Ubiquinol: Cytochrome c Oxidoreductase

EFFECTS OF INHIBITORS ON REVERSE ELECTRON TRANSFER FROM THE IRON-SULFUR PROTEIN TO CYTOCHROME b

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The effects of inhibitors on the reduction of the bis-heme cytochrome b of ubiquinol: cytochrome c oxidoreductase (complex III, bc1 complex) has been studied in bovine heart submitochondrial particles (SMP) when cytochrome b was reduced by NADH and succinate via the ubiquinone (Q) pool or by ascorbate plus N,N,N,N′-tetramethyl-p-phenylenediamine via cytochrome c1 and the iron-sulfur protein of complex III (ISP). The inhibitors used were antimycin (an N-side inhibitor), β-methoxyacrylate derivatives, stigmatellin (P-side inhibitors), and ethoxyformic anhydride, which modifies essential histidyl residues in ISP. In agreement with our previous findings, the following results were obtained: (i) When ISP/cytochrome c1 were prereduced or SMP were treated with a P-side inhibitor, the high potential heme bH was fully and rapidly reduced by NADH or succinate, whereas the low potential heme bL was only partially reduced. (ii) Reverse electron transfer from ISP/c1 to cytochrome b was inhibited more by antimycin than by the P-side inhibitors. This reverse electron transfer was unaffected when, instead of normal SMP, Q-extracted SMP containing 200-fold less Q (0.06 mol Q/mol cytochrome b or c1) were used. (iii) The cytochrome b reduced by reverse electron transfer through the leak of a P-side inhibitor was rapidly oxidized upon subsequent addition of antimycin. This antimycin-induced reoxidation did not happen when Q-extracted SMP were used. The implications of these results on the path of electrons in complex III, on oxidant-induced extra cytochrome b reduction, and on the inhibition of forward electron transfer to cytochrome b by a P-side plus an N-side inhibitor have been discussed.

The mitochondrial ubiquinol:cytochrome c oxidoreductase (complex III, bc1 complex) is a homodimer of monomer molecular mass of ~240 kDa (1–4). Each monomer is composed of 11 subunits, of which 3 are redox proteins. These three proteins are a bis-heme cytochrome b containing a low potential heme bH (E m = −190 mV), a [2Fe-2S] iron-sulfur protein (ISP, E m = +280 mV) with an EPR signal centered at g = 1.90, and a cytochrome c1 (E m = +230 mV) (1, 7). In the mitochondrial respiratory chain, complex III transfers electrons from the ubiquinone pool to cytochrome c in a reaction that is coupled to outward proton translocation with a stoichiometry of H+ /e = 2. When properly isolated, purified bovine heart complex III catalyzes the reduction of cytochrome c by ubiquinol-2 at a rate of 2500–3000 s⁻¹ at 38 °C (8, 9).

Recently, x-ray crystallographic data have been published at 2.9 Å resolution by Yu and co-workers (2, 10–12) for about 80% of the bovine enzyme, at 3.0 Å resolution by Zhang et al. (3) for the chicken (as well as cow and rabbit) complex III, and at a more refined 3.0 Å resolution by Iwata et al. (4) for the bovine enzyme. In bovine complex III, cytochrome b is largely membrane-interalcelated, with heme bH near the cytoplasmic surface of the molecule and 21 Å away from heme bL toward the matrix side. The iron-sulfur (FeS) cluster of ISP is 27 Å away from heme bH and 31 Å away from the c1 heme (2). With the chicken and the bovine complex III, Zhang et al. (3) noted that in the presence of the inhibitor stigmatellin the FeS cluster of ISP was located closer to bH than in the native crystals and 31 Å away from the c1 heme. They propose that the extramembraneous domain of ISP can assume two conformations, a proximal conformation with the FeS cluster near bH (or the Qo site of the Q cycle) and a distal conformation with the FeS cluster ~20 Å closer to the c1 heme. In a more recent report, Kim et al. (12) agree with the results and conclusions of Zhang et al. (3) regarding the effect of stigmatellin on the conformation of ISP. They further indicate that 5-undecyl-6-hydroxy-4,7-dioxobenzothiazol, but not myxothiazol, MOA-stilbene, and antimycin, also fix the conformation of ISP near bH.

We have shown recently in energized submitochondrial particles (SMP) that reverse electron transfer from ISP/c1 to cytochrome b is inhibited more by antimycin, which binds near bH, than by myxothiazol, which binds near bL (13). Antimycin also inhibited reverse electron transfer from ISP/c1 to b in Q-extracted SMP, which contained 0.06 mol Q/mol cytochrome b or c1 (200-fold less than the unextracted SMP) and was incapable of oxidizing NADH or succinate by molecular oxygen (13). We have also shown that when SMP were treated with antimycin, KCN, and ascorbate plus TMPD to reduce the high potential centers of complex III, subsequent addition of NADH or succinate resulted in rapid and complete reduction of bH, and only the reduction of bL became slow and partial when ISP/c1 were prereduced (14). These and other results reported in Refs. 13 and 14 are not compatible with the Q cycle hypothesis, but they are fully consistent with the x-ray diffraction data of the three different groups mentioned, especially because these

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1 In Refs. 5 and 6, the E m values of bL and bH are given as +40 mV and −90 mV, respectively.

2 The abbreviations used are: ISP, iron-sulfur protein of complex III; SMP, bovine heart submitochondrial particles; b, cytochrome or heme b; c1, cytochrome or heme c1; Q, oxidized ubiquinone; QH2, reduced ubiquinone; EPR, electron paramagnetic resonance; MOA-stilbene; (E)-methoxyacrylate; (9R,9S)-N,N,N,N′-tetramethyl-p-phenylenediamine; EFA, ethoxyformic anhydride; 3,3-dimethoxyazobenzene; TMPD, N,N,N,N′-tetramethyl-p-phenylenediamine; EFA, ethoxyformic anhydride.
data do not show the presence of any Q at the “Q₀ site” of the Q cycle.

As pointed out by Zhang et al. (3), the possible movement of the extramembranous domain of ISP between b₁ and c₁ has important mechanistic implications, because in its proximal position the FeS cluster of ISP would be 31 Å away from the c₁ heme, a distance incompatible with rapid electron transfer. However, whether in the absence of stigmatellin or 5-undecyl-6-hydroxy-4,7-dioxobenzothiazol the extramembranous domain of ISP can move so close to b₁ is not known. Nor is it known whether in the uninhibited enzyme the redox states of cytochrome b, ISP and c₁ affect the movement of the extramembranous domain of ISP between its proposed proximal and distal positions. This paper examines features of the bis-heme cytochrome b reduction in SMP in the absence and the presence of complex III inhibitors when it is reduced by the respiratory chain substrates, NADH and succinate, and when it is reduced by reverse electron transfer from ISP/c₁. The results confirm our previous data that Q is not an obligatory electron carrier between b and ISP and suggest that when ISP and c₁ are both reduced, reduced ISP inhibits the rapid and complete reduction of b₁ by respiratory substrates.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**Antimycin A, EFA, and carbonyl cyanide p-trifluoromethoxyphenylhydrazone were obtained from Sigma. Myxothiazol was from Boehringer Mannheim. Stigmatellin, ascorbic acid, and TMPD were from Fluka. NaN₃ was from CalBiochem. ATP was from American Pharmacia Biotech. Myoglobin was from Fisher Chemicals. MOA-stilbene was a generous gift of Dr. G. von Jagow (Universitätsklinikum, Frankfurt, Germany). The sources of other chemicals were as indicated elsewhere (13–16).

**Preparation of Bovine SMP—**SMP were prepared from bovine heart mitochondria in the presence of 1.5 mM ATP during sonication as described previously (16). Extraction of Q from SMP and determination of the Q content of SMP and Q-extracted SMP were the same as previously reported (13). Protein concentration was determined by the method of Lowry et al. (17).

**Treatment of SMP with EFA—**Treatment of SMP with EFA and the activity measurements were done essentially as described before (16). Briefer SMP at 10 mg/mg were incubated with 8.9 mM EFA at 0 °C for 30–40 min in a buffer containing 0.25 mM succinate and 10 mM Tris-HCl, pH 7.5. The succinate-cytochrome c reductase activity of SMP after EFA treatment was 5–10% of that of the untreated SMP. To prevent the inactivation of succinate dehydrogenase by EFA, either 10 mM malonate or 15 mM fumarate was added during incubation of SMP with EFA (18).

**Assays—**Reduction of cytochrome b was monitored spectrophotometrically at 563 minus 575 nm in a buffer containing 0.25 mM succinate, 5 mM MgCl₂, and 50 mM Tris-HCl, pH 8.0. SMP concentration was 1.1–1.14 mg/ml. Results were essentially the same at 565 minus 575 nm. Reduction of cytochromes c₆c₁c₁ was monitored at 550 minus 540 nm. At this wavelength pair, contribution from the reduction of the b hemes was negligible. KCN, ascorbic acid (neutralized with NaOH) and TMPD were added at 10, 1.0, and 0.1 mM, respectively. Inhibitors of complex III were added from an ethanolic solution at the final concentrations indicated: antimycin A (2 μM), myxothiazol (4 μM), stigmatellin (4 μM), and MOA-stilbene (4 μM). Ethanol concentration never exceeded 1%. Absorbance changes and spectra were recorded using an SLM DW2000 dual wavelength spectrophotometer. The data shown were collected and stored on a computer on line to the spectrophotometer. When the spectra of the cytochromes were recorded, addition of dithionite to SMP gave rise to a small increase in absorbance below 556 nm, which was not related to the absorbances of the cytochromes. Spectra recorded in the presence of dithionite were corrected for this absorbance increase. Assay temperature was 30 °C throughout.

**RESULTS AND DISCUSSION**

**Complete Reduction of b₁ and Incomplete Reduction of b₁—**As stated above, the x-ray diffraction data of complex III crystals from three different groups (2–4, 12) do not show any Q located near b₁ and ISP (i.e. the crucial Q₀ site of the Q cycle). Kim et al. (12) state that in their native complex III crystals the Q₆ binding pocket is empty and that when these crystals were soaked with either Q-6 or Q-10 difference density maps had their highest peaks at the antimycin-binding site, i.e. near b₁H. Moreover, Rich and co-workers (19) have concluded from their recent studies that there is no detectable ubisemiquinone at the Q₀ site, thus making the proposed chemistry of bifurcated QH₂ oxidation at the Q₀ site of the Q cycle hypothesis more complicated. These recent data agree with our previous results (13, 14) as well as with those of Palmer and co-workers (20, 21) that Q is not an obligatory electron carrier between cytochrome b and ISP/c₁. In the Q cycle terminology, the complex III inhibitors binding near b₁H (e.g. antimycin, fumiculosin, and 2-nonyl-4-hydroxyquinoline-N-oxide) are referred to as Q site inhibitors, and those binding near b₁L (e.g. myxothiazol, MOA-stilbene, meucidin, and stigmatellin) as Q₀ site inhibitors. However, in view of the absence of evidence for a Q binding site near b₁L and ISP, we shall refer, as before (13), to these two sets of inhibitors, respectively, as inside or N-side inhibitors, and outside or P-side inhibitors.

As mentioned above, we have shown that addition of NADH or succinate to SMP pretreated with antimycin, KCN, and ascorbate (± TMPD) results in the rapid and complete reduction of b₁H. Only the reduction of b₁L became slow and incomplete under these conditions (14). These results do not agree with the Q cycle hypothesis, which requires that blocking of the Q₀ site by antimycin and prereduction of ISP/c₁ by ascorbate should inhibit the reduction of both b₁L and b₁H by NADH or succinate. Treatment of SMP with myxothiazol, MOA-stilbene, or stigmatellin in the presence of the absence of KCN and ascorbate (± TMPD) resulted in a similar pattern of substrate reduction of b₁H and b₁L (14). Heme b₁H was rapidly reduced, whereas b₁L was only partially reduced. Furthermore, when SMP were pretreated with myxothiazol, KCN, and ascorbate and then succinate was added to reduce cytochrome b₁, subsequent addition of K₃Fe(CN)₆ to oxidize ISP/c₁ also resulted in the rapid oxidation of the partially reduced b₁L, but b₁H remained in rapid electronic communication with the reduced Q pool and could not be oxidized by ferricyanide (14).

Fig. 1A shows an example of the partial reduction of cytochrome b when NADH was added to SMP treated with MOA-stilbene. However, as seen in Fig. 1B, no complex III inhibitor is needed to obtain a partial b₁ reduction. In this experiment, the SMP were treated only with KCN, and no complex III inhibitor was added, but the pattern of b heme reduction upon addition of NADH was essentially the same as in Fig. 1A and the experiments described above. Cytochrome b₁ reduction was partial (Fig. 1B), and what was not reduced was a portion of b₁L (Fig. 1C). In this experiment, the partial reduction of b₁L is referable to the fact that addition of NADH results first in the reduction of the high potential electron carriers on the oxygen side of cytochrome b₁, and this condition inhibits the complete reduction of b₁H. Thus, there are essentially two conditions in SMP that inhibit the complete reduction of b₁L by NADH or succinate: (i) prereduction of ISP/c₁ and (ii) presence of a P-side complex III inhibitor. Neither condition inhibits the rapid and complete reduction of b₁L. As stated above, prereduction of ISP/c₁ in the presence of an N-side inhibitor (e.g. antimycin) results in a very slow and partial reduction of b₁L by NADH or succinate (14). This also applies to prereduction of ISP/c₁ in the presence of a P-side inhibitor, in the sense that the partial reduction of b₁L by NADH or succinate also becomes slower. However, the fact that both b hemes are rapidly and completely reduced by either succinate or NADH in antimycin-treated SMP (13) indicates that the partial reduction of b₁L under conditions (i) and (ii) is not an inherent feature of the system. Nor can we relegate the complete reduction of b₁L in the presence of
antimycin and its incomplete reduction in the presence of a P-side inhibitor, respectively, to favorable and unfavorable \( E_m \) changes effected by these inhibitors. According to Howell and Robertson (7), antimycin lowers the \( E_m \) of \( b_1 \) by 20 mV, myxothiazol raises it by 10–30 mV, and stigmatellin causes no detectable change. The reason for the incomplete reduction of \( b_1 \) under conditions (i) and (ii) must therefore rest in the changes effected on complex III by the P-side inhibitors or by reduced ISP\(_c\).

Reverse Electron Transfer from ISP\(_c\) to \( b \)—In SMP, cytochrome \( b \) can be reduced by reverse electron transfer via reduced ISP\(_c\) as the particles are treated with KCN and ascorbate plus TMPD (Fig. 2A). The reduction of cytochrome \( b \) is slow and partial, in keeping with the thermodynamically uphill nature of the process, and the electrons accumulate in the higher potential heme \( b_H \). As was shown previously, this reaction is strongly inhibited by antimycin and unaffected when the Q content of SMP was reduced 200-fold by extraction to \( \leq 0.06 \) mol Q/mol cytochrome \( b \) or \( c_1 \) (13). The rate of reverse electron transfer to \( b \) is also inhibited by myxothiazol and MOA-stilbene (81% inhibition, Fig. 2B) but to a much smaller extent than by antimycin (94% inhibition, Fig. 2C).3 We had shown earlier that in SMP complex III is inhibited by incubation of the particles with EFA and that the inhibition is completely reversed by subsequent treatment of SMP with hydroxylamine, indicating that EFA ethoxyformylates the imidazole nitrogen(s) of one or more essential histidyl residues (18). The inhibition appeared to block electron transfer between \( b \) and \( c_1 \), and subsequent studies with complex III and isolated ISP showed that EFA modifies ISP and alters its EPR spectrum (22). Stigmatellin also alters the EPR line shape of ISP in complex III. However, pretreatment of complex III with EFA completely prevented the effect of stigmatellin on the EPR signal of ISP, and pretreatment with stigmatellin largely prevented the effect of EFA, suggesting overlapping sites of ISP modification by the two inhibitors (22). As seen in Fig. 2D, treatment of SMP with EFA inhibits (by \( \approx 70\% \)) the initial rate of electron transfer from ascorbate/TMPD to \( b \), further confirming that this reaction involves ISP as an intermediate electron carrier.

As pointed out elsewhere (13), the stronger inhibition by antimycin than by myxothiazol (and MOA-stilbene, Fig. 2B) of reverse electron transfer from ISP\(_c\) to \( b \) does not agree with the Q cycle scheme. This is because in order for this reaction to be antimycin-sensitive in the Q cycle, the electrons from ISP would have to go to \( Q_p \) at the \( Q_p \) site. Then the reduced \( Q \) would have to be protonated from the P-side, move to the \( Q_p \) site, reduce \( b_H \) via the antimycin inhibition site, and deposit protons to the N-side. Clearly, this reverse proton translocation from the P-side to the N-side cannot take place in an unenergized system, and we have shown that the reverse electron transfer reactions shown in Fig. 2 and elsewhere (13) are unaffected in the presence of the uncoupler, carbonyl cyanide \( p \)-trifluoromethoxyphenylhydrozalone.

Recent x-ray crystallographic data of complex III have shown that, unlike myxothiazol and MOA-stilbene, stigmatellin fixes the extramembranous domain of ISP near \( b_1 \) (3, 12). Also in our reverse electron transfer experiments, stigmatellin showed a different effect than myxothiazol and MOA-stilbene. As seen in Fig. 3A, treatment of SMP with stigmatellin slowed down the rate of \( b \) reduction by reverse electron transfer but considerably increased its extent (compare with Fig. 2A). As in the case of Fig. 2, the reduced \( b \) in the presence of stigmatellin was also \( b_H \)
would inhibit electron flow from ISP/cytb because when the experiment of Fig. 4 was repeated with EFA- or antimycin-treated SMP were subsequently treated with stigmatellin, KCN, ascorbate, and TMPD. These results suggest that in the presence of stigmatellin the path of electrons from ISP/c1 to b remains unaltered. Therefore, the greater extent of b reduction in the presence of stigmatellin must somehow be a consequence of the increased proximity of the FeS cluster of ISP to b, even though the reported 250 mV increased E_m of ISP in the presence of stigmatellin is difficult to reconcile with the results. It should be pointed out, however, that these E_m measurements were made in detergent-treated and fractionated preparations (23, 24). Whether the E_m of ISP in intact SMP treated with stigmatellin is the same or different remains to be determined.

As seen in Fig. 4A, when b was reduced by addition to SMP of KCN, ascorbate, and TMPD, subsequent addition of antimycin resulted in partial reoxidation of the reduced b. The extent of reoxidation upon antimycin addition was greater when EFA-treated SMP was used (Fig. 4B), suggesting a balance between the rates of b reduction by reverse electron transfer and its reoxidation as a consequence of antimycin addition. Fig. 4C shows that the b heme (b_H, see Fig. 4E) reduced by reverse electron transfer in the presence of stigmatellin was also reoxidized upon addition of antimycin. These results suggested that the recipient of electrons from b_H is ubiquinone. Antimycin would inhibit electron flow from ISP/c1 to b via b_H (Fig. 5A) and also would lower the E_m of b_H by 20–40 mV (7), resulting in the oxidation of b_H by Q. This conclusion proved correct, because when the experiment of Fig. 4C was repeated with Q-extracted SMP, the reduction of b_H via ISP/c1 was unaltered, but subsequent addition of antimycin caused only a small degree of reoxidation (Fig. 4D). In Fig. 4E, trace 1 shows the absorption peaks of the hemes reduced (b_H heme, λ_max at 562 nm; c + c₁; λ_max at 553 nm) after the addition of ascorbate plus TMPD to SMP treated with stigmatellin and KCN (C). Trace 2 shows the difference in absorbance before minus after addition of antimycin A in C. EFA treatment and Q extraction of SMP were done as described under “Experimental Procedures.” Other conditions were the same as in Fig. 3. Asc, ascorbate; Ant A, antimycin A; Stig, stigmatellin.
that the stoichiometric inhibitors antimycin, myxothiazol, MOA-stiblene, and stigmatellin appear each to inhibit three redox reactions of the bis-heme cytochrome \( b \) (Fig. 5C), we proposed that these inhibitors act by causing conformational changes in cytochrome \( b \) (13), a suggestion that agreed with the results of others (26, 27). We now suggest further that the conformation changes effected by the combination of an N-side and a P-side inhibitor retards the off-rate of Q bound to cytochrome \( b \). In such a case, the reduction of \( b \) by NADH or succinate in SMP treated with, for example, antimycin and myxothiazol (or stigmatellin) would be inhibited, because the oxidized Q bound to \( b \) would not come off rapidly to be replaced by reduced Q. In the experiment of Fig. 4C, there would be sufficient oxidized Q bound to \( b \) to reoxidize the partially reduced \( b_L \).

As regards the inhibition of \( b_L \) reduction when ISP/\( c_1 \) are prereduced (Fig. 1B and Ref. 14), the following considerations may be of interest. As stated above, Zhang et al. (3) have suggested that the extramembranous domain of ISP moves during electron transfer between its proximal position near \( b_L \) and its distal position near \( c_1 \). These electron transfer steps may be graphically illustrated as follows, with the asterisked electron carriers indicating reduced species and the length of the lines between them indicating proximity: \([b_L^\ast – ISP – c_1] \rightarrow [b_L – ISP – c_1] \rightarrow [b_L – ISP – c_1^\ast] \rightarrow [b_L – ISP – c_1^\ast] \rightarrow [b_L^\ast – ISP – c_1] \rightarrow [b_L – ISP – c_1^\ast] \rightarrow [b_L^\ast – ISP – c_1^\ast]. \)

In other words, oxidized ISP would move near reduced \( b_L \), and reduced ISP would move near oxidized \( c_1 \). However, by prereduction of ISP/\( c_1 \), with ascorbate or as in the experiment of Fig. 1B where the oxidation of reduced ISP and \( c_1 \) was prevented by inhibiting cytochrome oxidase with KCN, we create a situation that does not exist under normal electron transfer conditions. We propose that when both ISP and \( c_1 \) are reduced, the reduced ISP is repulsed from the proximity of reduced \( c_1 \), that the proximity of reduced ISP to \( b_L \) inhibits the rapid and complete reduction of this heme. It is also possible that the P-side inhibitors binding near \( b_L \) exert a similar effect on its reducibility. Our new results and conclusions agree with the tentative electron transfer scheme published earlier (13, 14). In Fig. 5, this scheme has been expanded to incorporate (i) the conclusion of Zhang et al. (Ref. 3; see also Ref. 12) regarding the movement of the extramembranous domain of ISP between \( b_L \) and \( c_1 \) for facilitated electron transfer; (ii) our proposal that when ISP and \( c_1 \) are both reduced, reduced ISP is repulsed from the vicinity of reduced \( c_1 \), moves near \( b_L \), and interferes with the rapid and complete reduction of \( b_L \) via the Q pool; and (iii) our suggestion that the combination of an N-side and a P-side inhibitor alters the conformation of cytochrome \( b \) in such a manner that the off-rate of Q becomes inhibited.

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