Ubiquinol:cytochrome c Oxidoreductase

THE REDOX REACTIONS OF THE BIS-HEME CYTOCHROME b IN UNENERGIZED AND ENERGIZED SUBMITOCHONDRIAL PARTICLES

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The redox reactions of the bis-heme cytochrome b of the ubiquinol:cytochrome c oxidoreductase complex (complex III, bc1 complex) were studied in bovine heart submitochondrial particles (SMP). It was shown that (i) when SMP were treated with the complex III inhibitor myxothiazol (or MOA-stilbene or stigmatellin) or with KCN and ascorbate to reduce the high potential centers of complex III (iron-sulfur protein and cytochromes c + c1), NADH or succinate reduced heme b1 slowly and incompletely. In contrast, heme b566 was reduced by these substances completely and much more rapidly. Only when the complex III inhibitor was antimycin, and the high potential centers were in the oxidized state, NADH or succinate was able to reduce both b566 and b562 rapidly and completely. (ii) When NADH or succinate was added to SMP inhibited at complex III by antimycin and energized by ATP, the bis-heme cytochrome b was reduced only partially. Prereduction of the high potential centers was not necessary for this partial b reduction, but slowed down the reduction rate. Deenergization of SMP by uncoupling (or addition of oligomycin to inhibit ATP hydrolysis) resulted in further b reduction. Addition of ATP after b was reduced by substrate resulted in partial b oxidation, and the heme remaining reduced appeared to be mainly b566. Other experiments suggested that the redox changes of cytochrome b affected by energization and deenergization of SMP occurred via electronic communication with the ubiquinone pool. These results have been discussed in relation to current concepts regarding the mechanism of electron transfer by complex III.

Our recent studies (1) have revealed features of the electron transfer system of bovine mitochondrial ubiquinol:cytochrome c oxidoreductase (complex III, bc1 complex) that are incompatible with the Q cycle hypothesis (2–5). (i) It was shown that in SMP the reoxidation of the bis-heme cytochrome b of complex III could be inhibited by either antimycin or myxothiazol. The inhibition by either reagent was incomplete, and heme b562 (b566) was oxidized through the leak allowed by either inhibitor at least 10 times faster than heme b566 (b562). (ii) Cytochrome b of complex III could be partially reduced via cytochrome c1 and the Rieske iron-sulfur protein (ISP) by ascorbate, or faster and to a greater extent by ascorbate plus TMPD. This reaction was inhibited more strongly by antimycin than by myxothiazol (see also Ref. 6). (iii) Ascorbate or ascorbate plus TMPD could also partially reduce b in ubiquinone-depleted bovine heart SMP, in which the molar ratio of Qm to complex III monomer had been reduced 200-fold from 12.5 to 0.06, or in SMP from a Q-deficient yeast mutant. These results agreed with the finding of others that in bovine or yeast complex III the removal of Q by extraction did not impair the oxidation of b via ISPc1 by ferricyanide (7, 8). (iv) Our results also showed that antimycin and myxothiazol, which exert their maximal inhibition at concentrations stoichiometric to complex III monomer, each inhibited three reactions of the bis-heme cytochrome b, all incompletely. The strongest effect by either reagent was inhibition of electron transfer from heme b566 to heme b562; next was inhibition of the reoxidation of b566 via ISPc1; and least was inhibition of substrate (NADH or succinate) reduction of b, which is known to require the combined actions of both antimycin and myxothiazol (1, 2, 4). In these regards, HQNO (at 20–40 M) behaved similarly to antimycin, and MOA-stilbene and stigmatellin similarly to myxothiazol (1). It should be added here that the oxidation of reduced b566 and b562, or their reduction by reverse electron transfer via ISPc1 was not inhibited to a greater extent when SMP were treated with both antimycin and myxothiazol (1).

The above results were summarized in a scheme, which is reproduced in Fig. 1A. It was emphasized that this scheme serves only to present our new results in graphic form and should not be considered as a new hypothesis for the mechanism of electron transfer by complex III. The reason for emphasizing this point was that Fig. 1A does not explain an important feature of complex III, namely the oxidant-induced extra reduction of cytochrome b, which was a major consideration in the design of the Q-cycle (Fig. 1B) (9–11). This report addresses this feature of complex III.

EXPERIMENTAL PROCEDURES

Chemicals—NADH was obtained from Calbiochem. Ascorbic acid and TMPD were from Fluka. ATP was from Pharmacia Biotech Inc. Potassium ferricyanide was from Fisher Chemicals. Antimycin A and FCCP were from Sigma. Myxothiazol was from Boehringer Mannheim.

MOA-stilbene (12) was the generous gift of Dr. G. von Jagow, Universitätsklinikum, Frankfurt, Germany. The sources of other chemicals were as indicated elsewhere (1, 13, 14).

Preparation of Bovine SMP—SMP were prepared from bovine heart mitochondria in the presence of 1.5 mM ATP during sonication as
RESULTS

Reduction of \( b_H \) and \( b_L \) by Succinate or NADH in SMP Pretreated with KCN and Ascorbate—According to the Q-cycle hypothesis (Fig. 1B), \( Q_H \) at the \( Q_0 \) site delivers one electron to ISP, \( 2H^+ \) to the outside, and one electron to \( b_L \). Electron transfer to ISP must occur first, so that \( Q^- \), which is considered to be the electron donor to \( b_L \), is generated. Therefore, if ISP is prereduced and antimycin is added to prevent \( b \) reduction via the \( Q \) site, \( Q_H \) or a respiratory substrate should be unable to rapidly reduce the two \( b \) hemes (2, 4). However, as will be seen, our results are difficult to reconcile with this expectation. The experiments were carried out with bovine heart SMP for the reasons stated previously (1), and the reduction of cytochrome \( b \) was monitored at 563 minus 575 nm. The particles were treated with KCN to inhibit cytochrome oxidase, and 0.4 mM neutralized sodium ascorbate was added to reduce cytochromes \( c \) and \( c_1 \). In control experiments, the reduction of \( c + c_1 \) by ascorbate was monitored spectrophotometrically, and the time required after ascorbate addition to achieve complete reduction of \( c + c_1 \) determined before proceeding with the experiments reported here.

Fig. 2, traces A and B, show the results with antimycin as the complex III inhibitor. It is seen in Fig. 2A that addition of succinate to SMP, pretreated with KCN, ascorbate and antimycin, resulted in rapid reduction of cytochrome \( b \) followed by a slow further reduction. When shortly after the start of the slow phase, ferricyanide was added to oxidize \( c + c_1 \), the slow reduction phase was changed to a rapid one (Fig. 2B). This ferricyanide-induced effect is what is known as the oxidant-induced extra reduction of cytochrome \( b \), which was observed in the early studies on complex III (16, 17) and subsequently rationalized in the design of the Q cycle (2, 4, 9–11). It may be noted, however, that the initial rapid reduction of cytochrome \( b \) by succinate does not agree with the Q cycle.

Fig. 2C shows an experiment similar to that of Fig. 2B, except that the complex III inhibitor used here was myxothiazol, instead of antimycin. It is seen that upon addition of succinate a biphasic reduction of cytochrome \( b \) was observed, again with an initial rapid phase followed by a slow phase. When ferricyanide was added at the plateau of the slow reduction phase, a rapid partial oxidation of cytochrome \( b \) took place, corresponding in extent to the slow reduction phase. This was followed by a slow re-reduction (Fig. 2C). Spectral analyses showed that the succinate-induced rapid initial reduction phase was due mainly to the reduction of \( b_H \) (Fig. 3 dashed trace, \( \lambda_{\max} \) at ~563 nm), and that the ferricyanide-induced oxidized heme, in addition to \( c + c_1 \), was \( b_L \) (Fig. 3, solid trace, cytochrome \( b \) \( \lambda_{\max} \) at ~566 nm). When myxothiazol was omit-
ted in an experiment similar to that of Fig. 2C, addition of succinate to KCN + ascorbate treated SMP resulted in a biphasic b reduction essentially identical to the biphasic b reduction phase of Fig. 2C (data not shown). In addition, data similar to those of Fig. 2 were obtained when, instead of succinate, NADH was used as the reductant, except that in an experiment similar to that of Fig. 2B it was necessary for the molar equivalent of NADH to be greater than that of ferricyanide. Otherwise NADH was rapidly oxidized by ferricyanide at the level of complex I, resulting in b oxidation.

Another point of clarification regarding the effects of antimycin and myxothiazol on the redox reactions of the b hemes of complex III is afforded by the data of Fig. 4. In these experiments, no KCN and ascorbate were added. The oxidation of cytochrome b was blocked by antimycin in Fig. 4, trace A, and by myxothiazol in Fig. 4, trace B. It is seen that addition of succinate resulted in a rapid and complete reduction of the b hemes in the presence of antimycin, and in a biphasic and incomplete reduction of these hemes in the presence of myxothiazol. The difference in the spectra of the plateau regions of traces A and B, shown in Fig. 4, trace C, indicated that the heme incompletely reduced in the presence of myxothiazol was $b_1$ (Fig. 4, trace C, $\lambda_{\text{max}}$ at $-566$ nm).

Previous results of others have shown a slow reduction of both $b_1$ and $b_H$ in preparations pretreated with antimycin and ascorbate ($\pm$TMPD). In one case, a succinate-cytochrome c reductase preparation was used (18) (see also Ref. 19), which had been obtained by fractionating the mitochondria in the presence of 10 mg of cholate per ml (20). In another case, CO-treated mitochondria were used in the absence or presence of 6.7 mM ascorbate, with 0.3 mM succinate as the electron donor and 3–30 mM malonate, which greatly inhibited succinate oxidation (17). Respiration was then initiated by flash photolysis of the CO-inhibited cytochrome oxidase. We have also found that 5–10 times higher ascorbate concentrations than used in our experiments diminished the rate of $b_1$ reduction in experiments as in Fig. 2. However, as mentioned earlier, ascorbate concentrations higher than 0.4 mM were not necessary to achieve complete reduction of $c + c_1$ in the above experiments. Another point that requires further explanation here concerns the data of Fig. 2C, where it is seen that addition of ferricyanide to SMP, pretreated with KCN, ascorbate, myxothiazol, and succinate, resulted in reoxidation of the $b_1$ component of the reduced cytochrome b. In other experiments, it was found that this partial $b$ reoxidation by ferricyanide took place also with either MOA-stilbene or stigmatellin as the complex III inhibitor and regardless of whether the SMP were pretreated with KCN and ascorbate (data not shown). Furthermore, as seen in Fig. 5, when cytochrome b was reduced in myxothiazol-treated SMP, then antimycin was added to block electron transfer from succinate to b, subsequent addition of ferricyanide resulted in the reoxidation of both $b_1$ and $b_H$. Similar results were obtained with intact SMP in the absence of deoxycholate (0.05%, see legend to Fig. 2) in the reaction mixture. It is possible that modification of cytochrome b by myxothiazol, MOA-stilbene, or stigmatellin makes it susceptible to oxidation by ferricyanide. In addition, the data of Figs. 2C, 3, and 5 indicate that $b_H$ is in rapid electronic communication with complexes I and II regardless of whether the complex III inhibitor is antimycin or myxothiazol, even when $c + c_1$ are prereduced. In other words, the rate of $b_H$ reduction by succinate appears to supersede that of its oxidation (directly or indirectly) by ferricyanide. These data are in complete agreement with our previous results, which showed that the step most strongly inhibited by antimycin or myxothiazol is electron transfer from $b_1$ to $b_H$ (1). Together, the above results also indicate that only in the presence of antimycin is $b_1$ rapidly and completely reducible by substrates, and that rapid substrate reduction of $b_1$ in the presence of antimycin can be inhibited by prereduction of the high potential centers of complex III.

Effect of SMP Energization on the Reduction of $b_1$ and $b_L$ by NADH or Succinate—In an experiment such as that of Fig. 2B, it was necessary to treat SMP with a detergent to allow ferri-
cyanide access to $c + c_1$ on the interior surface of the SMP vesicles. Therefore, for uniformity, all the experiments of Figs. 2–5 were carried out in the presence of the minimal necessary amount (0.05%) of deoxycholate. Under these conditions, 0.4 mM ascorbate was sufficient to completely reduce $c + c_1$, and the addition of TMPD was not necessary. In the experiments to be reported in this section, intact, well coupled SMP were used. The addition of NADH resulted in partial reduction of cytochrome b563 minus 575 nm. It is seen that the addition of NADH with 0.4 mM ascorbate to achieve complete reduction.

As seen in Fig. 6, SMP were treated with KCN, antimycin, ascorbate, and TMPD. Then ATP was added to energize the system, and NADH was added to reduce the b hemes of complex III. Cytochrome b reduction was monitored, as before, at 563 minus 575 nm. It is seen that the addition of NADH resulted in partial reduction of cytochrome b, and subsequent addition of the uncoupler FCCP resulted in greater b reduction. As shown in Fig. 7A, the order of addition of ATP and NADH could be changed, with NADH added before ATP. A greater reduction of b was achieved when NADH was added in the absence of ATP, but subsequent ATP addition caused partial b oxidation, which was reversed upon addition of FCCP. Similar results were obtained when the respiratory substrate was succinate instead of NADH (Fig. 7B), or when the complex III inhibitor was myxothiazol instead of antimycin (data not shown), except that in these cases the extent of reoxidation of b induced by ATP addition was less. Spectral analysis of the plateau regions of Fig. 7A suggested that the component remaining reduced after ATP addition is mainly $b_1$ ($\lambda_{\text{max}}$ at 564.5 nm). Fig. 8, A and B, shows experiments similar to Fig. 7, A and B, except that myxothiazol was added where indicated before (Fig. 8A) or after (Fig. 8B) the addition of ATP. It is seen that the combination of antimycin and myxothiazol prevented the ATP-induced reoxidation (Fig. 8A) and the FCCP-induced reduction (Fig. 8B) of cytochrome b. In view of the known fact that the combination of antimycin and myxothiazol inhibits the rapid reduction of $b_1$ and $b_2$, by QH$_2$ (1, 2, 4), the data of Fig. 8 suggest that in the absence of myxothiazol ATP addition results in reverse electron transfer from the b hemes to the Q pool, and uncoupling in the reversal of this process.

The experiment of Fig. 6 is somewhat analogous to that of Fig. 2B in the sense that in an antimycin-treated system substrate-induced reduction of the cytochrome b of complex III was partially inhibited, then the partial inhibition was reversed by the addition of ferricyanide in Fig. 2B, and of FCCP in Fig. 6. However, the fact that prereduction of $c + c_1$ is not required for the ATP/uncoupler effect is shown in Fig. 9. In this experiment SMP were treated only with antimycin. Then ATP was added, followed by NADH. It is seen that NADH addition resulted in partial, but in this case more rapid, b reduction, and subsequent addition of oligomycin to inhibit ATP hydrolysis (or uncoupler, data not shown) resulted in further b reduction. Then, after exhaustion of NADH, cytochrome b was oxidized in a biphasic manner, as demonstrated previously (1, 21).

Therefore, by analogy to the phenomenon of oxidant-induced extra reduction of cytochrome b, which is rationalized by the design of the Q cycle, one could term the results of Fig. 6 and 9 as deenergization-induced extra reduction of cytochrome b. However, as will be discussed below, it is also difficult to rationalize the data of Figs. 6–9 on the basis of the Q cycle hypothesis.

**DISCUSSION**

The results of two sets of experiments have been reported here. One set concerns substrate reduction of hemes $b_1$ and $b_2$ in SMP under conditions that b oxidation was inhibited by antimycin or myxothiazol, and the high potential centers of complex III (ISP and $c + c_1$) were prereduced by ascorbate. In
The partially reduced when the inhibitor was myxothiazol, ferricyanide oxidized only substrate reduction of NADH resulted in rapid and complete reduction of myxothiazol was the complex III inhibitor addition of succinate or prereduced or not. The ferricyanide reoxidation data of Fig. 2 indicated that when the inhibitor was myxothiazol, ferricyanide oxidized only the partially reduced $b_{1L}$, regardless of whether or not $c + c_1$ were prereduced (Figs. 2C and 4B). Similar results on the substrate reduction of $b_{1H}$ and $b_1$ were obtained in the absence of myxothiazol when $c + c_1$ were prereduced (data not shown). The ferricyanide reoxidation data of Fig. 2C indicated that when the inhibitor was myxothiazol, ferricyanide oxidized only the partially reduced $b_{1L}$, but not $b_{1H}$. However, when antimycin was added after succinate (Fig. 5), and the combination of myxothiazol and antimycin inhibited electron transfer from succinate to $b$, then ferricyanide rapidly oxidized both $b_{1L}$ and $b_{1H}$. These results suggested that, under the conditions of Fig. 2C, ferricyanide was still capable of oxidizing both $b$ hemes. However, in this case the rate of $b_{1H}$ reduction, but not of $b_{1L}$ reduction, by succinate superseded its rate of oxidation (directly or indirectly) by ferricyanide. This interpretation of the data of Figs. 2C, 3, and 5 agrees with our previous finding that the strongest inhibition exerted by myxothiazol (or antimycin) is on electron transfer from $b_{1L}$ to $b_1$ (1).

The biphasic reduction of the $b$ hemes in the experiment of Fig. 2C, including the partial reduction of $b_{1L}$, can also be explained by the Q cycle, because with only myxothiazol as the complex III inhibitor (or when myxothiazol was absent, but $c + c_1$ were prereduced) the Q site of the Q cycle would still be open for reduction of the $b$ hemes, and the lower $E_{QH}$ of $b_{1L}$ would be responsible for its slow and partial reduction via $b_{1H}$. However, when SMP were pretreated with KCN, ascorbate and antimycin (the Q i site inhibitor of the Q cycle), addition of succinate or NADH also resulted in a much faster reduction of $b_{1H}$ than $b_{1L}$ (Fig. 2A). These results are still compatible with those shown in Fig. 1A, but not with the Q cycle (Fig. 1B), because under the conditions of Fig. 2A both the Qo and the Q, pathways for $b$ reduction would be inhibited, and neither $b_{1L}$ nor $b_{1H}$ should have been rapidly reduced.

In summary, the results of the first set of experiments suggest that prereduction of ISP/c1 results mainly in inhibition of the reduction of $b_{1L}$ by NADH or succinate. This effect is similar to that of myxothiazol (or MOA-stilbene or stigmatellin). It differs from the effect of myxothiazol in that the combination of myxothiazol and antimycin severely inhibits substrate reduction of $b_{1H}$ as well, but the combination of prereduced ISP/c1 and antimycin does not (Fig. 2A). Comparing Fig. 2, A and C, it also seems that the combination of ISP/c1 prereduction and antimycin addition has a greater (additive?) effect on the inhibition of $b_{1H}$ reduction than the combination of ISP/c1 prereduction and myxothiazol addition.

The second set of experiments (Figs. 6–9) have shown that in antimycin-treated SMP energization by ATP alters the manner in which NADH or succinate reduces the $b$ hemes of complex III. Only partial $b$ reduction occurred in ATP-energized SMP, but subsequent addition of an uncoupler or oligomycin (to inhibit ATP hydrolysis) resulted in further $b$ reduction (Figs. 6 and 9). Prereduction of $c + c_1$ was not necessary for partial $b$ reduction in ATP-energized SMP, nor for further $b$ reduction upon SMP deenergization. However, prereduction of $c + c_1$ did lower the rate at which $b$ was reduced by substrates. The effect of SMP energization and deenergization on the extent of $b$ reduction could also be seen after the $b$ hemes had been reduced by NADH or succinate (Fig. 7). Furthermore, addition to the antimycin-treated SMP of myxothiazol before or after ATP addition inhibited the subsequent effects of energization and deenergization (Fig. 8), suggesting that energization results in partial $b$ oxidation by the Q/QH2 pool and deenergization in further $b$ reduction by the same source. Analysis of the spectra of the plateau regions of the experiment of Fig. 7A before and after ATP addition suggested that the $b$ heme remaining reduced after ATP addition was mainly $b_{1L}$ (major $\alpha$ peak $\lambda_{\text{max}}$ at 564.5 nm), which agrees with early reports that energization of mitochondria by ATP raises the apparent $E_{QH}$ of $b_{1L}$ to $+245$ mV at pH 7.0 (22, 23). The altered redox properties of cytochrome $b$ in energized SMP merit detailed investigation, because this transmembranous component of complex III may be involved in proton translocation (Fig. 1A).

The results shown in Figs. 6–9 are difficult to reconcile with the Q cycle hypothesis. One would expect on the basis of the Q cycle that substrate reduction of $b$ would be inhibited in SMP treated with antimycin to block the Q site; with KCN, ascorbate and TMPD to reduce ISP/c1 and prevent the single-elec-
tron oxidation of QH₂; and with ATP which by creating a protonmotive force should inhibit QH₂ deprotonation at the QO site. However, our results shown in Fig. 6 (see also Figs. 7 and 8) are not compatible with this expectation. Furthermore, one would expect from the Q cycle that in a system such as above reverse electron transfer from b would also be inhibited. This expectation is again not fulfilled by our results (Figs. 7 and 8). By contrast, the results of Figs. 6–8 are not inconsistent with Fig. 1A, because in the presence of either antimycin or myxothiazol the path of electrons between the Q pool and the b hemes would be open, and ATP-induced reverse electron transfer would be expected to register on the redox state of cytochrome b.

In conclusion, the data presented here make it difficult to rationalize the phenomenon of oxidant-induced extra reduction of cytochrome b on the basis of the Q cycle hypothesis. This phenomenon appears to result from two conditions: (a) presence of antimycin (or HQNO), because only under this condition can both bH and bL be completely reduced by substrates and (b) oxidized state of ISP/c₁ (or absence of myxothiazol, MOA-stilbene, or stigmatellin), because otherwise b₁ would be slowly and incompletely reduced by substrates. Therefore, when condition (a) is fulfilled and restriction (b) is removed, bL (as well as bH) becomes reduced by substrate faster and to a greater extent. The reason for the partial and slow reduction of bL when c + c₁ are prereduced or when complex III is treated with myxothiazol, MOA-stilbene or stigmatellin remains to be investigated. So does the reason for partial b reduction under the energized conditions of Fig. 6. Therefore, although Fig. 1A is more consistent than the Q cycle (Fig. 1B) with the results presented here and previously (1), much remains to be done before a complete picture emerges. The anticipated crystal structure of bovine complex III (24) should pave the way toward this goal.

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