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IMPLICATIONS FOR STRUCTURE AND CATALYTIC MECHANISM

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The respiratory chain enzyme ubiquinol-cytochrome $c$ oxidoreductase, or $bc_1$ complex, transfers electrons from ubiquinol to cytochrome $c$ (1). The eukaryotic enzyme is embedded in the inner mitochondrial membrane and couples electron transfer to proton translocation across this membrane. This enzyme complex may consist of up to 11 subunits, as in the bovine heart complex (2), but only three subunits contain prosthetic groups and are directly involved in electron transfer. These subunits are cytochrome $b$, cytochrome $c_1$, and the Rieske Fe-S protein and they form the “minimal,” or “core” complex. In many prokaryotes the $bc_1$ complex is constituted of only these three subunits (1). The $bc_1$ complex from the yeast Saccharomyces cerevisiae contains at least seven subunits lacking a prosthetic group, their function is still poorly defined. A very similar situation holds for the other proton-pumping respiratory complexes, complex I, cytochrome $c$ oxidase, and ATP synthase. A role that is generally attributed to these “accessory” subunits is that they may contribute to the structural stability of these complexes (1, 5).

$\alpha$-Phenanthroline (OP)$^*$ is a metal chelator, capable of binding divalent cations. Because of its membrane-permeable nature, it is commonly used as an inhibitor of enzymes that require such a metal atom for catalytic activity, like the family of mitochondrial metalloproteases (6, 7) and the matrix-processing peptidase (8). OP was found to be a noncompetitive inhibitor of complex I in bovine submitochondrial particles (9). The inhibition site of OP is similar to that of rotenone, namely between all the Fe-S clusters of complex I and the ubiquinone pool. The sensitivity toward OP of isolated complex I is very similar to that of submitochondrial particles, indicating that complex I is the main site of inhibition by OP in the respiratory chain (9). OP also inhibits electron transfer in the bacterial photosynthetic reaction center by displacing the secondary quinone from the $Q_B$ site (10). OP binds to the $Q_B$ binding pocket by hydrogen bonding to the histidine at position 190 of the $L$-chain (HisL190) (11).

In this study we report the effect of OP on the yeast $bc_1$ complex and its use as probe for the structural stability of the complex. In addition, the OP-ferrous sulfate complex (ferroin) is found to have an inhibitory effect on the $bc_1$ complex but of a different type. The characteristics of this latter type of inhibition are discussed in relation to the dimeric structure of the $bc_1$ complex.

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1 The abbreviations used are: OP, $\alpha$-phenanthroline (1,10-phenanthroline); $Q_{H_2}$, 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone; $MP$, $m$-phenanthroline (1,7-phenanthroline); $P$, $p$-phenanthroline (4,7-phenanthroline); ferroin, 1,10-phenanthroline-ferrous sulfate complex; $Q_{out}$, ubiquinol oxidation site.
**EXPERIMENTAL PROCEDURES**

**Preparation of Mitochondria and Solubilized bc1 Complex—** Yeast strain DLI1 (mat α, his3–11, 15, leu2–3, 112, ura3–251, 328, 372) (12) was grown in lactate medium (0.5% yeast extract, 0.2% w/v magnesium sulfate, 0.6% w/v ammonium phosphate, 2% sodium lactate (70% w/v), 1.3% lactic acid (75% w/v), pH 4.5) until late logarithmic phase. Mitochondria were isolated as described previously (13) and resuspended in 0.6 M sorbitol; 25 mM KPi; pH 7.4; 1 mM EDTA; 1 mM MgCl₂. Protein concentrations were determined with the Lowry method (14). Mitochondrial membranes were solubilized with 1 mg/mg protein lauryl maltoside (dodecyl-β-D-maltoside) at a final concentration of 5 mg/ml mitochondrial protein. Partly purified bc1 complex, used for EPR analysis, was prepared according to (15), except for the final purification step.

**Pre-steady-state Kinetics—** Solubilized mitochondria were centrifuged at 100,000 × g for 90 min. The supernatant was used in the pre-steady-state measurements. Spectral analysis before the measurements indicated that less than 10% of the cytochromes was in the reduced state because of endogenous substrate. Spectra taken after pre-steady-state reduction indicated that complete reduction is achieved, despite the absence of KCN, an inhibitor of cytochrome c oxidase. These spectra further indicated that the amount of cytochrome c was significantly reduced.Stopped-flow measurements were performed using the RX-1000 Rapid Kinetics Spectrometer Accessory (Applied Photophysics, Leatherhead, U. K.), connected to the DW2000 spectrophotometer. Rapid mixing of enzyme and substrate (ratio 1:1) is achieved by a manual drive. At the end of a drive, a trigger is given to the spectrophotometer which initiates the measurement. Reduction of cytochrome b was monitored at 562 versus 575 nm on a DW2000 spectrophotometer at 4 °C. As a substrate 0.5 mM duroquinol was used (final concentration 0.25 mM) in 50 mM Tris, pH 8; 250 mM NaCl; 0.1 mM dodecyl maltoside.

**EPR Analysis—** EPR spectra at X-band (9 GHz) were obtained with a Bruker ECS 106 EPR spectrometer at a field modulation frequency of 100 kHz. Cooling of the sample was performed with an Oxford Instruments ESR 900 cryostat with an ITC4 temperature controller. The magnetic field was calibrated with an AEG Magnetic Field Meter. The X-band frequency was measured with an HP 5350B Microwave Frequency Counter.

**Chemicals—** 1,10-Phenanthroline, 1,7-phenanthroline, 4,7-phenanthroline, and 1,10-phenanthroline-ferrous sulfate complex (standard concentration 180–200; pH 7; 510 nm) were from BDH Chemicals Ltd., Poole, U. K.

**RESULTS**

**Inactivation of the bc1 Complex Using o- and m-Phenanthroline—** We have found that when OP is added to lauryl maltoside-solubilized mitochondria, it acts as an inactivator of the bc1 complex. This inactivation is time-dependent, increasing progressively over a period of 1–2 h.

To quantitate this inactivation, solubilized mitochondria were incubated with 0.6 µM of OP/mg of protein at 0 °C, 25 °C, and 30 °C (dilution experiments showed that not the concentration of inhibitor, but the rate of the reaction to bc1 complex is the relevant factor). As can be seen in Fig. 1, inactivation is temperature-dependent, the complex being fully stable for up to 70 min at 0 °C. At 25 and 30 °C inactivation is rapid, activity being reduced by 50% within 40 min at 25 °C and within 25 min at 30 °C. In contrast, no inactivation occurs within 60 min when solubilized mitochondria are incubated at 25 °C in the absence of OP or when intact mitochondria are incubated with OP at 25 °C. This implies that the site of action of OP on the bc1 complex becomes exposed or at least more accessible after solubilization of the membrane.

To determine whether the chelating activity of OP is responsible for the inactivation of bc1 activity, we included the nonchelating isomers m-phenanthroline (MP) and p-phenanthroline (PP) (see Fig. 8). MP was found to inhibit bc1 activity as well, with approximately the same rate as OP (Fig. 1). In contrast, PP did not have any (significant) effect on electron transfer activity of the bc1 complex (data not shown). The fact that MP also inhibits bc1 activity indicates that the chelating activity is not responsible for inactivation.

**Inactivation by OP Is Inhibited by Fe^{2+}—** Chelation of a metal ion by OP affects the structure of this compound and thus may affect its capacity to inactivate the bc1 complex. To test this, bc1 complex was incubated with 0.6 µM of OP/mg of protein in the presence of increasing amounts of FeSO4 at 25 °C. In the course of this experiment it was found that ferroin itself, which is formed when OP chelates iron, has an inhibitory effect on the bc1 activity (see also below). The fact that a fraction of OP is converted into ferroin when FeSO4 is added may seem to complicate the analysis of the inhibitory effect of OP in response to increasing amounts of FeSO4. However, ferroin was found to affect bc1 activity directly after addition, whereas OP requires a certain incubation time and does not show any (measurable) inhibition directly after addition (see Fig. 1, at 1 min). In other words, any inhibition observed directly after addition of OP + FeSO4 is directly attributable to the ferroin that is formed, whereas the inhibition observed after 1 h of incubation is a summation of the effects of ferroin and OP (Fig. 2A). The difference between the amount of inhibition after 1 h and directly after addition provides the net inhibitory effect of OP. It can be seen that FeSO4 reduces the inhibitory effect of OP, which can be completely abolished when sufficient amounts of FeSO4 are added (Fig. 2B).

To examine further the effect of ferroin, solubilized bc1 complex was titrated with this agent. It can be seen from Fig. 3 that increasing amounts of ferroin linearly inhibit the electron transfer activity to around 50% at 1 µM of ferroin/mg of protein. Surprisingly, a further increase of the amount of fer-
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**Fig. 2.** The inhibitory effect of OP can be prohibited by Fe\(^{2+}\). A, mitochondria were solubilized in lauryl maltoside (1 mg/mg of mitochondrial protein) and 0.6 \(\mu\)mol OP/mg of protein was added plus FeSO\(_4\) at the concentration indicated on the x axis. The percentage of inhibition of electron transfer activity was measured directly (□) and after incubation at 25 °C for 60 min (○). B, the percentage of inhibition by OP at the given conditions in relation to the ratio FeSO\(_4\)/OP. Since directly after addition OP has not caused any inhibition, the net contribution of OP can be determined by subtracting the percentage of inhibition after 60 min minus that directly after addition.

**Fig. 3.** Ferroin inhibits half of the bc\(_1\) electron transfer activity. Increasing amounts of ferroin were added to solubilized bc\(_1\) complex, and electron transfer activity was assayed.

Ferroin did not result in a further inhibition. It was found that the inhibitory effect of ferroin is not dependent on the incubation temperature.

Ferroin thus also inhibits solubilized bc\(_1\) complex, but in a different manner than OP. The inhibition by ferroin is instantaneous, in contrast to that by OP, and ferroin can only reduce the bc\(_1\) activity by 50%.

**The Cytochromes of the bc\(_1\) Complex Are Not Affected by OP—**Although inactivation of the bc\(_1\) complex by OP is not the result of its chelating activity, we noticed that after a certain time of incubation of bc\(_1\) complex with OP the sample turned red. This red stain was identified as ferroin, based on its absorption maximum at 510 nm. The formation of ferroin in the sample can be explained in two ways: either iron was present in the sample which is not related to the bc\(_1\) complex, or OP first binds to the bc\(_1\) complex, thereby causing inactivation, and in a second stage chelates one of the iron atoms of the complex. To test this second possibility, the integrity of the prosthetic groups of the bc\(_1\) complex after OP inactivation was examined.

The cytochromes of the bc\(_1\) complex, the \(b\) and the \(c\), contain an iron atom and are putative targets for OP. The upper trace of Fig. 4 shows the absorption spectrum of the cytochromes of fully active bc\(_1\) complex and cytochrome c oxidase. For the lower trace, solubilized mitochondria were incubated with OP until complete inactivation of bc\(_1\) activity was achieved. The peak at 510 nm in the lower trace is because of ferroin present in higher amounts in the sample cuvette than in the reference cuvette. This is because dithionite reduces additional iron not related to the bc\(_1\) complex present in the mitochondria, which is subsequently bound by OP.

Having excluded that OP chelates an iron of one of the cytochromes, the remaining prosthetic group, that of the Rieske Fe-S protein, may be the target of OP.

**OP Chelates an Iron Atom from the Rieske Fe-S Cluster—**The effect of OP on the Rieske Fe-S cluster was analyzed by EPR after reduction of solubilized bc\(_1\) complex with ascorbate/CN\(^-\). First, wild type mitochondria were solubilized and incubated with OP. The decrease in bc\(_1\) activity in time was followed, and an EPR sample was prepared at 100, 50, and 10% of enzyme activity by immediately freezing the sample in liquid nitrogen. Fig. 5 shows EPR spectra of the Rieske Fe-S cluster of the three samples. Comparison of the traces corresponding to 100 and 10% enzyme activity indicates that the components of the spectrum of the Fe-S cluster (at \(g = 1.75, 1.90,\) and 2.02) all are strongly decreased. The spectrum at 50% enzyme activity, however, is more complicated: the signal at \(g = 1.75\) clearly is reduced, while the other two components are not. The signal at \(g = 1.90\) may even be slightly more intense than for the fully active sample. Note that the signal of the radical at \(g = 2.00\), attributable to the semiquinone form of the covalently bound flavin of the succinate dehydrogenase, is not affected by OP.

It can be concluded from this experiment that OP binds an iron atom from the Rieske Fe-S cluster but that this does not parallel the inactivation rate. It thus seems that OP binds to the bc\(_1\) complex in close vicinity of the Rieske Fe-S cluster, thereby possibly affecting the midpoint potential of the cluster, which causes inactivation of electron transfer activity. Furthermore, binding seems to cause an alteration of the EPR line shape, implied by the differences between the EPR spectra corresponding to 100 and 50% electron transfer activity. In a later stage, the Rieske Fe-S cluster disintegrates when OP chelates an iron atom. This view is consistent with the bottom
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**Fig. 4. OP does not affect the cytochromes of the bc1 complex.** Dithionite-reduced minus ferricyanide-oxidized difference spectrum of mitochondria before (upper trace) and after inactivation of the bc1 complex with OP (lower trace). Spectra were recorded at room temperature; the protein concentration was 2.5 mg/ml.

trace in Fig 5 where the signal at \( g = 1.90 \) is 30% of that of the top trace while electron transfer activity is only 10%.

Interpretation of the dual effect of OP, first binding to the bc1 complex causing inactivation and then chelation of an iron atom from the Rieske Fe-S cluster, is slightly hampered by the fact that the two effects partially overlap. As a control, EPR spectra were taken before and after inactivation using MP, the nonchelating isomer of OP. The EPR spectrum after inactivation was identical to that before inactivation (data not shown), thereby confirming that inactivation of bc1 activity and disruption of the bc1 Fe-S cluster are two distinct processes.

Since OP can only chelate an iron atom in its reduced state, we tested whether the inactivation rate is increased when the Rieske Fe-S cluster is kept in its reduced state. The incubation with OP was performed in the presence of 10 mM succinate + KCN. The inactivation rate, however, was found to be virtually the same as for the bc1 complex in the oxidized state. This result further indicates that the chelation of an iron atom of the Rieske Fe-S cluster is not the step determining the inactivation rate.

Pre-steady-state Reduction of Cytochrome b after Ferroin and OP Inactivation—The different mechanisms by which ferroin and OP inactivate the bc1 complex have been studied in more detail by analyzing the pre-steady-state reduction kinetics of cytochrome b. Under normal conditions there are two pathways for reduction of cytochrome b, as described by the proton motive Q-cycle (19, 20). Reduction can either be achieved via center P, on the positive side of the membrane, and center N on the negative side of the membrane. At center P ubiquinol, or in this case the artificial substrate duroquinol, is oxidized by the Rieske Fe-S protein, thereby generating a ubisemiquinone anion, which in turn reduces cytochrome \( b_{552} \). In the other pathway, via center N, electrons are transferred to cytochrome \( b_{562} \). Since both b cytochromes are electronically connected, both can be reduced via either pathway. By using specific inhibitors for either reaction center, reduction of cytochrome b can be restricted to only one pathway. Antimycin blocks reduction via center N, thus only allowing reduction of cytochrome b via the center P pathway (21). Myxothiazol functions in the opposite way, blocking the center P pathway and only allowing reduction via the center N pathway (22).

Reduction of cytochrome b without inhibitors added and without inactivation of any kind is biphasic when duroquinol is used as electron donor, the fast phase having a half-time (\( t_{1/2} \)) of 40 ms (see Fig. 6A). Note that the experiment was carried out at 4°C, thereby reducing the reaction rate by a factor of 8–10 compared with the \( in vivo \) conditions at 28°C. The addition of KCN had no effect on the reduction of cytochrome b. Spectral analysis indicated that cytochrome c content was strongly diminished in the preparation used. Furthermore, solubilization causes a strong “dilution” of cytochrome c, thereby virtually blocking electron transfer from the bc1 complex to cytochrome c. Spectral analysis further indicated that all cytochrome b in the presence of duroquinol in the pre-steady-state measurements get reduced. Reduction of cytochrome b via center N in the presence of myxothiazol is monophasic and rapid (Fig. 6C). Binding of myxothiazol is known to increase the rate of reduction of cytochrome b via center N (23), thereby explaining the monophasic reduction behavior, in contrast to the biphasic reduction kinetics observed when no inhibitor is present. Reduction via the other side of the complex in the presence of antimycin, via center P, is also monophasic, but is much slower, having a \( t_{1/2} \) of 200 ms.

When ferroin is added, thereby inhibiting steady-state activity to 50%, pre-steady-state reduction of cytochrome b in the absence of any inhibitor is also biphasic. The first, rapid phase is not changed, but the second phase is much slower than obtained with the fully active enzyme. Analysis of the reduction kinetics using inhibitors indicates that reduction in the presence of myxothiazol is unaltered, whereas in the presence of antimycin the reduction rate is reduced compared with the fully active enzyme. In the time course of the measurement (1 s) b reduction does not run to completion. The experiment was
therefore repeated on a larger time scale (5 s), which indicates that all cytochrome b still is reducible under these conditions (data not shown). This trace also makes it possible to determine the half-time of b reduction in the presence of antimycin, which was found to be ~450 ms. However, the 1-s experiment implies that reduction of cytochrome b in the presence of antimycin after inhibition by ferroin is biphasic, which makes it difficult to draw conclusions about the mechanism of inactivation by ferroin (see "Discussion"). This experiment nevertheless shows that the reaction rate via center P is roughly half of that for the fully active enzyme. It can thus be concluded that ferroin acts on the outside of the bc1 complex, leaving reduction of cytochrome b via center N unaltered while reducing the reaction rate via center P by 50%.

To study the effect of inactivation by OP on the reduction kinetics of cytochrome b, solubilized bc1 complex was incubated with OP under the same conditions as used in Fig. 1. When steady-state electron transfer activity was reduced to 30%, pre-steady-state reduction of cytochrome b in the presence of antimycin was determined. As can be seen in Fig. 6, only a fraction of cytochrome b is still reducible, corresponding to the 30% residual steady-state activity. After this, using the same sample, cytochrome b reduction in the presence of myxothiazol was measured. Meanwhile, steady-state reduction was reduced to 20%. It can be seen in Fig. 6 that also via center N not all cytochrome b can be reduced. However, the 50% of cytochrome b which is reduced via this pathway does not correspond to the residual fraction of steady-state activity. Thus, a part of the bc1 complex which can still be reduced via center N is already inactivated, indicating a time lag between inactivation and loss of reducibility of the b cytochromes via center N.

We conclude that these results are in agreement with the outcome of the EPR analysis of the Rieske Fe-S cluster. OP binds at the outside of the bc1 complex thereby blocking reduction of cytochrome b via center P. In a later stage, the Rieske Fe-S cluster is disrupted, resulting in destruction of the entire complex, thereby also abolishing reduction of cytochrome b via center N.

Similar to the outcome of the EPR analysis, the pre-steady-state analysis indicates that the two effects of OP partially overlap. Therefore, again MP was included as a control to make a clear distinction between inactivation and distortion of the complex. Solubilized bc1 complex was incubated with MP under the same conditions as used in Fig. 1. When steady-state electron transfer activity was reduced to 20%, pre-steady-state reduction of cytochrome b was measured (Fig. 7). Note that the time scale in Fig. 7 is different from that in Fig. 6. After inactivation by MP, cytochrome b can still be reduced in the presence of myxothiazol, whereas in the presence of antimycin only a fraction of b is reducible, which corresponds to the residual steady-state activity. Thus, after inactivation center P is blocked, but reduction via center N can still occur.

**DISCUSSION**

We have shown that OP and MP both inhibit the electron transfer activity of the yeast bc1 complex. The inhibitory effect is time- and temperature-dependent and is only observed with solubilized mitochondria. The implication of this second obser-
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**FIG. 7.** Pre-steady-state reduction of cytochrome b through centers P and N after MP inactivation. Reduction of cytochrome b by duroquinol in the absence of inhibitor (A) or in presence of antimycin (B) or myxothiazol (C) was recorded at 562–575 nm at 4 °C. Solubilized bc₁ complex was incubated with MP at 25 °C (same conditions as used in Fig. 1) until electron transfer activity was reduced to 20%, before pre-steady-state reduction was measured. Note that, in contrast to the experiment in Fig. 6, no stopped-flow device was used, and the reduction of cytochrome b is recorded on another time scale.

The site of interaction of OP and MP with the bc₁ complex is normally buried in the lipid bilayer and only becomes accessible upon solubilization. This site of action is subsequently identified as the Rieske Fe-S cluster on the basis of experiments performed with OP- and MP-inactivated bc₁ complex.

It has been reported by Finel and Majander that complex I is the main site of action of OP in the inner mitochondrial membrane (9). This is not in contradiction with the data presented here: the studies on complex I were performed with submitochondrial particles, and, as we show here, OP has no effect on the bc₁ complex activity when present in the membrane. The exact site of inhibition of OP on complex I could not be elucidated using EPR spectroscopy, only that it must be between the Fe-S clusters and the ubiquinone pool. It should be noted, however, that the number of Fe-S clusters in complex I is still undefined (24, 25), and an inhibition mechanism involving destruction of one of the Fe-S clusters therefore cannot be completely excluded.

In the case of the bacterial reaction center the binding site of OP was precisely determined by crystallization of the protein in the absence of inhibitor (11). OP binds at the bottom of the pocket for ubiquinol is bordered by two histidines of the Rieske center P (31). In this model, the back of the proposed binding pocket for ubiquinol is bordered by two histidines of the Rieske Fe-S protein which coordinate the Fe-S cluster. We propose that the two nitrogen atoms of OP form a shared hydrogen bond with the imidazole nitrogen of one of the histidines coordinating the Fe-S cluster, identical to the binding mode in the bacterial reaction center. The same binding mode to the bc₁ complex has also been proposed for the center P inhibitor stigmatellin (32). In fact, stigmatellin is also able to bind to the Qₐ site of the bacterial reaction center via a hydrogen bond to residue His-L190 (33), the same residue that is hydrogen-bonded by OP.

One of the other phenanthroline compounds, MP, was shown to inhibit bc₁ activity in a fashion similar to OP, in contrast to the third compound PP. Comparison of the three compounds (see Fig. 8) shows that the structures are very similar and suggests that all three fit into the same binding pocket. The orientation is such that the two nitrogen atoms of OP are facing the histidines coordinating the Fe-S cluster. On this side of the molecule MP contains one nitrogen atom, whereas PP contains none. Since MP is able to inhibit bc₁ activity, it has to be assumed that one nitrogen atom is sufficient for interaction with one of the histidines of the Rieske Fe-S protein, although possibly in a slightly different manner than OP. Lack of inhibition by PP can now easily be explained by the absence of a nitrogen atom at this side of the molecule. The structure of ferroin is entirely different (see Fig. 8). This complex consists of an iron atom surrounded by three OP molecules and is unlikely to fit into the same binding pocket as OP and MP.

The rate of inactivation by OP was found to be only slightly higher when the Rieske Fe-S cluster was kept in the reduced state. In this respect, binding of OP to the bc₁ complex differs from that of stigmatellin, since this inhibitor binds 4 orders of magnitude more tightly to the reduced form than to the oxidized form (34). However, since OP only binds to solubilized bc₁ complex and since the binding rate is temperature-dependent, it is clear that it is not the formation of a hydrogen bond which determines the inactivation rate, but the accessibility for OP to its binding site.

In a second step OP binds an iron atom from the Rieske Fe-S cluster, thereby distorting the structure of the Rieske protein. Pre-steady-state reduction of cytochrome b in the presence of myxothiazol after inhibition by OP indicated that disintegration of the Rieske Fe-S cluster is followed by destruction of the remainder of the complex, as demonstrated by the lack of reduction of cytochrome b via center N. This is in agreement with the finding that the Rieske Fe-S protein from the yeast bc₁ complex cannot be removed without destruction of the remainder of the complex (35). In contrast, the Rieske Fe-S protein from bovine heart can be removed reversibly from the bc₁ complex, leaving the remainder of the complex intact (36, 37). These findings together with the data presented here support the view that the Rieske Fe-S protein from the yeast bc₁ com-
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plex is relatively strongly associated with the complex, and as a consequence, distortion of this subunit affects the integrity of the entire complex.

Ferroin shows an intriguing effect on the bc$_1$ complex: electron transfer is inhibited in parallel with the amount of ferroin added up to 50% at a concentration of 1 μmol of ferroin/mg of protein. However, a further increase in amount of this inhibitor does not further reduce enzyme activity. This pattern can be explained in two ways: either ferroin acts only on half of the bc$_1$ complexes, or ferroin inhibits electron transfer in all complexes by 50%. In the latter case, an effect on the reduction rate of the Rieske Fe-S cluster should be observed, since this rate is determining for the catalytic activity of the bc$_1$ complex (38, 39). The 50% maximal inhibition would thus be fortuitous. An effect on the rate determining step should be caused by either a decrease in midpoint potential of the Fe-S cluster or a reduction in the rate of deprotonation of ubiquinol upon binding of ferroin to the complex. One of the histidine ligands of the Fe-S cluster is important both for determining the midpoint potential as well as for deprotonation of ubiquinol (31). However, since the nitrogen atoms of OP, which are capable of hydrogen bonding with histidine, are not available in ferroin (see Fig. 8), it seems unlikely that this complex could cause such an effect. Furthermore, such an effect results in a decrease of the rate of cytochrome $b$ reduction via center P by a factor of 2. The pre-steady-state kinetics of $b$ reduction in the presence of antimycin would be expected to be monophasic, while a biphasic reduction is observed. Taken together, we consider this mechanism of inactivation by ferroin not very likely, although it cannot completely be excluded.

If, on the other hand, ferroin acts only on half of the bc$_1$ complexes, this effect should be considered in relation to the finding that the purified bc$_1$ complex is a structural dimer (40, 41) and the belief that this is also the case in mitochondrial membranes in vivo (42). This makes it tempting to speculate that ferroin can bind to only one of the two monomers per dimer, which implies that the maximum inhibition by 50% is an intrinsic property of the mechanism. A possible physiological function for the dimeric structure was given by Schmitt and Trumpower (42), who suggest a regulatory mechanism by which the bc$_1$ activity reversibly can be reduced to 50% by temporarily “silencing” one of the two monomers. This mechanism thus would permit regulation of the rate of respiration at the level of the bc$_1$ complex. Such a mechanism requires that the two monomers are not completely identical and that small structural differences must exist.

Attractive as this proposal is, this mechanism of inactivation by ferroin can only be fitted with the data presented here if redox equilibration occurs, i.e. the two $b_{558}$ cytochromes in a dimer are electrically connected. Nieboer and Berden (43) concluded that the bc$_1$ dimer must consist of electrically interacting protomers to account for the inhibitor titrations found when using a combination of antimycin, myxothiazol, and dicyclohexylcarbodiimide. In addition, the preliminary structure of the bc$_1$ complex from bovine heart shows that the distance between two $b_{558}$ cytochromes in a dimer is only 21 Å, the same distance as between the two $b$ cytochromes within one monomer (44). If indeed electron transfer between the two protomers can occur, allowing redox equilibration, the reduction kinetics found here support a model in which ferroin inactivates one of the monomers in the bc$_1$ dimer, in analogy with the half-of-the-sites-reactivity mechanism postulated by Schmitt and Trumpower (42). The biphasic reduction behavior of cytochrome $b$ in the presence of antimycin after ferroin inactivation (see Fig. 6) does fit with such a mechanism. The first phase in this model would correspond to reduction of cytochrome $b$ of the monomer unaffected by ferroin; the second, slower phase, would be indicative for the redox equilibration with cytochrome $b$ in the other, ferroin-inhibited, monomer.

We do not fully understand why the second phase of the pre-steady-state reduction of cytochrome $b$ without any inhibitor present and after ferroin inactivation is so much reduced (see Fig. 6). However, it should be noted that it is not certain whether the main contribution of this second phase is via center N or center P.

The rate of inhibition of bc$_1$ activity by OP is dependent on the incubation temperature and on the state (membraneous versus soluble) of the bc$_1$ complex. This rate can be quantitated using the half-life, which thus provides an indication of the structural stability of the complex. It can be used to study further the effect of different conditions (ionic strength, pH, temperature) or different detergents on this respiratory chain complex. Another possible application is the analysis of the stability of mutant complexes relative to the wild type enzyme.

Since the inactivation rate by OP is dependent on accessibility of the complex to the agent, mutations causing structural modifications should result in reduced stability, indicated by a reduced half-life. We have used this method in a study on bc$_1$ complexes containing mutated versions of subunit VIII and indeed found reduced half-lives,2 thus confirming the applicability of this method.

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