purchased from Aldrich. Myxothiazol was a product of Boehringer Anraku (University of Tokyo).

Concentrates (Woodside, Long Island, NY) and partially purified acylglycerides from R. sphaeroides were purchased from A. M. Biochemical Corp., and 2-methyl-6-(N,N,N-trimethylammonium)pyrazin-3-one hydrochloride (MCLA) was a gift from Dr. M. Anraku (University of Tokyo).

The mitochondrial cytochrome $b_{562}$ complex was purified as described earlier (19). The R. sphaeroides cytochrome $b_{562}$ complex was prepared by a method involving Triton X-100 solubilization followed by DEAE-Bio-Gel A and DEAE-Sepharose 6B column chromatography in the presence of 0.01% dodecyl maltoside. This method (17) was developed by adaptation of those of Yu and Yu (20) and Ljungdahl et al. (21). Quinone-depleted mitochondrial cytochrome $b_{562}$ complex was prepared by dialysis of the above mixture, removing TMPD, and reducing phospholipids and quinone, and subsequent asadectin replenishment (22). The specific activity of mitochondrial $b_{562}$ complex at room temperature was better than 10 μmol of cytochrome $c$ reduced per min/nmol of $b$ heme. Whereas the activity of quinone- and phospholipid-depleted preparations was less than 10% that of the intact mitochondrial $b_{562}$ complex, the activity of the phospholipid-replenished preparation was better than 90%. The activity of the bacterial $b_{562}$ complex was more than 2 μmol of cytochrome $c$ reduced per min/nmol of $b$ heme. Activity assay mixtures contained 50 mM sodium/potassium phosphate buffer, pH 7.0, 0.3 mM EDTA, 50 μmol of cytochrome $c$ and 25 μmol of Q$_1$C$_6$H$_2$.

Reconstitution of potassium-loaded proteoliposomes was performed by the cholate dialysis method (13, 23, 24). To prepare mitochondrial $b_{562}$ complex, apo-cytochrome $c_1$ was reconstituted with a pH 7.0 buffer solution containing 100 mM KCl, 10 mM MOPS, pH 7.4, and 100 μM NaCl. The reaction was initiated by adding 0.3 μg of valinomycin. To collapse the gradient 1 μg of nigericin was added. The absorbance was followed at 525 nm.

To prepare samples for the recording of potential induced cytochrome $c$ reduction, 100 μl of proteoliposomes were diluted four times in 0.5-mM cuvettes with the corresponding NaCl-containing dialysis buffer and, if not otherwise stated, reduced for 5 min with 10 mM ascorbate or for 30 min with 10 mM ascorbate plus 100 μM TMPD. Inhibitors and uncouplers solutions were made in ethanol. Unless otherwise stated, the antimycin- and myxothiazol-to-cytochrome $c$ ratios were 4:1 and 8:1, respectively. Transmembrane diffusion potentials were induced by adding 0.3 μg of valinomycin to 0.4 ml of proteoliposomes. Spectra were taken every 10 s in the 540–580-nm region. The time necessary to record one spectrum was 6 s; the slit width was 2 nm; and the sampling interval varied from 0.05 to 0.2 nm. Deconvolution of the spectra was performed using extinction coefficients from West et al. (7). The ethanol concentration in samples was kept at less than 1%.

**RESULTS**

Response of Mitochondrial Cytochrome $b_{562}$ Complex Reconstituted in Liposomes to an Induced Diffusion Potential—The mitochondrial cytochrome $b_{562}$ complex reconstituted in liposomes had a high oxidation control ratio, 10.15, and in the presence of the uncouplers valinomycin plus nigericin, the specific activity of the reconstituted system was close to that of the detergent-dispersed complex. The effect of uncouplers is to dissipate the membrane potential that otherwise inhibits the energy-coupled electron transfer activities of redox enzyme complexes (23). Since it was possible to restore full activity using uncouplers without completely disrupting the membrane, and the inner part of the membrane is not accessible to the cytochrome $c$ used as electron acceptor in the activity assay, most of the reconstituted $b_{562}$ complex must have been oriented at the inner membrane with its cytochrome $c$ domains facing inward.

In the absence of ion exchange, both sides of the proteoliposome membrane have approximately equal electric potential. This was tested by safranine, an optical probe for the membrane potential (13, 25) (see “Experimental Procedures”). Valinomycin acts selectively as a potassium cation unipporter. Rapid diffusion of cations through the membrane (along the concentration gradient) creates an excess of positive charges on the outer side of the membrane and an excess of negative charges on the inner side of the membrane. A valinomycin-induced electrical potential (diffusion potential), negative on the inside, shifts the equilibrium in those reactions in which charges are moved across the membrane. The diffusion potential caused reduction of cytochrome $b$ in vesicles pretreated with ascorbate (Fig. 1). The direct cytochrome $b$ reduction by ascorbate in the absence of induced diffusion potential was negligible, less than 5% of cytochrome $b$ was reduced after a 30-min incubation of vesicles with 10 mM ascorbate. In agreement with Miki et al. (13), nigericin or excess valinomycin collapsed the diffusion potential and reversed the cytochrome $b$ reduction.

Contrary to the report of Miki et al. (13), we observed reduction of both hemes $b_{557}$ and $b_{562}$. At the beginning of the reaction, reduction of $b_{562}$ was more obvious, the absorption peak being at 563 nm, with a characteristic shoulder at 558 nm. As reduction proceeds, the peak position shifts to a shorter wavelength, indicating an increasing contribution of reduced $b_{557}$ heme. After potential induced $b_{557}$ reduction was complete (about 40–45% of total dithionite-reducible cytochrome $b$), the peak maximum was 562.3 nm. Deconvolution of this final ab-
sient energy-dependent cytochrome c(b562) and little in center

The presence of low concentrations (up to 2 Q/cytochrome b) of exogenous quinone (Q0C10) enhances cytochrome b reduction to 60–65% of the dithionite-reducible cytochrome b (Fig. 2). The reduction rate is significantly greater than in the absence of exogenous Q. At Q/cytochrome b ratios greater than 1, transient energy-dependent cytochrome c1 partial reoxidation is clearly seen (Fig. 3a), and cytochrome b566 reduction is more obvious (Fig. 3). Cytochrome b reduction, as well as transient cytochrome c1 reoxidation, is totally abolished by the addition of antimycin (not shown). Cytochrome c1 oxidation is an indirect indication for the quinone being reduced at center o. The purified cytochrome b-c1 complex contains less than 1 mol of quinone per mol of cytochrome c1, most of the quinone molecules may be located at center i (or associated with cytochrome b562) and little in center o. Added exogenous Q will bind to center o (or to cytochrome b566) and this Q may be able to accept the first electron from cytochrome b562 via b566 and the second electron from ISP-cytochrome c1 upon induction of diffusion potential.

At higher Q/cytochrome b ratios (more than 2), a decrease of the potential induced cytochrome b reduction was observed (Fig. 4). Miki et al. (13), who first reported the effect, ascribed it to direct reoxidation of reduced cytochrome b562 by oxidized quinone in center i. We think that this effect is more likely caused by a limited rate of electron transfer from ascorbate to cytochrome c1 or ISP. As the excess exogenous Q in center o may accept more electrons from cytochromes b upon induction of diffusion potential, it acts as an electron sink and thus decreases the extent of cytochrome b562 reduction. Since to complete the Q reduction at the Qo site it is necessary to receive an electron from ISP/cytochrome c1 and reduction of ISP/cytochrome c1 by ascorbate is not rapid enough, a transient oxidation of cytochrome c1 is expected. This factor, together with the limited lifetime of the diffusion potential, causes the apparent inverse dependence of potential induced cytochrome b reducibility upon quinone concentration at high Q/cytochrome b ratios.

Unfortunately the transient nature of cytochrome c1 reoxidation makes correlation of the increase of c1 reoxidation with the decrease of cytochrome b reduction difficult. To further investigate the reason for diminished cytochrome b reduction in the presence of an excess of quinone, we studied the dependence of cytochrome b reduction on the quinone/cytochrome b
Fig. 4. Effect of exogenous quinone concentration on the potential induced cytochrome b reduction. In the presence of 5 mM ascorbate; \( \mathcal{O} \), 10 mM ascorbate; \( \triangle \), 20 mM ascorbate. Total cytochrome b (100%) was 1.16 \( \mu \text{M} \), and 1- \( \mu \text{l} \) aliquots of \( Q_{562} \), \( Q_{566} \) were added in ethanol to give indicated quinone/cytochrome b ratio. Other conditions were as in Fig. 1.

The studies of Gopher and Gutman (5) and of Konstantinov et al. (6) were performed on submitochondrial particles (SMP) poised with succinate/fumarate. Therefore, we compared potential dependent reactions of antimycin-inhibited proteoliposomes in the presence and absence of a 10 \( \mu \text{M} \) fumarate/1 \( \mu \text{M} \) succinate/poising medium containing a catalytic amount of succinate-ubiquinone reductase. We found that the energy-dependent partial reoxidation of high potential heme \( b_{562} \) was blocked by antimycin plus myxothiazol inhibition. For comparison, we examined the effect of myxothiazol plus antimycin on succinate-ubiquinone reductase. We found that the energy-dependent partial reoxidation of high potential heme \( b_{562} \) together with partial reduction of low potential heme \( b_{566} \) in the reconstituted proteoliposomes was not changed upon addition of 10 \( \mu \text{M} \) fumarate/1 \( \mu \text{M} \) succinate to the medium. Deconvolution of difference spectra using the extinction coefficients from West et al. (7) gives a \( b_{562} \) oxidized/\( b_{566} \) reduced ratio about 1.15 (average of three experiments).

In the succinate/fumarate poised SMP, Konstantinov et al. (6) observed an energy-linked reduction of cytochrome \( b_{566} \) via center i, induced by the addition of ATP, with no further reduction of \( b_{562} \). Later West (26) showed that membrane potential, created by aeration of a suspension of mitochondria in the presence of ascorbate and hexammineruthenium chloride, forces electrons from the Q pool through center i into \( b_{566} \), with no change in the redox state of \( b_{562} \). Because of the redox state of \( b_{562} \), antimycin could not be artificially driven into the heme center (27). However, in the presence of myxothiazol and antimycin, the system could be driven into the heme center by the addition of succinate/fumarate.

In the myxothiazol-inhibited and succinate-ubiquinone reductase (sucinate/fumarate/succinate-ubiquinone reductase) in the reconstituted liposomes, the data obtained from the myxothiazol-inhibited proteoliposomes are consist-
ent with the data obtained with SMP and mitochondria (6, 26).

In the absence of TMPD, the \(b_{562}\) was not reduced, and addition of valinomycin to myxothiazol-inhibited proteoliposomes caused a AA-sensitive reduction of \(b_{566}\), while \(b_{562}\) remained oxidized (Fig. 6). Under similar conditions, reduction of cytochrome b was observed by Miki et al. (13), but these authors did not analyze the species of cytochromes b reduced and erroneously assumed the reduced cytochrome to be \(b_{562}\). This low potential heme \(b_{566}\) reduction was readily abolished by adding nigericin. An excess of exogenous quinone, which serves as “redox buffer,” increases the level of cytochrome \(b_{566}\) reduction to 20–25% of the cytochrome with no observable \(b_{562}\) reduction. Hence, myxothiazol inhibits the potential dependent reduction, but not the potential dependent \(b_{566}\) reduction.

Quinone-depleted Cytochrome b–c\(_1\) Complex—Removal of phospholipids from the cytochrome b–c\(_1\) complex has been shown to abolish the enzymatic activity and significantly decrease the stability (22). Delipidation also removes quinone from the complex. However, immediate replesishment of phospholipids to the complex restores almost all activity. The reversibility of b–c\(_1\) complex deactivation suggests that the replesimated complex is similar to the intact complex. Although the ubinon–cytochrome c reductase activity of quinone-depleted complexes, after reconstituting into liposomes and using \(Q_{10}\) as substrate, was more than 80% of that of the intact complex, the degree of the reduction of cytochrome b by the induced diffusion potential was only about 60–65% of that observed in the intact proteoliposomes in the presence of equimolar amounts of \(Q_{10}\). This less than 100% potential induced reduction of the \(Q_{10}\)-replenished system can be explained by the decreased efficiency of \(Q_{10}\), compared with natural \(Q_{10}\). Proteoliposomes of quinone-depleted cytochrome b–c\(_1\) complex, in the absence of added quinone, show only small potential induced cytochrome b reduction, about 10% of that observed in intact liposomes under the same conditions (13, 22). This is consistent with the 10–15% residual quinone content of the depleted preparation (22). That Q-depleted cytochrome b–c\(_1\) complex liposomes do not have the diffusion potential induced cytochrome b reduction is simply due to the lack of reducing species.

Ascorbate plus TMPD reduced the cytochrome \(b_{562}\) in quinone-depleted liposomes much like in the intact liposomes. Potential development in Q-depleted cytochrome b–c\(_1\) liposomes showed potential dependent electron movement, from high to low potential hemes (Fig. 7, a and b). The reduction pattern, in the absence of quinone, is similar to that of the antimycin- and myxothiazol-treated Q-sufficient complex. The average \(b_{562}\)-oxidized/\(b_{566}\)-reduced ratio is 1.1. According to the Q-cycle model (1–4), since quinone depletion of the b–c\(_1\) complex blocks electron transfer in both centers i and o, no additional influence of antimycin and myxothiazol on Q-depleted preparation should be expected.

However, in the presence of antimycin, ascorbate plus TMPD-reduced cytochrome b underwent partial reoxidation upon potential development in the Q-depleted preparation (Fig. 7c). Difference spectra between valinomycin-treated and valinomycin-free samples show a negative absorption peak at 562.5 nm, with no \(b_{566}\) reduction. The oxidation of high potential b heme occurred rapidly, within the time required to obtain a spectrum. Addition of nigericin restored the initial cytochrome b spectrum. Surprisingly, myxothiazol had a similar effect on energized, quinone-depleted vesicles. In the presence of myxothiazol, oxidation of \(b_{562}\), with no apparent effect on \(b_{566}\), was observed with a negative absorption peak at 561.5 (Fig. 7d). The combined effect of myxothiazol and antimycin was the same as that of antimycin alone (Fig. 7e). A possible explanation is that, at least in Q-depleted preparations, there exists an electron pathway, sensitive to antimycin and myxothiazol, that is not compatible with the Q-cycle model. However, it is not known whether or not this hypothetical pathway only appears in the presence of antibiotic under the altered phospholipid environment.

Using MCLA, an indicator of superoxide (28), did not result in a change in luminescence upon adding valinomycin to the sample containing either Q-depleted or Q-sufficient proteoliposomes that were prereduced by ascorbate plus TMPD and antimycin-inhibited (data not shown). Preliminary flushing of ascorbate plus TMPD-reduced, Q-depleted proteoliposomes with argon did not change their response to potential development in the presence of antimycin or myxothiazol. Thus generation of \(O_2^\bullet\) in the system can be excluded and oxygen cannot be considered as possible electron acceptor.

The remaining quinone in Q-depleted preparations is not...
In the lack of detailed direct structural data on the spatial organization of the b-c1 complex, the study of the potential dependent reactions plays an important role for clarification of not only energy transduction mechanisms, but also of the closely related problem of the geometric arrangement of the complex across the membrane. According to Gopher and Gutman (5) and Konstantinov et al. (6), energy-linked oxidation of prereduced \( \text{b}_{562} \) in antimycin-inhibited submitochondrial particles occurs through center \( o \), while \( \text{b}_{566} \) is in equilibrium with the succinate/fumarate pair. This is consistent with a P side location for \( \text{b}_{566} \). On the other hand, based on the facts that high potential heme reduction in center i is energy-independent (6, 26) and that high potential heme \( b \) was reduced by membrane-impermeable redox agents from the N side (8, 12), Konstantinov (9) places \( b_{562} \) electrically on the inner side and \( b_{566} \) in the middle of the membrane. According to his model, antimycin A, an inhibitor of electron transfer between Oi (center i) and high potential \( b_{562} \), also inhibits proton extrusion in center \( o \). This can explain the energy-independent redox state of heme \( b_{566} \) in antimycin-inhibited SMP (5, 6).

Some structural data on the arrangement of the b hemes became available after the study of the spin-spin interactions of paramagnetic probes with redox active sites of reconstituted b-c1 complex (11). The data obtained by Ohnishi et al. (11) exclude the location of the low potential \( b_{566} \) heme in the middle of the membrane and the location of the high potential \( b_{562} \) heme close to the matrix side surface. Nevertheless, the low potential b heme is somewhat buried within the b-c1 complex, the effective distance from the heme to the outside surface being approximately 1.6 nm.

Recently Miki et al. (13) studied the energy-linked reduction of cytochrome b in the reconstituted potassium-loaded proteoliposome system. They proposed an alternative model of potential-linked reversed electron transfer in the beef heart cytochrome b-c1 complex reconstituted into potassium-loaded phospholipid vesicles, which can be described in terms of the redox-loop mechanism. The conclusions made by these authors imply that the high and low potential hemes are at approximately equal distances from N and P sides of the membrane, respectively. However, no study of potential dependent reactions in antimycin- and/or myxothiazol-inhibited reconstituted proteoliposomes was performed to confirm this model.

Our results described in this paper support a proton motive mechanism that is a compromise between two extremes, a pure redox-loop mechanism and a pure proton-pump mechanism. The possibility of this compromise was stressed before (1, 6, 9, 29), and the influence of antimycin on the electrogenic reactions within the frame of the corresponding model was discussed (9, 12).

We assume that both antimycin and myxothiazol not only inhibit the electron transfer in the centers i and o, but also change the conformation of the cytochrome b subunit comprising both high potential and low potential b hemes, thus affecting redox-linked electrogenic proton movement. The ability of antimycin to modify the \( E_m/pH \) dependence of cytochrome b and to affect the electrogenic proton extrusion from center o has been already discussed (9, 12, 14). We obtained some insight concerning the effect of myxothiazol on the properties of cytochrome b when EPR spectra of inhibited and uninhibited R. sphaeroides cytochrome b-c1 complexes were studied. Both \( b_{562} \) and \( b_{566} \) EPR signals (\( g = 3.49 \) and \( g = 3.76 \) respectively) were shifted upfield in the presence of myxothiazol, suggesting that the inhibitor strongly affects both b cytochromes (17).

The evidence for a conformation change caused by antimycin and/or myxothiazol is also provided by our observation of energy-

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**Cytochrome b-c1 Complexes**

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**DISCUSSION**

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**Fig. 8. Energy-dependent reactions in reconstituted bacterial b-c1 complex.** 0.3 \( \mu \)g of valinomycin was added to 0.4 ml of potassium-loaded proteoliposomes (0.95 \( \mu \)M cytochrome b) prereduced by 10 mM ascorbate (a and b) or 10 mM ascorbate plus 0.1 mM TMPD (c and d) in 10 mM MOPS/NaOH, pH 8.0, 300 mM NaCl, at room temperature. After the potential induced reaction is completed, a difference spectrum is recorded with the valinomycin-free sample as a reference; a, diffusion potential reduced cytochrome b; b, difference spectrum between potential reduced and ascorbate + TMPD-reduced proteoliposomes; c, potential induced reoxidation of \( b_{562} \) in antimycin-inhibited reconstituted bacterial b-c1; d, potential induced reoxidation of \( b_{562} \) in antimycin plus myxothiazol-inhibited, reconstituted bacterial b-c1.

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likely to account for the observed phenomenon since it is not likely to be moving rapidly between Q-depleted and Q-sufficient complexes. The b-c1 complex is roughly a cylinder, one-third part of which is buried in the phospholipid bilayer (11). If we neglect differences in density between the complex and the membrane, the average distance between neighboring complexes is approximately 11 complex diameters, much greater than the distance between hypothetical centers o and i. Besides, in reconstituted mixtures of intact and quinone-deficient b-c1 complexes, cytochrome b reducibility is linearly proportional to the intact complex percentage (data not shown).

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R. sphaeroides Cytochrome b-c1 Complex—Since differences in the spatial organization of the mitochondrial and bacterial cytochrome b-c1 complexes remain unclear, it is appropriate to try to reconstitute the latter in asolectin potassium-loaded vesicles and investigate the possibility of energy-linked cytochrome b reduction in the bacterial system. Dialysis in the presence of high salt concentrations made it possible to observe antimycin-sensitive cytochrome b reduction after potential development (Fig. 8a). The maximum of potential-reduced cytochrome b absorption was at 560 nm. The simultaneous reduction of low potential heme \( b_{566} \) was clearly seen in the difference spectrum between potential and ascorbate plus TMPD-reduced complexes (Fig. 8b). Potential induced cytochrome b reduction was approximately 18% of the total. Reduction of both cytochromes b was completely inhibited by antimycin and is not observed in 0.3 mM potassium buffer.

In the presence of myxothiazol and antimycin, or antimycin alone, \( b_{566} \) in bacterial cytochrome b-c1 proteoliposomes was reduced by ascorbate plus TMPD as in the case of the mitochondrial complex. The prereduced cytochrome b was oxidized upon potential development (Fig. 8, c and d). Although no measurement of heme \( b_{566} \) reduction was carried out, because of high turbidity, the reduction of cytochrome \( b_{566} \) was expected. The electron transfer sequence within the bacterial complex appears to be identical to that of the mitochondrial system.
dependent \( b_{562} \) oxidation in the presence of myxothiazol and/or antimycin in Q-depleted b-c\(_{1} \) complexes. According to the Q-cycle model (2–4), the first electron of quinol is passed to iron-sulfur protein (ISP) and then passed to cytochrome c\(_{1}\). The resulting semiquinone is able to reduce the low potential cytochrome b. The low potential cytochrome b transfers the electron to high potential cytochrome b, which reduces quinone in center i. Low potential cytochrome b is not able to directly exchange electrons with ISP. If it did, the electron would not recycle through the high potential cytochrome b but would transfer from \( b_{566} \) to the iron-sulfur protein which has a more positive midpoint potential. According to this model, in the absence of quinone there should be no communication between the cytochromes b and other parts of the electron transfer chain. The only possible electron movement is between transmembranous b hemes.

Contrary to the Q-cycle model, we observed potential-linked \( b_{562} \) reoxidation, with no change in the \( b_{566} \) redox state, in Q-depleted antimycin- and/or myxothiazol-inhibited proteoliposomes. A possible explanation is \( b_{566} \)-ISP short-circuiting. If the high potential cytochrome b is rapidly equilibrating with an external redox medium through \( b_{566} \) and ISP, an electron will move from reduced heme \( b_{562} \) along the electric diffusion potential, to \( b_{566} \) located near the P side, which immediately passes the electron to an external acceptor. The short-circuiting is a consequence of conformational changes caused by the presence of inhibitors (antimycin and/or myxothiazol) and Q deficiency, and it is observed only if both factors are present. Conformational change affecting both cytochromes b, in Q-depleted preparations, has been clearly demonstrated by EPR studies (30).

The electron pathway and redox coupling to proton translocation in the Q-sufficient b-c\(_{1} \) complex can be illustrated as follows (Fig. 9). The model includes some of the features proposed by Konstantinov and co-workers (6, 9, 12), and it is consistent with the structural findings made by Ohnishi et al. (11). This model implies the existence of at least two redox-linked ionizable groups (1, 12, 14, 29), one closer to the outside and another closer to the inside, the pK values of which depend on the redox states of both hemes b and, possibly, on the redox states of quinone in centers i and o. We assume that myxothiazol and antimycin significantly change the responses of these groups to the redox processes in the b-c\(_{1} \) complex.

Fig. 9A shows the events postulated to take place during the potential induced reverse electron transfer in uninhibited proteoliposomes. The reduced quinone in center i reduces heme \( b_{562} \), and an ionizable group in the vicinity of the heme accepts a proton from the quinone molecule. Simultaneously, proton uptake from the P side by another redox-linked ionizable group occurs, coupled to the heme \( b_{562} \) reduction by quinol in center i. The diffusion potential drives an electron from high potential heme \( b_{562} \) to low potential heme \( b_{566} \). The low potential heme passes the electron and the proton from the redox-linked ionizable group to the quinone molecule, and proton release to the N side, coupled to the low potential heme oxidation in center o by quinone, occurs. The second electron and the second proton necessary to completely reduce Qo come from the P side through cytochrome c\(_{1} \)-ISP chain. The quinone reduced in center o freely diffuses to center i. As a result of multiple turnovers of the Q-b-b'-Q loop with three electrogenic processes involved, proton uptake from the P side, proton release to the N side, and electron movement from heme \( b_{562} \) to heme \( b_{566} \), the overall Q-pool and cytochrome b redox potential lowers, and the cytochrome b reduction induced by the diffusion potential is observed. The electrogenic proton uptake from P side is included on the picture because a marked distance (1.6 nm) between the low potential heme and the outside surface was reported (11).

In the antimycin-inhibited proteoliposomes, electron transfer from center i to \( b_{562} \) is blocked, and proton uptake from the P side is inhibited. Apparently, proton release to the N side is
also inhibited by antimycin (Fig. 9B). Heme $b_{562}$ rapidly exchanges electron with Q/QH$^+$ pair in center o. However, since significant components of the electrogenic span are lost, the oxidation of low potential heme $b_{566}$ by Q to yield QH$^+$ does not occur to a measurable extent due to the more negative midpoint redox potential of the Q/QH$^+$ pair, and we observe reduction of low potential heme together with partial oxidation of high potential heme $b_{562}$ after the diffusion potential development. In SMP the Q/QH$^+$ pair is rapidly equilibrated with the Q-pool by stoichiometric amounts of succinate-ubiquinone reductase, which forms a supercomplex with the b-c$_1$ complex (31). Since the electron transfer from $b_{566}$ to Q/QH$^+$ is not accompanied by proton translocation, the redox potential of the heme $b_{566}$ in energized SMP is equal to that established by the succinate/fumarate pair (5, 6).

We observed that $b_{566}$ remains oxidized while cytochrome $b_{566}$ is partially reduced after potential development in the myxothiazol-inhibited ascorbate-reduced proteoliposomes. Since the electron donor (ISP) is blocked by the inhibitor, the reducing power is apparently supplied by the Q/QH$^+$ pair. The excess exogenous quinone creates a “redox buffer,” and the chemical potential of the Q/QH$^+$ pair will remain essentially constant during the course of the reaction. In the presence of exogenous quinone, 20–25% of cytochrome $b_{562}$ can be reduced with little or no $b_{566}$ reduction. A rough estimate of the apparent electric potential difference between the two b hemes is 160 mV or more, with $b_{566}$ heme being more reducible than $b_{562}$ heme in the presence of a diffusion potential. On the other hand, other experiments with antimycin- or myxothiazol- plus antimycin-inhibited, or quinone-depleted preparations, show that the induced potential difference between the two hemes is less than 100 mV, with $b_{566}$ heme being less reducible than $b_{562}$ heme. The difference cannot be accounted for by a myxothiazol influence on the heme chemical midpoint potentials because both b cytochromes titrate in the presence of myxothiazol with $E_m$ values 15–30 mV more positive than in its absence (16).

Our explanation for the effect is illustrated in Fig. 9C. In the myxothiazol-inhibited b-c$_1$ complex, the electron transfer from Q to high potential heme is electroneutral. The electron is passed to the high potential heme together with a proton, which binds to a redox-linked ionizable group in the vicinity of the $b_{562}$ heme. It is likely that myxothiazol affects a redox-linked ionizable group, located in the vicinity of center o, and thus no proton is taken up from the P side. Even if the proton uptake from P side is not inhibited by myxothiazol, its contribution to the total electrogenic span may be rather small, since the low potential heme is close to the P side. On the next step, $b_{562}$ heme is oxidized by $b_{566}$ heme. In the uninhibited b-c$_1$ complex, the proton that came from the QI remains with the $b_{566}$ heme and is released to the N side only on the subsequent step, when $b_{566}$ heme is reoxidized by Qo (Fig. 9A). The electron transfer from $b_{566}$ heme to $b_{562}$ heme is not electrogenic enough to reduce $b_{562}$ heme to a greater extent than $b_{566}$ heme (see experiments with antimycin- plus myxothiazol-inhibited proteoliposomes and Q-depleted proteoliposomes). Probably, due to the influence of myxothiazol, the proton bound near the $b_{562}$ heme is released to the N side during the electron transfer from high potential to low potential heme. This proton movement from the deeply buried $b_{562}$ heme to the N side accounts for more than 60 mV of apparent electric potential difference between the b hemes and contributes to low potential heme reduction with $b_{562}$ heme remaining significantly oxidized.

The important aspect of this work was to analyze the influence of antimycin and myxothiazol on the potential dependent reactions in the b-c$_1$ complex. The results obtained show that these inhibitors not only block electron conductivity between the Q-pool and cytochromes b, but they affect the conformation of the cytochrome b subunit and, possibly, the coupling of proton translocation to electron transfer in the b-c$_1$ complex. The observation of a potential induced cytochrome b reduction, in reconstituted bacterial b-c$_1$ complexes, is consistent with there being significant structural similarity between mitochondrial and bacterial ubiquinol-cytochrome c oxidoreductases. Further detailed study of the bacterial b-c$_1$ response to membrane potential in reconstituted proteoliposomes is in progress in our lab.

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REFERENCES

1. Mitchell, P. (1976) J. Theor. Biol. 62, 327–367
2. Trumpower, B. L. (1990) Biochim. Biophys. Acta 1018, 138–141
3. Konstantinov, A. A. (1990) Biochim. Biophys. Acta 1018, 138–141
4. Von Jagow, G., Ljungdahl, P. O., Graf, P., Ohnishi, T., and Trumpower, B. L. (1984) J. Biol. Chem. 259, 6318–6326
5. Gogor, A., and Gutman, M. (1988) Bioenerg. Biomembr. 12, 349–367
6. Konstantinov, A. A., Kunz, W. S., and Kamenisky, Y. A. (1981) in Chemoswitching Proton Circuits in Biological Membranes (Skulachev, V. P., and Hinkle, P. C., eds) pp. 123–146, Addison-Wesley, Reading
7. West, I. C., Mitchell, P., and Rich, P. R. (1988) Biochim. Biophys. Acta 933, 35–41
8. Kunz, W. S., Konstantinov, A. A., Tschina, L. M., and Liberman, E. A. (1984) FEBS Lett. 172, 261–266
9. Konstantinov, A. A. (1990) Biochim. Biophys. Acta 1018, 138–141
10. Robertson, D. E., and Dutton, P. L. (1988) Biochim. Biophys. Acta 935, 273–291
11. Ohnishi, T., Schagger, H., Meinhardt, S. W., LoRusso, R., Link, T. A., and von Jagow, G. (1989) J. Biol. Chem. 264, 735–744
12. Konstantinov A. A., and Popova E. (1988) in Cytochrome Systems: Molecular Biology and Bioenergetics (Papa, S., ed) pp. 751–765, Plenum Publishing Corp., New York
13. Miki, T., Miki, M., and Orii, Y. (1994) J. Biol. Chem. 269, 1827–1833
14. Kamensky, Yu. A., Arztabazar, V. Yu., Shewchenko, D. V., and Konstantinov, A. A. (1990) Dokl. Acad. Nauk SSSR 249, 994–997
15. Kamensky, Y., Konstantinov, A. A., Kunz, W. S., and Surkov, S. (1985) FEBS Lett. 181, 95–99
16. Kunz, W. S., and Konstantinov, A. A. (1983) FEBS Lett. 155, 237–240
17. McCurley, J. P., Miki, T., Yu, L., and Yu, C.-A. (1990) Biochim. Biophys. Acta 1020, 176–185
18. Sone, N., Yoshida, M., Hirata, H., and Kagawa, Y. (1977) Biochim. Biophys. Acta 81, 519–528
19. Yu, C.-A., and Yu, L. (1980) Biochim. Biophys. Acta 591, 409–420
20. Yu, L., Mei, Q.-C., and Yu, C.-A. (1984) J. Biol. Chem. 259, 5752–5760
21. Ljungdahl, P. O., Penney, J. D., Robertson, D. E., and Trumpower, B. L. (1987) Biochim. Biophys. Acta 891, 227–241
22. Yu, C.-A., and Yu, L. (1988) Biochemistry 19, 5715–5720
23. Leung, K. H., and Hinkle, P. C. (1975) J. Biol. Chem. 250, 8467–8471
24. Beattie, D. S., and Villalobos, A. (1982) J. Biol. Chem. 257, 14745–14752
25. Gutwenger, H., Massari, S., Beltrame, M., Galoiu, R., Vereze, P., and Ziche, B. (1977) Biochim. Biophys. Acta 493, 236–244
26. West, I. C. (1988) in Cytochrome Systems: Molecular Biology and Bioenergetics (Papa, S., ed) pp. 679–680, Plenum Publishing Corp., New York
27. Nielsen, B. D., and Gellerfors, P. (1974) Biochim. Biophys. Acta 357, 358–364
28. Nakano, M. (1990) Methods Enzymol. 186, 227–232, 585–591
29. Von Jagow, G., Link, T. A., and Ohnishi, T. (1986) Biochim. Biophys. Acta 1020, 175–179
30. Yu, C.-A., and Yu, L. (1987) in Bioenergetics: Structure and Function of Energy Transducing Systems (Ozawa, T., and Papa, S., eds) pp. 81–99, Japan Scientific Society Press, Tokyo:Springer-Verlag, Berlin
31. Gwak, S.-H., Yu, L., and Yu, C.-A. (1988) Biochemistry 25, 7675–7682