A Common Mechanism for the Interaction of Nitric Oxide with the Oxidized Binuclear Centre and Oxygen Intermediates of Cytochrome c Oxidase

Jaume Torres, Chris E. Cooper, and Michael T. Wilson†
From the Department of Biological Sciences, University of Essex, Wivenhoe Park, CO4 3SQ Colchester, Essex, United Kingdom

The reactions of nitric oxide (NO) with fully oxidized cytochrome c oxidase (O) and the intermediates P and F have been investigated by optical spectroscopy, using both static and kinetic methods. The reaction of NO with O leads to a rapid (≈100 s⁻¹) electron ejection from the binuclear center to cytochrome a and CuA. The reaction with the intermediates P and F leads to the depletion of these species in slower reactions, yielding the fully oxidized enzyme. The fastest optical change, however, takes place within the dead time of the stopped-flow apparatus (≈1 ms), and corresponds to the formation of the F intermediate (580 nm) upon reaction of NO with a species that we postulate is at the peroxide oxidation level. This species can be formulated as either Fe³⁺⁺ = O Cu₂⁺⁰ or Fe⁴⁺⁺ = O Cu³⁺³⁺, and it is spectrally distinct from the P intermediate (607 nm). All of these reactions have been rationalized through a mechanism in which NO reacts with CuB⁻²⁻, generating the nitrosyl species CuB⁻¹⁺⁻⁰⁻, which upon hydration yields nitrous acid and CuB⁺⁻. This is followed by redox equilibration of CuB with Fe₅/Cu₅ or Fe₆a₃ (in which Fe₅ and Fe₆a₃ are the iron centers of cytochromes a and a₃, respectively). In agreement with this hypothesis, our results indicate that nitrite is rapidly formed within the binuclear center following the addition of NO to the three species tested (O, P, and F). This work suggests that nitrosylation at Cu₅⁻²⁻ instead of at Fe₆a₃⁻²² is a key event in the fast inhibition of cytochrome c oxidase by NO.

Cytochrome c oxidase (ferrocytochrome c oxidoreductase, EC 1.9.3.1), the terminal enzyme in the mitochondrial respiratory chain, catalyzes the reduction of molecular oxygen to water (1). This process is coupled to proton translocation across the inner membrane. The enzyme contains four redox-active centers. Electron entry from cytochrome c, the natural substrate, occurs via a diatomic copper center, CuA. After rapid equilibrium with Fe₅⁺⁻¹, the electron is transferred to CuB and Fe₆a₃⁻⁺⁻²⁺, which together constitute the binuclear center, where oxygen is reduced.

Although oxygen binds with low affinity (10³ M⁻¹) to reduced Fe₆a₃⁻⁻, rapid electron transfer from the two reduced metals comprising the binuclear center to molecular oxygen ensures that oxygen remains bound as a peroxy species. In this way, oxygen is kinetically trapped and further reduction can take place (3, 4). The successive steps leading to the formation of water have been studied by different spectroscopic techniques (see Ref. 5 for review). The spectral signatures of two of these intermediates, which exhibit bands at 607 and 580 nm in the difference spectrum with respect to the oxidized enzyme, were first reported by Wikström (6), by reversing the electron transfer reaction, and further characterized by Wikström and Morgan (7). These authors assigned the spectral signatures at 607 and 580 nm to a ferric peroxo (P) and ferryl oxo (F) species, respectively. These assignments, however, have been challenged by a number of authors (8–10). For example, Resonance Raman results obtained by Proshlyakov et al. (10) suggest that the 607 nm band originates from an oxoferryl structure. On the other hand, the same authors have been unable to identify the putative peroxy species in their system in turnover sustained by H₂O₂ (11). However, irrespective of the assignments, there seems to exist a general agreement that compound F (580 nm) is a ferryl oxo species and that it is one electron more reduced than compound P (607 nm) (11, 12).

One of the ways to solve the problem of the identity of the P intermediate could perhaps be through the use of a suitable probe. The possibility that nitric oxide (NO), a powerful reversible inhibitor of cytochrome c oxidase (13–15), can be used as a probe for the binuclear center has been suggested by recent results using enzyme in slow turnover (16–18). In these experiments, an electron was ejected from the binuclear center, partially reducing cytochrome a. In addition, depletion of the intermediates P and F was also observed on mixing with NO (16, 17). As suggested in these papers, these reactions could be mediated via reaction of NO with Cu₅, as it has been shown that in addition to binding to reduced Fe₆a₃⁻⁻, NO binds to both Cu₅⁻⁻¹⁺ and Cu₅⁻⁺⁻²⁺, albeit with different affinities (19–21). On the other hand, we have suggested previously (15) that binding of NO to Cu₅⁻⁻¹⁺ could be a key to understanding the mechanism of inhibition of CeO by NO, although this hypothesis was based solely on steady-state kinetic considerations.

A mechanism describing the interaction of NO with Cu₅, leading to the partial reduction of cytochrome a, could be the reverse of that postulated for the reduction of nitrite to NO by non-heme nitrite reductases, which contain only Cu as a metal (see Ref. 22 for review). In this mechanism, depicted in Scheme 1 (solid arrows), Cu¹⁺ of nitrite reductase (A) binds nitrite, photodissociation from the fully reduced CO complex in the presence of oxygen.

© 1998 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.
Interaction of NO with Intermediates ofCcO

Scheme 1. This eventually yields nitrite and CuB

\[ \text{Cu}^{2+} + \text{NO}_2 \rightarrow \text{Cu}^{2+} \text{NO}_2 \rightarrow \text{Cu}^{2+} \text{NO}^{+} \rightarrow \text{Cu}^{2+} \text{NO}^{+} + 2\text{H}^{+} \rightarrow \text{Cu}^{2+} \text{NO}^{+} + \text{H}_2 \text{O} \rightarrow \text{Cu}^{2+} \text{NO}^{+} \] (A)

Forming a complex (B), which after abstraction of one oxygen atom gives an electrophilic nitrosium (C). This species is analogous to the nitrosium (Fe3+ NO+), detected by Fourier transform infrared spectroscopy in the heme-containing cytochrome cd1 nitrite reductase of Pseudomonas stutzeri, obtained by incubating the oxidized enzyme with NO (23). The donation of one electron from the metal gives Cu2+ NO (D), which then dissociates as NO and oxidized Cu (E).

We suggest that inCcO, NO interacts with the oxidized enzyme at the copper present in the binuclear center, CuB2−, forming a complex analogous to that shown at point D in Scheme 1. This eventually yields nitrite and CuB1− (Scheme 1, dotted arrows, E through A). As depicted in Scheme 2, the reduced CuA could be reoxidized by equilibration with the other redox centers, i.e., Fea, CuA, and Fea+. In those cases in which oxygen intermediates are present in the binuclear center, an obvious possible outcome of the internal electron transfer from CuA to Fea+ would be the transition of the oxygen intermediate to the next intermediate in the catalytic cycle, i.e., P → F or F → O, representing the oxidized binuclear center of CeC (Fe3+ + Cu5+). Alternatively, Cu3+ could bind NO, forming a relatively stable complex, Cu3+ NO. The formation of this complex could ultimately be responsible for the inhibition of the enzyme.

In this study, we have investigated in detail these reactions with a view to test the hypotheses formulated in Schemes 1 and 2. We provide evidence for the mechanisms depicted in these schemes, which are able to explain many of the features encountered in the reaction of NO withCcO.

MATERIALS AND METHODS

Cytochrome c oxidase was prepared by the method of Soulmane and Buse (24), which yields highly active enzyme (maximal turnover number, 600 s−1). The buffer used throughout was 0.1 M HEPES, 0.5% Tween 80, pH 7.4. Because of the sluggish reactivity of the binuclear center of this enzyme as preparation with CO, H2O, (used to form P and F, respectively), and NO, the enzyme was “pulsed” by reduction and reoxidation. This was necessary both to prepare the intermediates P and F and to investigate the reactivity of the oxidized enzyme with NO.

Pulsed Oxidized Enzyme O—CcO (~45 μM) was fully reduced by incubation with 1 mM sodium dithionite for 2 h at 4 °C. Then, the enzyme was reoxidized by passage through a Sephadex G-25 column equilibrated with the same buffer but containing no dithionite. To ensure that the enzyme was fully reoxidized, the column was loaded, immediately before the addition of the enzyme, with a band of 20 mM potassium ferricyanide. Formation of peroxide upon reoxidation was avoided by addition of 50 μl of 40 mM catalase to the reduced enzyme prior loading onto the column. Following this procedure, the maximum of the Soret band corresponding to the pulsed enzyme was at 423 nm, identical to the maximum observed in the oxidized enzyme as prepared. Formation of oxygen intermediates of the enzyme (e.g., peroxy), due to either incomplete reoxidation or the formation of peroxide, would induce a red shift of the Soret band. The absence of a significant shift in this band after pulsing is indicative of the absence of such oxygen intermediates. The pulsed oxidized enzyme (O) thus obtained was used to prepare the intermediates P and F.

Compounds P and F—Compound F was formed by incubating O (see above) in 1 mM H2O2, and the concentration of F was determined spectroscopically using 5800–630 nm = 5,500 M−1 cm−1 (7) relative to O. Compound P was obtained by two different methods. In the first method, P was formed when CO gas was bubbled for a short time (≤10 s) through a solution containing typically ~4 μM O (see above) as described previously (25). After about 10 min, formation of P reached a steady state, with conversion ~50% of the enzyme to this form. In the second method, the oxidized enzyme (pulsing was not necessary) was incubated with CO overnight at 4 °C to obtain the mixed valence-C0 complex. After briefly degassing the solution, the sample was exposed to an intense flash of white light in the presence of oxygen. The amount of compound P formed using this method was typically ~80% of the total enzyme, as determined using the extinction coefficient 5600–630 nm = 11,000 M−1 cm−1 (7) relative to O.

Static Experiments—In the static experiments, an aliquot of a solution of NO (30 μM of 2 mM NO) was added to 1.5 ml of a solution containing compound P (500 μM), F (580 μM), or O (fully oxidized enzyme). A difference spectrum relative to O was recorded immediately in a Cary 5E UV-Vis-NIR spectrophotometer. The amount of cytochrome c reduced was calculated as follows: 5800 (reduced minus oxidized) = 17,500 M−1 cm−1.

Stopped Flow—Compounds P, F, and O were rapidly mixed with saturated (~2 mM) or diluted solutions of NO, prepared by diluting the stock saturated solution with different volumes of anaerobic buffer, in a stopped flow apparatus (model SX-18MV Applied Photophysics, Leatherhead, UK). The spectra were collected using a photodiode array detector with a time resolution of 3.3 ms. A global fitting analysis program (Global Analysis, Applied Photophysics) using singular value decomposition of the data was used to model the reactions, the spectra of the kinetic components, and the corresponding rate constants. The spectra of the components constructed in this way were consistent with difference spectra obtained by subtracting spectra taken at different time points during the course of the reaction. The amount of CuA reduced after mixing O with NO was obtained by comparing the amplitude of the change at 830 nm with that obtained after the addition of ditionite (18).

Nitrin Oxide—Nitric oxide (NO) was obtained with a Kipp apparatus by mixing 1 mM sulfuric acid with sodium nitrite, essentially as described by Torres and Wilson (26). The NO in the stock solution (usually ~2 mM) and in the dilutions was measured with an NO electrode (Iso-NO Mark II, World Precision Instruments). The level of nitrite in the NO stock solution was also monitored (usually less than 0.1 mM) in the same electrode, by measuring the NO formed after acidification with H2SO4 in the presence of KI.

RESULTS

Static Optical Spectroscopy

Reaction of NO with Species O—Addition of NO to 0 generated a difference spectrum (Fig. 1A) that, in the Soret region, exhibited a peak at 445 nm and a trough at 430 nm. At longer wavelengths, a positive band at 605 nm was also observed. Both the positions of the bands and the relative intensities are indicative of reduction of cytochrome a (~80% of the total). These changes were accompanied by a bleached region from 620 to 660 nm.

Reactions of NO with Species F and P—Addition of NO to P or F resulted in the rapid depletion of these intermediates. The spectrum resulting from the addition of NO to P (Fig. 1B) has a peak at 415 nm and a trough at 430 nm in the Soret region, with a region from ~550 to 630 nm also bleached. From this scheme presented above (see Scheme 1, dotted arrows) predicts the formation of nitrite in the binuclear center of CeC, we tested this prediction by adding nitrite to O and comparing the final spectrum to that obtained after addition of NO to F. Fig. 1C shows that exposure of O to a high concentration of nitrite (10 mM) generated the same spectral changes as obtained when
FIG. 1. Spectral changes occurring on mixing species O, F, and P with 20 μM NO. All difference spectra were recorded with respect to O. A, addition of NO to O; B, a sample containing 80% F before (——) and after (-----) the addition of NO; C, spectra after the addition of 10 mM sodium nitrite to O (——) and after the addition of NO to species F (-----) (the visible region has been expanded (×3)); D, a sample containing ~80% P before (——) and after (-----) the addition of NO; E, comparison between the spectra (visible region) after addition of NO to the samples O (——), P (-----), and F (-----). Concentration of CcO, 4.7 μM. Other details are given under “Materials and Methods.”
NO (20 μM) was added to F. The affinity of the enzyme for nitrite is low and required a relatively high nitrite concentration to induce spectral changes of measurable amplitude. The fact that the same effect was obtained with a much lower NO concentration suggests that when NO is added to F, nitrite is formed within the binuclear center and interacts with the oxidized binuclear center (Ob) formed as a result of the conversion F → Ob.

When NO was added to P (Fig. 1D), the depletion of this intermediate was accompanied, as for O, by the appearance of bands at 605 and 442 nm, indicative of cytochrome a reduction. However, the extent of the reduction was not, as with O, 40% of the total enzyme, but rather 40% of the P present before the addition of NO (e.g., for a sample containing 50% P, the extent of cytochrome a reduction would be 20%).

The features observed when adding NO to F (Fig. 1C), assigned to the interaction of nitrite with the oxidized binuclear center, Ob, were also present after addition of NO to the samples O and P (see Fig. 1E). Indeed, the spectrum resulting after addition of NO to O or P can be shown to be a superposition of two components: (a) interaction of nitrite with Ob, and (b) the reduction of cytochrome a (i.e., transition Feₐ³⁺ → Feₐ²⁺). Accordingly, the subtraction of the spectral contribution of nitrite gives a residual identical to the difference spectrum corresponding to reduced minus oxidized cytochrome a (27) (data not shown). We postulate that NO converts P and F to Ob, and NO reacts with Ob, reducing cytochrome a (Fig. 1A). However, it is clear that we did not observe cytochrome a reduction after mixing F with NO (Fig. 1B). This was due to the presence of H₂O₂ in the sample, because when we repeated the experiment adding catalase prior to mixing F with NO, we observed reduction of cytochrome a simultaneous with the decay of F (not shown). This indicates that H₂O₂ either impedes the interaction of NO with the binuclear center or reoxidizes cytochrome a as soon as it becomes reduced. It is also worth mentioning that even though CO and H₂O₂ are still present in solution, neither P nor F were reformed after the reaction of NO with these intermediates. This is consistent with the observed sluggish reactivity of the binuclear center of CcO with H₂O₂ and with CO (which in certain conditions lead to formation of F and P, respectively; see “Materials and Methods”) after NO has been added to O (not shown).

Thus, these experiments confirm three predictions derived from Schemes 1 and 2. First, the product of the reaction of NO with O, P, or F displays a spectrum consistent with the presence of nitrite interacting with the oxidized binuclear center...
Second, the interaction of O_b with NO ejects an electron to cytochrome c oxidase containing F with NO. These reactions were fast and no time courses could be observed. We therefore performed similar experiments in a stopped flow apparatus equipped with a diode array detector.

Stopped Flow Spectrophotometry

**Reactions of Compound P with NO**

The reaction of a sample containing compound P (80%) with NO led, as expected (see Fig. 1B), to the rapid decay of this compound (Fig. 2, curves a and b). The time course was fitted to a biexponential decay, and the rates of the two phases were essentially proportional to the NO concentration (see Fig. 2 legend). Difference spectra relative to O are shown in Fig. 2.

Reactions within the Dead Time (1.4 ms)—When a sample containing a high proportion of compound P (80%) was mixed...
with NO in the stopped flow spectrophotometer, some spectral changes took place in the dead time of the apparatus (~1.4 ms), whereas the band at 607 nm remained unchanged in this time frame (Fig. 3, arrows). The final spectrum after ~1 s is consistent with that shown in Fig. 1D (static experiments), showing reduction of cytochrome $a$ and depletion of P. The difference spectrum corresponding to the changes which occurred during the dead time is given in Fig. 4A, solid line (and also in Fig. 3, inset, solid line). This spectrum (Fig. 4A, solid line) shows two peaks in the Soret region at about 425 and 445 nm and a trough around 410 nm. At longer wavelengths, a positive band at 605 nm and a shoulder at 600 nm, and a negative region centered at ~615 nm can also be observed. These features are reminiscent of the formation of compound F, possibly accompanied by some reduction of cytochrome $a$. However, both the changes in the Soret and the $a$ regions are slightly different from those expected for simultaneous formation of F and reduction of cytochrome $a$. For example, although a band at 445 nm is clearly present in the Soret region, there is no concomitant increase in the $a$ band at ~605 nm. Only a shoulder at 600 nm is observed. We can discount spectral contributions from a transition of the type P → F, because the increase at 580 nm is not paralleled by a decrease at 607 nm of an amplitude approximately twice as large (see the extinction coefficients given under “Materials and Methods”) (Fig. 4A, solid line). In addition, even in case that reduction of cytochrome $a$ (increase at 605 nm) compensates for the decrease at 607 nm, the change at 445 nm in the Soret region (which is 3 times larger in amplitude than at 605 nm; see Fig. 1A) would have to be about twice as large than observed in Fig. 4A.

Thus, a transition of the type O → F seems more plausible, except that the peak in the Soret region, which should appear at ~435 nm (9), appears to be shifted to 425 nm. These differences indicate that other changes, possibly attributable to the effect of nitrite (see Fig. 1C), distort the composite spectrum arising from the formation of compound F and the reduction of cytochrome $a$. In fact, when the contribution of the interaction of nitrite with O$_b$ (Fig. 4A, dotted line) was subtracted, the spectral features conformed more closely to those expected from the transitions O → F and Fe$_a^{3+}$ → Fe$_a^{2+}$ (Fig. 4B). Further, when the contribution of the reduction of cytochrome $a$ was also subtracted (see Fig. 4 legend for details), the resulting spectrum was very similar to that of F relative to O (see Fig. 1B, solid line). This analysis reveals that when NO is mixed
with samples containing P, spectral changes occur within the dead time of the stopped flow spectrometer consistent with the following events: (a) formation of species F from a species spectrally similar to O, not from P, (b) formation of nitrite within the binuclear center, and (c) reduction of some cytochrome a.

When P was generated from mixed valence-CO enzyme (≈80% P), the percentage of compound F formed in the dead time was only 20% of the total enzyme. However, when P was generated by bubbling CO in the presence of oxygen (≈40% P), the percentage of F generated was larger (40–50%) (28). This indicates that the precursor of F is not P itself. This finds further support from experiments in which compound P, generated from the mixed valence-CO preparation, was allowed to decay at room temperature (not shown) prior to mixing with NO. Addition of NO after a long period (≈1 h) produced a larger fraction of F formed (30–40%) in the dead time. Only after about 2 h was a fast partial reduction of cytochrome a (k ≈ 100 s⁻¹), which corresponded to 20% of the enzyme (not shown), also observed, consistent with a proportion of the molecules having returned to O. This shows that the species absorbing at 607 nm does not decay directly to O but rather to another species that is O-like in spectral properties but that generates F rapidly on mixing with NO.

Reactions Following the Dead Time—Following the dead time, species P (607 nm) and the newly formed species absorbing at 580 nm decayed simultaneously (Fig. 5), with a rate that was found to depend on the NO concentration (Fig. 5, inset). These changes were complete in ≈800 ms at 1 mM NO, and together with those changes observed in the Soret region, they are compatible with the decay of compounds P and F. The amplitude of the decrease at 580 nm from 1.4 to 800 ms was found to be identical, within our experimental error, to the amplitude of the increase observed within the dead time (Fig. 3, inset). This indicates that the compound P that decays from 1.4 to 800 ms corresponds to that formed within the dead time. The amplitude of the decrease at 607 nm, however, was smaller than the corresponding amplitude of the P present before mixing (Fig. 3). In fact, one of the troughs in Fig. 5 is centered at 612 nm, not at 607 nm. This apparent shift can be explained by a simultaneous decrease at 607 nm and an increase at 605 nm, which, together with the presence of a positive band at 442 nm in the difference spectrum, indicates that some reduction of cytochrome a takes place simultaneously with the decay of the P and F intermediates. Accordingly, the reduction of cytochrome a and the decay of P and F resulted in a single kinetic transition in the global analysis (see "Materials and Methods").

Reactions of O with NO

As for compound P, when NO was mixed with O some changes occurred within the instrumental dead time (<1.4 ms) (Fig. 6A). The difference spectrum recorded (t = 1.4 ms minus t = 0 ms) is compatible with the appearance of approximately equal percentage (≈15%) of reduced cytochrome a and compound F. Although the reduction of cytochrome a observed may be expected (in ≈1.4 ms, a process with k = 100 s⁻¹ completes ≈15% of its total amplitude), the formation of F was not anticipated. We attribute the formation of F to heterogeneity in the sample, suggesting that preparations of O (after reduction and reoxidation; see "Materials and Methods") contain a small proportion of the O-like species described above. After the dead time, about 40% of cytochrome a became reduced (k = 100 s⁻¹), as indicated by bands at 442 and 605 nm (intensity ratio, 3:1) (Fig. 6B), in agreement with the results obtained in the static spectral experiments (Fig. 1A). Reduction of cytochrome a was accompanied by reduction of 15–20% Cu₆₃ (not shown) (18).

In a slower phase (Fig. 6C), a decay at 580 nm and a simultaneous increase at 605 nm was also observed (k = 8 s⁻¹). Both these changes and those detected in the Soret region are indicative of simultaneous decay of F (formed in the dead time) and reduction of cytochrome a. This slow process is similar to that seen when mixing P and NO, when reduction of cytochrome a

Because the spectra of P and reduced cytochrome a are similar in the a band (P has a band at 607 nm and cytochrome a at 605 nm) the simultaneous decay of P and a smaller increase of reduced cytochrome a led only to a small perturbation in this region (see extinction coefficients under "Materials and Methods").

Other authors have previously suggested the presence of heterogeneities even in "fast" oxidase preparations, based on the presence of two phases in the kinetics of ligand binding (32), especially formate.
accompanied the decay of the bands corresponding to P (607 nm) (Fig. 5). This can be clearly seen in Fig. 7, where the difference spectrum produced 50 ms after mixing O with NO (i.e. at the end of the process with $k = 100 s^{-1}$) (see Fig. 6B), is compared with the difference spectrum of the final product (after ~800 ms) obtained when mixing P with NO (i.e. following...
the process with \( k \sim 8 \text{ s}^{-1} \) (from Fig. 3). It is clear from this figure that the same optical change is obtained in both cases, regardless of the initial species (P or O), although with different kinetics. Together with the results in Fig. 6C, these results indicate that the reduction of cytochrome \( a \) is rate limited by the decay of P or F when these oxygen intermediates are present.

DISCUSSION

The results presented above may be rationalized by reference to a simplified reaction mechanism presented in Scheme 3. The basic feature of this scheme is that the different reactions, observed upon mixing NO with derivatives of CeO, can all be explained by a single electron donation from NO to the binuclear center. Each step in Scheme 3 comprises a sequence of events analogous to those indicated by dotted arrows in Scheme 1, i.e. NO binds to CuO\(^{2+}\) and results in the formation of HNO\(_2\) and reduction of CuB. The latter would permit electron transfer from CuB either to cytochrome \( a/CuA \) or to oxygen intermediates bound to Fe\(_{56}\). A detailed reaction mechanism is given in Scheme 4, in which the two parts, A and B, differ in the chemical assignment of P. We have assumed in part A that the species P (607 nm) and F (580 nm) are the ferric peroxy and oxoferryl derivatives of Fe\(_{56}\), respectively, as suggested previously by Wikström and Morgan (7).

Because we propose that NO provides the binuclear center with one reducing equivalent, it follows that the precursor of the F formed during the dead time (spectrally O-like and labeled X in Scheme 4) is at the formal oxidation level of peroxide. Therefore, if P (PM) is the ferric peroxy species, then X could contain either CuB\(^{3+}\), with the iron as a ferryl oxo species (Fe\(^{5+}\) = O), or alternatively as CuB\(^{2+}\), with Fe\(^{6+}\) = O (see Scheme 4). The presence of either of these species, suggested previously by other authors (9–12, 29, 30), would explain the fast formation of compound F solely by reduction of CuB\(^{3+}\) to CuB\(^{2+}\) or Fe\(^{6+}\) to Fe\(^{5+}\). Our data suggest that PM coexists with species X, possibly in an equilibrium, because they are at the same oxidation level.

An analogous and equally fast reaction may be expected to occur between NO and P\(_M\), generating a peroxy species with CuB reduced. Such a species, postulated previously, has been termed P\(_F\) in the literature (31). The conversion from P\(_M\) to P\(_R\) would not result in observable optical changes, because these species have similar spectra (31). This is in agreement with our results, because the band at 607 nm corresponding to P remains unchanged on addition of NO during the dead time (Fig. 3). Thus, after mixing a sample containing P (PM and X) with NO, we obtained two different products: one was P\(_R\), from the transition PM \( \rightarrow \) PR, and the other was F, from the transition X \( \rightarrow \) F. The mechanism in Scheme 4 accounts, therefore, for the spectral changes that occur within the dead time after mixing P with NO, namely, the apparent nonreactivity of P\(_M\) (Fig. 5) and the formation of F from a species spectrally similar to O.

Furthermore, our results show that the species F (absorbing at 580 nm) and P\(_R\) (assumed to absorb at 607 nm) (31) decay simultaneously after mixing with NO (Fig. 5). This suggests that they are in rapid equilibrium, one of them reacting with NO and recruiting the other into the reactive form. From our experiments (Fig. 1B), it is clear that it is compound F that reacts with NO yielding O, and we thus incorporated this into Scheme 4. Thus, P\(_R\) would react not with NO per se, but through an equilibrium established between P\(_R\) and F. For the reaction between F and NO, a reaction analogous to that described for PM and species X was suggested, after which, an internal electron transfer from CuB could facilitate the transi-

---

\(^{4}\) P\(_R\) is the form obtained after photolysis, in the presence of oxygen, of the Fe\(_{56}\)–CO bond in CO-bound fully reduced oxidase. In this case, the second reducing equivalent for oxygen is provided directly from cytochrome \( a \), and not by CuB, which remains reduced. In contrast, the species obtained from CO-bound mixed valence enzyme, in which the second reducing equivalent for oxygen is provided through oxidation of CuB, is called P\(_M\) (31).
tion $F \rightarrow O_\delta$. Support for the role of $Cu_\delta$ in this reaction comes from the fact that although $F$ is known to react with CO (33) and other ferryl groups (e.g., in myoglobin) react with NO (34), these processes are much slower than those presented here. After the formation of species $O$, a further electron donation from NO would then lead to the observed reduction of cytochrome $a$. In agreement with this model, our results show that this process is fast when mixing $O$ with NO ($k = 100$ s$^{-1}$) but is rate-limited by the decay of $P$ and $F$ ($-8$ s$^{-1}$) when NO reacts with these intermediates. This is expected if the substrate for this reduction ($O_\delta$) is the product of the reaction of $P$ and $F$ with NO, as Scheme 4 shows.

**The Identity of Compound X**—It seems generally agreed that $F$ (580 nm) has an oxo ferryl structure, containing $Cu_\delta$, but the assignment of $P$ (607 nm) is more problematic. Basically, the discussion in the literature revolves around whether the oxygen $O-O$ bond can be broken following addition of only two electrons to the enzyme. This could be achieved by means of an additional electron, recruited from elsewhere in the protein, to produce an oxo ferryl derivative. Four main sources for this electron have been suggested, i.e., the porphyrin ring, an amino acid, the copper atom $Cu_\delta$, or the iron atom itself. The donation of an electron from the porphyrin ring or an amino acid, leading to the formation of a radical, as observed in other enzymes (e.g., peroxidases and catalases), seems to be unlikely (9, 29, 31), but there is no need for reversible $O-O$ cleavage. Clearly, this equilibrium implies that the splitting of the $O-O$ bond is reversible in these conditions, and this poses a problem from the thermodynamic point of view.

This problem can be avoided as shown in Scheme 4 (part $B$), where $P_M$ and $P_R$ are assigned the structures $Fe^{5+} = O$ with $Cu_{\delta}^{2+}$ and $Cu_{\delta}^{1+}$, respectively, instead of ferric peroxide species. In this case, $P_R$ and $F$ are also in the same oxidation state, but there is no need for reversible $O-O$ cleavage. Clearly, this model lacks the ferric peroxide form, in contradiction of previous assignments and observations made using synthetic model compounds in which a peroxo species was detected during the reduction of oxygen to water (41).

The results presented indicate that a species $X$ spectrally similar to $O$ is in equilibrium with the 607 nm species in the sample containing compound $P$. In our view, this could clarify some of the apparent inconsistencies concerning the structural assignments of the intermediates $P$ and $F$. For example, the cytochrome iron structure in compound $P$ has been described either as a ferric peroxo or an iron oxo ($Fe^{4+} = O$ or $Fe^{5+} = O$) by different authors (see references under “Introduction”). This discrepancy could be explained if a ferric peroxo and an iron oxo (either $Fe^{4+} = O$ or $Fe^{5+} = O$, as shown in Scheme 4, part

---

**Scheme 4**

![Scheme 4](image.png)
mechanism by which inhibition could take place is suggested as a consequence rather than a cause of the inhibition. A possible real inhibitory site. Alternatively, NO may transfer rapidly by NO for CcO—

Our finding that nitrite is rapidly formed in the interaction of NO with Intermediates of CcO

4. Verkhovsky, M. I., Morgan, J. E., and Wikström, M. (1996) Biochim. Biophys. Acta 1223–12239

5. Ferguson-Miller, S., and Babcock, G. T. (1996) FEBS Lett. 354, 50–54

6. Weng, L. C., and Baker, G. M. (1991) Biochemistry 30, 5720–5725

7. Wikström, M., and Morgan, J. E. (1992) J. Biol. Chem. 267, 10266–10273

8. Weng, L. C., and Baker, G. M. (1991) Biochemistry 30, 5720–5725

9. Fabian, M., and Palmer, G. (1995) Biochemistry 34, 13802–13810

10. Proshlyakov, D. A., Ogura, T., Shizawa-Itoh, K., Yoshikawa, S., Appelman, E. H., and Kitagawa, T. (1996) Biochemistry 35, 8580–8586

11. Blackmore, R. S., Greenwood, C., and Gibson, Q. H. (1991) J. Biol. Chem. 266, 19245–19249

12. Verkhovsky, M. I., Morgan, J. E., and Wikström, M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12235–12239

13. Cleeter, M. W. J., Cooper, J. M., Darley-Usmar, V. M., Moncada, S., and Schapira, A. H. V. (1994) FEBS Lett. 354, 50–54

14. Brown, G. C., and Cooper, C. E. (1994) FEBS Lett. 356, 295–298

15. Torres, J., Darley-Usmar, V. M., and Wilson, M. T. (1995) Biochem. J. 312, 169–173

16. Torres, J., Cooper, C. E., and Wilson, M. T. (1996) Biochem. Soc. Trans. 24, 4505

17. Wilson, M. T., Torres, J., Cooper, C. E., and Sharpe, M. (1997) Biochem. Soc. Trans. 25, 905–909

18. Cooper, C. E., Torres, J., Sharpe, M. A., and Wilson, M. T. (1997) FEBS Lett. 414, 281–284

19. Stevens, T. H., Brudwig, G. W., Bocian, D. F., and Chan, S. I. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 3320–3325

20. Brudwig, G. W., Stevens, T. H., and Chan, S. I. (1980) Biochemistry 19, 5275–5285

21. Zhao, J. X., Sampath, V., and Caughey, W. S. (1994) Biochem. Biophys. Res. Commun. 204, 537–543

22. Averill, B. A. (1996) Chem. Rev. 96, 2951–2964

23. Yang, Y., and Averill, B. A. (1996) J. Am. Chem. Soc. 118, 3972–3973

24. Weng, L. C., and Baker, G. M. (1991) Biochemistry 30, 5720–5725

25. Nicholls, P., and Chanady, G. A. (1981) Biochem. Biophys. Acta 634, 256–265

26. Torres, J., and Wilson, M. T. (1996) Methods Enzymology 260, 3–11

27. Liao, G. L., and Palmer, G. (1996) Biochim. Biophys. Acta 1274, 109–111

28. Torres, J., and Wilson, M. T. (1997) Biochem. Soc. Trans. 25, 4028

29. Ogura, T., Hirota, S., Proshlyakov, D. A., Shizawa-Itoh, K., Yoshikawa, S., and Kitagawa, T. (1996) J. Am. Chem. Soc. 118, 5443–5449

30. Babcock, G. T. (1996) Biochim. Biophys. Acta 1223–12239

31. Cleeter, M. W. J., Cooper, J. M., Darley-Usmar, V. M., Moncada, S., and Schapira, A. H. V. (1994) FEBS Lett. 354, 50–54

32. Brown, G. C., and Cooper, C. E. (1994) FEBS Lett. 356, 295–298

33. Torres, J., Darley-Usmar, V. M., and Wilson, M. T. (1995) Biochem. J. 312, 169–173

34. Torres, J., Cooper, C. E., and Wilson, M. T. (1996) Biochem. Soc. Trans. 25, 905–909

35. Morgan, J. E., Verkhovsky, M. I., and Wikström, M. (1996) Biochemistry 35, 12235–12240

36. Moody, A. J., Cooper, C. E., and Rich, P. R. (1991) Biochim. Biophys. Acta 1059, 189–207

37. Proshlyakov, D. A., Ogura, T., Shizawa-Itoh, K., Yoshikawa, S., Appelman, E. H., and Kitagawa, T. (1996) Biochemistry 35, 76–82

38. Varotsis, C., Zhang, Y., Appelman, E. H., Babcock, G. T. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 237–241

39. Varotsis, C., and Babcock, G. T. (1995) J. Am. Chem. Soc. 117, 11260–11269

40. Sucheta, A., Georgiadis, K. E., Einsardtöter, O. (1997) Biochemistry 36, 554–565

41. Collman, J. P., Fu, L., Herrmann, P. C., and Zhang, X. (1997) Science 275, 948–951

42. Giuffre, A., Sarti, P., D’Itti, E., Base, G., Soulimane, T., and Brunori, M. (1996) J. Biol. Chem. 271, 33404–33408

43. Borutaite, V., and Brown, G. C. (1996) Biochem. J. 315, 295–299

44. Proshlyakov, D. A., Ogura, T., Shizawa-Itoh, K., Yoshikawa, S., and Kitagawa, T. (1996) Biochemistry 35, 8580–8586

45. Morgan, J. E., Verkhovsky, M. I., and Wikström, M. (1996) J. Biol. Chem. 271, 1843–1852

46. Proshlyakov, D. A., Ogura, T., Shizawa-Itoh, K., Yoshikawa, S., and Kitagawa, T. (1996) Biochemistry 35, 76–82

47. Varotsis, C., Zhang, Y., Appelman, E. H., Babcock, G. T. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 237–241

48. Varotsis, C., and Babcock, G. T. (1995) J. Am. Chem. Soc. 117, 11260–11269

49. Sucheta, A., Georgiadis, K. E., Einsardtöter, O. (1997) Biochemistry 36, 554–565

50. Collman, J. P., Fu, L., Herrmann, P. C., and Zhang, X. (1997) Science 275, 948–951

51. Giuffre, A., Sarti, P., D’Itti, E., Base, G., Soulimane, T., and Brunori, M. (1996) J. Biol. Chem. 271, 33404–33408

52. Borutaite, V., and Brown, G. C. (1996) Biochem. J. 315, 295–299

53. Blackmore, R. S., Greenwood, C., and Gibson, Q. H. (1991) J. Biol. Chem. 266, 19245–19249