Reaction of Nitric Oxide with the Turnover Intermediates of Cytochrome *c* Oxidase: Reaction Pathway and Functional Effects†

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ABSTRACT: The reactions of nitric oxide (NO) with the turnover intermediates of cytochrome *c* oxidase were investigated by combining amperometric and spectroscopic techniques. We show that the complex of nitrite with the oxidized enzyme (**O**) is obtained by reaction of both the "peroxy" (**P**) and "ferryl" (**F**) intermediates with *stoichiometric* NO, following a common reaction pathway consistent with **P** being an oxo-ferryl adduct. Similarly to chloride-free **O**, NO reacted with **P** and **F** more slowly [$k \approx (2-8) \times 10^4$ M^{-1} s^{-1}] than with the reduced enzyme ($k \approx 1 \times 10^8$ M^{-1} s^{-1}). Recovery of activity of the nitrite-inhibited oxidase, either during turnover or after a reduction-oxygenation cycle, was much more rapid than nitrite dissociation from the fully oxidized enzyme ($t_{1/2} \approx 80$ min). The anaerobic reduction of nitrite-inhibited oxidase produced the fully reduced but uncomplexed enzyme, suggesting that reversal of inhibition occurs in turnover via nitrite dissociation from the cytochrome a_3-Cu_B site: this finding supports the hypothesis that oxidase may have a physiological role in the degradation of NO into nitrite. Kinetic simulations suggest that the probability for NO to be transformed into nitrite is greater at low electron flux through oxidase, while at high flux the fully reduced (photosensitive) NO-bound oxidase is formed; this is fully consistent with our recent finding that light releases the inhibition of oxidase by NO only at higher reductant pressure [Sarti, P., et al. (2000) *Biochem. Biophys. Res. Commun. 274,* 183].

Nitric oxide (NO) is a signaling molecule involved in many biological processes [smooth muscle relaxation, inflammation, neurotransmission, apoptosis, etc. (*1*)]. This versatile second messenger is enzymatically produced by the NO-synthase(s) also in the mitochondrion (*2*), where it attacks a number of targets, including cytochrome *c* oxidase (COX)[1] (see refs *3* and *4* for reviews). The interaction with COX leads to an efficient but reversible inhibition of cell respiration, which may have patho-physiological relevance also in view of the recent finding that NO endogenously produced in cultured cells controls mitochondrial energization even in the absence of exogenous stimuli (*5*).

Cytochrome *c* oxidase, the terminal enzyme of the respiratory chain, catalyzes the reduction of oxygen to water, a reaction coupled to proton translocation across the inner mitochondrial membrane (see refs *6* and *7* for reviews). Two of the four redox centers of COX, Cu_A and cytochrome *a*, are involved in delivering the electrons donated by cytochrome *c* to the cytochrome a_3-Cu_B binuclear site, which binds O_2, CO, and NO in the reduced state. A number of species have been identified during the reaction with O_2 and characterized by time-resolved Resonance Raman spectroscopy (*8*, *9*, and see ref *10* for a review): beside the extreme species, having both metals (a_3-Cu_B) either in the oxidized (**O**) or in the reduced (**R**) state, other intermediates with only one electron in the binuclear site (**E**) or with partially reduced oxygen bound [so-called "peroxy" (**P**) and "ferryl" (**F**)] have been characterized. Recently, the term peroxy for the **P** intermediate was found to be incorrect, since evidence was provided that **P** is an oxoferryl adduct (*11*−*14*) stabilized by a nearby protein radical, presumably on Tyr-244 (*15*); the latter residue, required for catalysis (*16*), is cross-linked to one of the Cu_B ligands, His-240, as revealed by crystallographic (*17*−*18*) and peptide sequencing data (*19*).

Two general problems concerning the interaction of NO with COX are under active investigation: (i) the mechanism of inhibition by NO (*20*−*21*) and, perhaps equally important from a physiological viewpoint, (ii) the involvement of COX in the metabolism of NO via a reductive or an oxidative pathway. In this context, we showed that eukaryotic COX is unable to catalyze the reduction of NO to N_2O under reducing anaerobic conditions (*22*), contrary to *Thermus thermophilus* oxidases (*23*). To be fully addressed, however, these issues require a detailed characterization of the reaction of NO with the catalytic intermediates of COX, and this is basically the object of this study.

Until 1997, fully reduced COX (**R**) was the only species known to react rapidly with NO by binding to ferrous cytochrome a_3 very rapidly [$k = 1 \times 10^8$ M^{-1} s^{-1} (*24*)], and with high affinity ($K_d = 2.5 \times 10^{-11}$ M, at 20 °C). The scenario changed when it was discovered that NO can readily react also with oxidized Cu_B^{2+} in the **O** species (*25*), provided the enzyme was freed from Cl^- (*26*); this reaction is much

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[1] Abbreviations: COX, cytochrome *c* oxidase; Hb, human hemoglobin.

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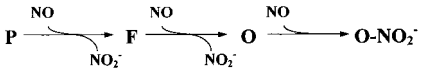
Scheme 1



slower ($k = 2 \times 10^5$ M^{-1} s^{-1}) than combination of NO to ferrous cytochrome a_3 and involves donation of 1 electron from NO, with conversion of NO into NO^+ and thereby NO_2^- by reaction with OH^-. Wilson and co-workers (*27*) reported that NO also reacts with **P** and **F** and suggested the overall reaction shown in Scheme 1.

This model is of general interest for cell physiology because it implies a specific role of COX in the oxidative degradation of NO into harmless nitrite. All together, the above findings have suggested that two different inhibition pathways may exist, one based on the formation of the cytochrome a_3^{2+}-NO derivative, the other on the accumulation of the nitrite-bound enzyme. In addition, experimental evidence has been very recently collected showing that these two inhibition mechanisms can both be operative, depending on the electron pressure on COX (*28*). With the aim of fully understanding the mechanism(s) by which COX is inhibited by NO, we have investigated the reaction of NO with the catalytic intermediates of COX by spectroscopic and amperometric techniques. We provide evidence that, contrary to prediction of Scheme 1, the reaction of either **P** or **F** with stoichiometric NO leads to the nitrite-bound COX in the oxidized inhibited state. A modified version of Scheme 1 is therefore proposed, based on the recently discovered identity of **P** as an oxo-ferryl adduct (*11*−*14*). Since we show that nitrite is a reversible inhibitor of oxidase, the possible physiological relevance of these reactions is also discussed.

EXPERIMENTAL PROCEDURES

Materials. Horse heart cytochrome *c*, ascorbate, ruthenium(III) hexamine, nitrite, and EDTA were from Sigma (St. Louis, MO); dodecyl-β-D-maltoside from Biomol (Hamburg, Germany); hydrogen peroxide from Merck (Darmstadt, Germany). Concentration of hydrogen peroxide was determined using the extinction coefficient $\epsilon_{240} = 40$ M^{-1} cm^{-1}. Stock solutions of NO (Air Liquide, Paris, France) were prepared equilibrating degassed water with the pure gas at 1 atm and at 20 °C: the concentration of NO in solution was 2.1 ± 0.1 mM. Experiments were carried out in 0.1 M K^+/HEPES pH 7.3 + 0.1% dodecyl-β-D-maltoside and at 20 °C (unless otherwise stated).

Cytochrome *c* oxidase was purified from beef heart according to Soulimane and Buse (*29*). Prior to use, Cl^- was removed by chromatography on a G25 column at 4 °C immediately after dithionite reduction and subsequent reoxygenation of COX in the presence of 1 mM ferricyanide (see ref *26*): the resulting chloride-free oxidized enzyme is called **O** throughout. The enzyme concentration was determined using $\Delta\epsilon_{444(red-ox)} = 156$ mM^{-1} cm^{-1} and is expressed as a functional unit (aa_3). Human hemoglobin, used in some experiments as a NO scavenger, was purified according to ref *30*; its concentration was determined spectrophotometrically ($\epsilon_{555} = 12.5$ mM^{-1} cm^{-1} in the deoxygenated state) and is expressed on the heme basis. The concentration of reduced cytochrome *c* was measured using $\Delta\epsilon_{550(red-ox)} = 18.7$ mM^{-1} cm^{-1}. Static spectra were recorded with a double-beam spectrophotometer (Jasco V-570) with a light path of 1 cm.

*Formation of **P** and **F**.* Formation and stability of these species were assessed spectrophotometrically. **P**[2] was generated by oxygenation under intense illumination of the mixed valence−CO enzyme (*31*), as obtained by anaerobic incubation of COX under 1 atm CO for >3 h. The yield of **P** was determined using the extinction coefficient $\Delta\epsilon_{(607-630)} = 11$ mM^{-1} cm^{-1} relative to **O** (*32*) and was always ~90%. In HEPES 0.1 M, pH 7.3, containing 0.1% (w/v) dodecyl-β-D-maltoside, **P** decays to the **O** state with a half time of ~15 min.

F was formed by addition of 1 mM H_2O_2 to **O**; the yield of **F** (about 70−80%) was determined using the extinction coefficient $\Delta\epsilon_{(580-630)} = 4$ mM^{-1} cm^{-1} relative to **O** (*33*). Addition of 50 nM catalase to rapidly scavenge free H_2O_2, prevents the production of **P** occurring at low H_2O_2 concentration (*33*), and allows the observation of the **F** → **O** decay, with a half time ~4 min.

When necessary, COX samples were made anaerobic by diluting (≥10-fold) concentrated (air-equilibrated) **O**, **P**, and **F** in a N_2-equilibrated buffer; the final solution was then rapidly degassed and further N_2-equilibrated. As assessed spectrophotometrically, dilution of COX down to 200 nM did not change the redox state of the enzyme.

Time-Resolved Spectroscopy. Stopped-flow experiments were carried out either collecting entire spectra with a Durrum-Gibson instrument equipped with a diode-array (TN6500, Tracor Northern, Madison, WI) or following time courses at single wavelength (DX.17MV, Applied Photophysics, Leatherhead, U.K.). The diode array has a light path of 2 cm and can acquire up to 80 spectra of 1024 elements with an acquisition time of 10 ms/spectrum. The single-wavelength instrument (light path = 1 cm) can work in a sequential mixing mode, allowing two sequential mixing events with a pre-set delay time in between. Curve fitting was performed with the software MATLAB (MathWorks) and analysis of time-resolved spectra with the singular value decomposition (SVD) algorithm (*34*). Experimental spectra were fitted to linear combinations of reference spectra by using the pseudoinverse algorithm.

NO Electrode. Amperometric measurements of NO in solution have been performed under anaerobic conditions with a Clark-type electrode (ISO-NO, World Precision Instruments, U.K.). The electrode was calibrated with subsequent additions of NO-saturated water and, after injection of COX in the different states (**O**, **P**, or **F**), the NO concentration in the reaction chamber was monitored as a function of time.

Kinetic Simulations. Kinetic simulations were carried out using a differential equations solver algorithm implemented by Dr. Eric Henry (National Institutes of Health, Bethesda, MD) running on a Silicon Graphics work station.

RESULTS AND DISCUSSION

We have extended previous investigations on the reaction between the turnover intermediates of COX and NO (*27*), by exploring the effect on enzymatic activity. In particular, we attempted to answer the following questions: (i) what is the stoichiometry, the kinetics and the product of the reaction of the intermediates **P** and **F** with NO, and (ii) do these

[2] This intermediate is actually called $\mathbf{P_M}$ (see ref *7* for details).

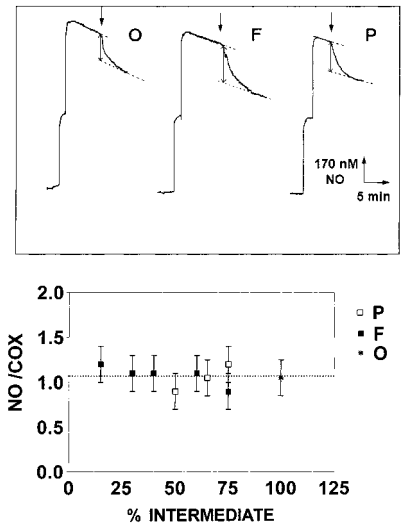


FIGURE 1: Stoichiometry of the reaction of NO with the O_2-intermediates. Amperometric measurements. (Top) 2 × 0.5 μM NO is added to the reaction chamber followed by anaerobic addition of 150 nM **O**; 200 nM **F**, and 150 nM **P**. In each case approximately stoichiometric (1:1) NO disappears from solution after enzyme addition. (Bottom) The above measurements are repeated over the decay time of either **P** or **F** (both plus and minus catalytic amounts of catalase). The apparent stoichiometry observed in the reaction with NO is plotted as a function of the occupancy of each intermediate at the time of the addition to the NO solution. Occupancy of COX intermediates has been determined spectrophotometrically in parallel with amperometric measurements. Despite their decreasing occupancies both **P** and **F** react with NO according to a 1:1 stoichiometry, similarly to **O** (*26*).

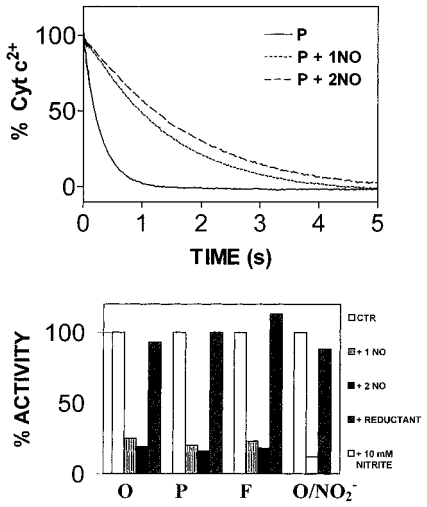


FIGURE 2: Stoichiometric NO inhibits **O**, **P**, and **F**. (Top) The activity of 1 μM COX in the **P** state (before and after addition of NO) is measured by mixing in the stopped-flow apparatus with 20 μM cytochrome c^{2+} (plus 6 μM oxy-Hb to scavenge free NO, if present). In the absence of NO, cytochrome c^{2+} is oxidized at $k' = 3.5\ s^{-1}$ as monitored at 550 nm. When stoichiometric NO is added to **P** (~5 min after its formation), the activity drops by a factor of 5 ($k' = 0.7\ s^{-1}$), and further addition of NO has very little additional effect ($k' = 0.55\ s^{-1}$). (Bottom) The experiment shown in the top panel is repeated starting with either **O** or **F**. For each intermediate, addition of either stoichiometric (1:1) or super-stoichiometric (2:1) NO yields a 5−6-fold inhibition of COX, which is fully reverted by reduction (either with dithionite or ascorbate) and reoxygenation of NO-treated COX. Interestingly, when 10 mM NO_2^- is added to **O**, the enzyme shows a very similar inhibition pattern.

reactions lead to a reversible or irreversible inhibition of COX? On the basis of kinetic measurements carried out in the presence of excess NO, it was proposed by Torres et al. (*27*) that, depending on the starting intermediate, the reaction with NO proceeds with different stoichiometries (and kinetics), as summarized in scheme 1. According to this model, (i) NO behaves as a one-electron donor for **P** and **F**, promoting the **P** → **F** and **F** → **O** transitions, respectively, (ii) NO reacts with **P** and **F** with maximal stoichiometry of 3:1 and 2:1, respectively, and (iii) the nitrite formed in the direct reaction of NO with **P** or **F** (but not with **O**), should dissociate from the enzyme.

Over the past few years, convincing evidence has been provided that the so-called **P** intermediate is an oxo-ferryl adduct (*11*−*14*), stabilized by a nearby protein radical [presumably on Tyr-244 (*15*)]. Therefore, the one-electron reduction of **P** by NO may in principle regenerate directly the **O** state (and not **F**), provided that the electron is transferred to the Fe^{IV}═O complex (and not to the nearby radical); one proton should also been supplied to maintain electroneutrality (see ref *35*). With this in mind we have investigated the stoichiometry and the product of the reaction of the COX intermediates **P** and **F** with NO.

*Reaction of **O**, **P**, and **F** with NO: The Stoichiometry and the Product.* The experiments reported in Figure 1 show that **P** and **F** react with NO with a 1:1 stoichiometry similarly to what demonstrated for **O** (*26*). The same result is obtained when probing **P** and **F** during their spontaneous decay to **O** [**F** being measured both in the presence and absence of catalytic catalase to prevent formation of **P** by residual H_2O_2 (*33*)]. When dealing with mixtures of **O**, **P**, and **F**, the apparent stoichiometry is always about 1:1 (Figure 1, bottom panel). The finding that each one of these species (**O**, **P**, or **F**) reacts with just 1 NO is consistent with **P** and **F** being both oxo-ferryl species.

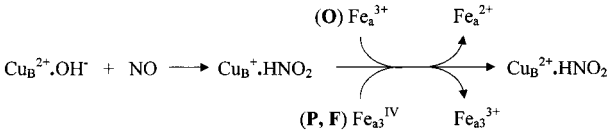
To assess the effect of the reaction on the catalytic activity of **P** or **F**, the rate of cytochrome *c* oxidation was measured before and after addition of NO. In these experiments, the enzyme carried out about five turnovers and Hb-O_2 was added to the cytochrome c^{2+} syringe to scavenge possible free NO. As shown in Figure 2 (top panel), addition of stoichiometric NO to **P** inhibits COX, the activity dropping to ~20%. A similar inhibition was obtained when starting with **F** or **O**, as well as after nitrite binding to the active site (Figure 2, bottom panel). These results suggest that the endpoint species generated in the reaction of **P**, **F**, or **O** with stoichiometric NO may be in all cases the fully oxidized enzyme complexed with nitrite, in line with the 1:1 stoichiometry measured amperometrically. Consistently, we have observed that starting with NO- or nitrite-treated COX, the time course of cytochrome *c* oxidation displays an initial lag phase, compatible with a release of inhibition associated to turnover. Under all conditions, initial activity was fully restored by a reduction-reoxygenation cycle of the inhibited enzyme (Figure 2).

To substantiate the hypothesis that the product of the reaction of stoichiometric NO with **P**, **F**, or **O** is the complex of nitrite with oxidized cytochrome a_3, we compared the difference spectra (**O**-nitrite *minus* **O**) obtained statically by incubating Cl-free **O** with large excess nitrite (the reaction was completed in ~10 min), with those observed kinetically by mixing **O**, **P**, and **F** with stoichiometric NO (Figure 3). After subtraction of the contribution due to reduced cytochrome *a*, the resulting kinetic difference spectrum matches

in shape the static one, accounting for ~80−90% of the expected absorbance change. Thus, we conclude that, upon reaction of stoichiometric NO with **O**, **P,** and **F**, nitrite is formed by one-electron oxidation of NO to NO^+ and subsequent hydroxylation. The smaller reduction of cytochrome *a* observed upon addition of NO to **P** and **F** can be rationalized assuming that electron-transfer involves the reduction of the oxo-ferryl adduct and thus, contrary to what happens with **O**, cytochrome *a* is not reduced. In so far as this interpretation is correct, we assign the small reduction of cytochrome *a* observed with **P** and **F** (Figure 3) to the unavoidable contamination of **O** in these samples.

On the basis of the results reported above and of the reaction between Cu_B^{2+} and NO proposed by Torres et al. (*27*), the following scheme for the reaction of **O**, **P**, and **F** with NO may be written:

Scheme 2



The novelty here is that addition of *stoichiometric* NO to each one of the three states (**P**, **F**, or **O**) leads to the formation of COX with nitrite bound to cytochrome a_3^{3+}, preventing further reaction with NO. The primary redox event is likely to be common to every intermediate and involves the one-electron reduction of Cu_B^{2+} by NO (*25*−*27*). The fate of this electron, however, is different: in the case of **P** or **F**, it is transferred to the oxo-ferryl adduct, whereas starting from **O**, the electron is back transferred from Cu_B^+ to cytochrome *a* (*25*−*27*).

The reaction pathway proposed for **F** seems plausible also in light of the recent observation that an intermediate with reduced Cu_B and oxo-ferryl cytochrome a_3 is transiently populated during the reaction between mixed-valence COX and H_2O_2, as investigated by flow-flash (*36*). Interestingly, it was also shown that in this intermediate a very rapid electron transfer ($\tau \approx 90$ μs) from Cu_B^+ to the oxo-ferryl complex takes place yielding the oxidized form of the enzyme. On the other hand, Scheme 2 poses a problem concerning the reaction of NO with **P**, given that this species obtained by oxygenation of the mixed-valence CO enzyme (called $\mathbf{P_M}$) is not a peroxy intermediate, but an oxo-ferryl species (*11*−*14*), stabilized by a protein radical [presumably Tyr-244 (*15*)]. This being the case, two electron equivalents would be demanded to convert $\mathbf{P_M}$ into **O**, one to bleach the radical Tyr-244 and the other to reduce the oxo-ferryl. This view is consistent with the data by Verkhovsky et al. (*37*) who claimed, using a photoinducible reductant, that one-electron reduction of $\mathbf{P_M}$ yields **F**, which in turn is converted into **O** by donation of a second electron. On the other hand, Fabian and Palmer (*38*) reported that addition of only 1-1.3 equiv of cytochrome c^{2+} to **P** leads to formation of **O**. A possible explanation is that the radical Tyr-244 may be unstable over a time scale of minutes, being possibly reduced by the protein matrix (*39*). If so, the redox state of the **P** intermediate probed by Fabian and Palmer and in the actual study may be not defined, the experiments being performed from 1 to 15 min after oxygenation of the mixed-valence CO enzyme. We cannot exclude, however, that a single

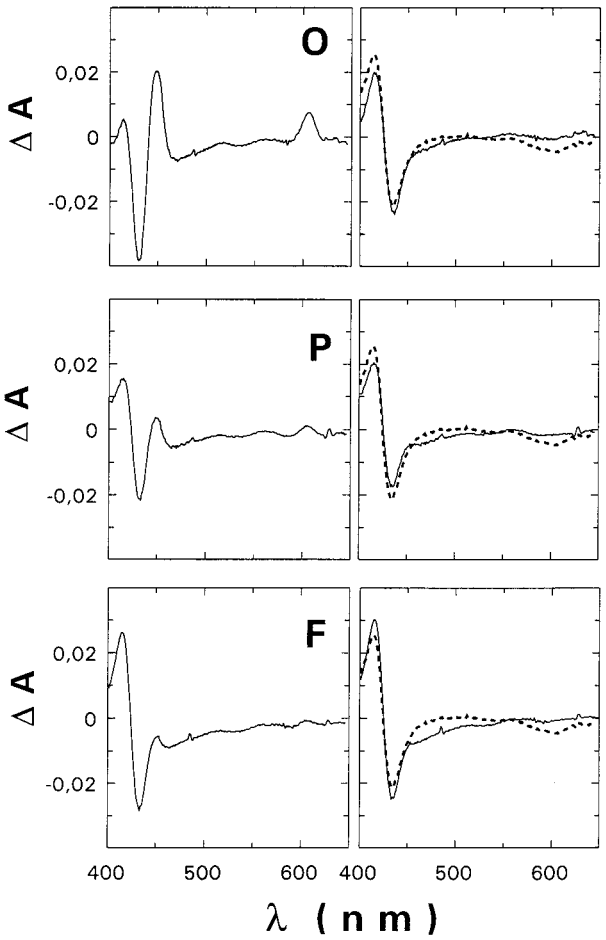


FIGURE 3: Reaction product is nitrite-bound oxidized COX. (Left panels) Difference spectra obtained by mixing NO with **O**, **P**, and **F**. Baseline: spectrum of **O**. [COX] = 2 μM. [NO] = 2 μM (with **O** and **P)** and 4 μM (with **F**). (Right panels) The same spectra after subtracting the contribution of cytochrome *a* (solid line), as compared to the spectrum of **O** after ~10 min incubation with 10 mM nitrite (dashed line). The fraction of reduced cytochrome *a* subtracted, as estimated by the pseudoinverse method, was: 22% (**O**), 7% (**P**), and 4% (**F**). The data suggest that in all cases the product of the reaction is nitrite-bound COX.

electron transfer from NO reduces preferentially the oxo-ferryl cytochrome a_3; if so, the nitrite-bound reaction product might still have Tyr-244 in the radical state.

Kinetics of the NO Reaction with COX Intermediates. Since the reaction of one molecule of NO with **O**, **P**, or **F** yields the nitrite-inhibited oxidase, to understand the mechanism of COX inhibition during turnover the rate of these reactions should be assessed and compared with direct NO binding to cytochrome a_3^{2+} [$k \approx 1 \times 10^8$ M^{-1} s^{-1} (*24*)]. COX inhibition may in principle proceed through anyone of these alternative pathways. As shown by Giuffrè et al. (*26*), NO reacts with the chloride-free **O** species at $k \approx 2 \times 10^5$ M^{-1} s^{-1}, thus much more slowly than with reduced cytochrome a_3. The **P** species, as originated by oxygenation of the mixed valence−CO enzyme (so-called $\mathbf{P_M}$), was proposed to be very reactive toward NO, the reaction being optically silent and lost within the dead-time of a stopped-flow instrument, i.e., 1−2 ms (see Scheme 4 in ref *27*). We have therefore measured the kinetics of the onset of inhibition of **P** by NO performing a sequential mixing stopped-flow experiment. As shown in Figure 4, at 5 μM NO, COX is inhibited at a relatively slow rate ($k' = 0.4$ s^{-1}, bottom panel

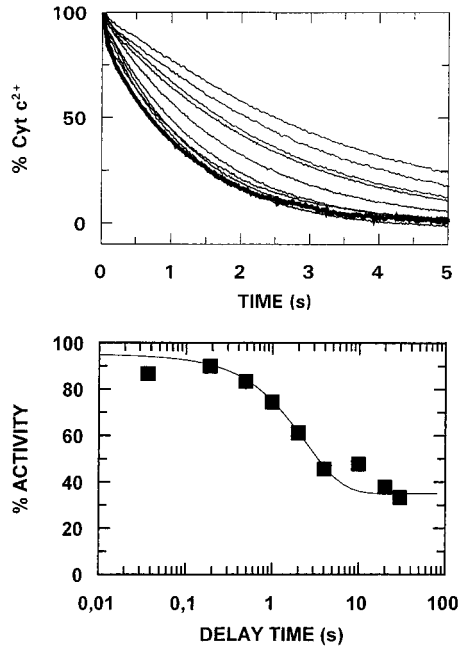


FIGURE 4: Onset of inhibition of **P** by NO. COX (1 μM) in the **P** state is premixed with 10 μM NO, and after a delay time, this mixture is mixed with 20 μM cytochrome c^{2+} plus 15 μM oxy-Hb (to scavenge free NO when present). (Top) The time courses at 550 nm collected at the different delay times (from 40 ms to 40 s) are shown (from bottom left to top right) with reference to the control trace in the absence of NO (heavy trace). As expected, the longer the delay time, the lower is the enzyme activity. The whole experiment has been carried out within 15 min from **P** formation. (Bottom) The observed time courses were fitted to single exponential decays and the corresponding rate constant plotted as a function of the delay time between the two sequential mixing events. Activity drops following an exponential time dependence with $k' = 0.4$ s^{-1}, yielding a bimolecular rate constant $k \approx 8 \times 10^4$ M^{-1} s^{-1} at NO = 5 μM after first mixing. Noteworthy, ~90% activity is retained up to 200 ms after mixing **P** with NO, with no evidence for a very fast inhibition.

of Figure 4) and, therefore, it is still ≥80% active after 0.5 s. This experiment allows to calculate a bimolecular rate constant for the reaction of **P** with NO of $k \approx 8 \times 10^4$ M^{-1} s^{-1}, quite similar to that estimated for **O** [$k \approx 2 \times 10^5$ M^{-1} s^{-1} (*26*)]. A similar experiment repeated starting with **F** yielded a bimolecular rate constant of $k \approx 2 \times 10^4$ M^{-1} s^{-1}, in agreement with Torres et al. [$k \approx 1 \times 10^4$ M^{-1} s^{-1} (*27*)]. The bottom line is that **O**, **P**, and **F** react with stoichiometric NO with a bimolecular rate constant which, though much lower than that with cytochrome $a_3{}^{2+}$ [$(0.1-1) \times 10^5$ M^{-1} s^{-1} versus 1×10^8 M^{-1} s^{-1}], still accounts for formation of an inhibited form of the enzyme. This information is essential to assess the primary target of NO inhibition during turnover, as discussed below.

Reversal of Inhibition and the Fate of Nitrite. The nitrite-inhibited enzyme, as obtained either by supplying excess nitrite to **O** or by reaction of **O**, **P**, or **F** with stoichiometric NO is not completely dead, having a residual 20% cytochrome *c* oxidase activity in a five-turnovers assay (Figure 2, bottom panel). Activity can be completely restored by a reduction-reoxygenation cycle. Moreover, the time course of cytochrome *c* oxidation shows under these conditions an initial lag phase (Figure 2, top panel). All together, these features suggest that nitrite, either exogenously added to **O** or formed at the binuclear site, is a reversible inhibitor of COX and that during turnover a fraction of the total enzyme

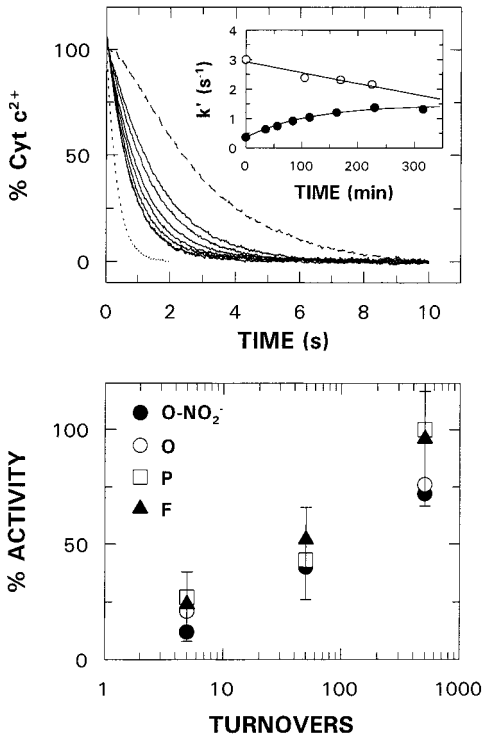


FIGURE 5: Inhibition is reversible. (Top) Time courses of oxidation of 10 μM cytochrome c^{2+} by 0.5 μM COX with and without nitrite. When COX in the **O** state is incubated with 10 mM NO_2^- for 10 min and then mixed with cytochrome c^{2+}, the enzyme is considerably inhibited ($k' = 0.36$ s^{-1}, dashed), as compared to the control ($k' = 3$ s^{-1}, dotted). If excess nitrite is removed with a G25 column and COX activity measured as a function of time (solid traces from top right to bottom left), the enzyme reactivates spontaneously at the rate of nitrite dissociation (as optically measured in parallel to the stopped-flow measurements). (Inset) Traces have been fitted to single exponential decays and the observed rate constant for the NO_2^--treated (●) and the untreated COX (○) plotted as a function of time after column elution. Enzyme reactivation proceeds with a $t_{1/2} \approx 80$ min. (Bottom) Cytochrome c^{2+} (20 μM) is mixed with 1 μM COX in the state **O** (or **P** or **F**) and preincubated with 5 μM NO: oxy-Hb (5 μM) was added to the oxidase syringe before mixing with reduced cytochrome *c* to scavenge free NO. The activity of NO-treated COX was measured as such or after dilution to 100 nM or 10 nM to make 5, 50, or 500 turnovers, respectively. The plot of residual activity as a function of the number of turnovers points to a release of the enzyme inhibition upon turning over. Interestingly, NO_2^--bound COX (incubated with 10 mM NO_2^- for 10 min) behaves similarly to the NO-treated enzyme.

is reactivated. If so, cytochrome *c* oxidase activity measured starting with the nitrite-inhibited COX should depend on the number of catalytic cycles performed. This expectation has been fulfilled by the results shown in the bottom panel of Figure 5: here, the nitrite-COX (obtained either by nitrite binding to **O** or treating **O**, **P**, or **F** with close-to stoichiometric NO) is probed for its residual cytochrome *c* oxidase activity at increasing number of turnovers (from 5 to 500) with recovery of full activity. Since in these experiments nitrite-COX is diluted manifold, to exclude that the observed effect is due to thermal dissociation of nitrite from the oxidized enzyme, we measured the intrinsic dissociation rate constant of nitrite from **O**. Starting with the nitrite complex, activity was assessed as a function of time after removal of excess nitrite by gel filtration. As shown in Figure 5 (top panel), and confirmed spectrophotometrically following the dissociation of nitrite from **O** (data not shown), the spontaneous recovery of activity is very slow (hours), with a half-

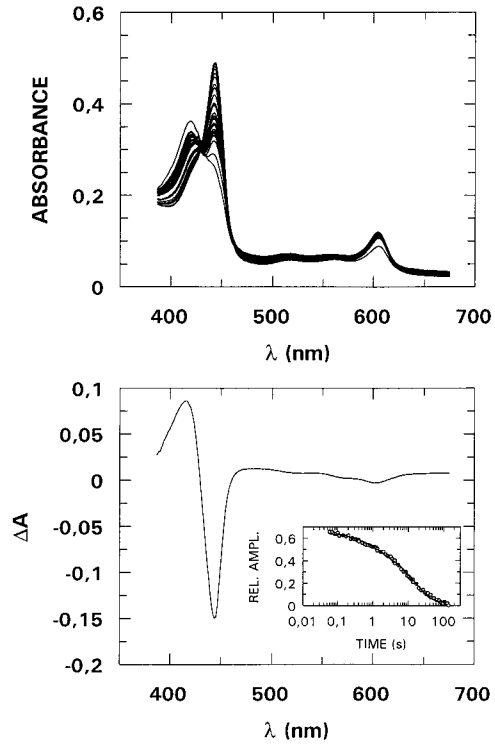
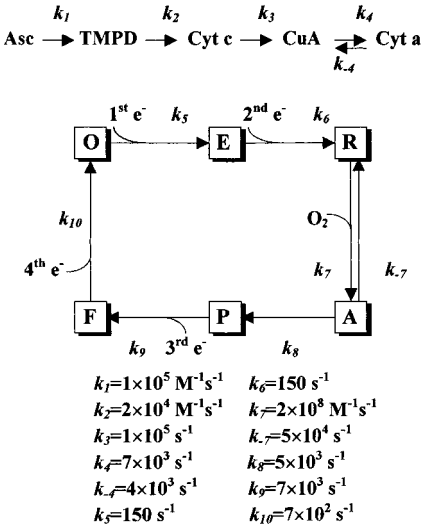


FIGURE 6: Dissociation of nitrite on COX reduction. COX in the **O** state was incubated with 10 mM NO_2^- for 15 min; afterward excess nitrite was removed with a G25 column and immediately after elution nitrite-bound COX was diluted to 2.5 μM with O_2-free buffer and mixed in the diode array with 20 mM ascorbate plus 200 μM ruthenium hexamine (at 35 min from loading onto the column). Nitrite dissociation from **O** has been monitored spectrophotometrically in parallel with the stopped-flow measurement. (Top) Absorption spectra collected from 10 ms to 140 s after mixing according to a logarithmic acquisition mode. Reduction of cytochrome *a* was the fastest event observed and was over within 50 ms. Notice that the endpoint species is the fully reduced unbound enzyme. (Bottom) Absorption changes from 60 ms after mixing on have been processed by singular value decomposition. Analysis reveals only one optical transition corresponding to cytochrome a_3 reduction and proceeding with biphasic time course: $k_1 = 0.9\ s^{-1}$ and $k_2 = 0.06\ s^{-1}$ (corresponding to 25 and 75% of total amplitude, respectively). (Inset) The best fit of the first **V** column of the SVD output.

time of ~80 min. The results reported above clearly show that during turnover, nitrite dissociates in a few seconds, i.e., much more rapidly than intrinsic k_{off} from **O** ($t_{1/2} \approx 80$ min). The question is whether nitrite dissociates as such or after rereduction to NO. Only in the former case, COX would "catalyze" the oxidative degradation of NO to NO_2^-. To address this problem, we optically monitored the time course of reduction of the nitrite-COX complex under anaerobic conditions (Figure 6). Given the high affinity of NO for cytochrome a_3^{2+}, we assumed that in the absence of O_2, in loco production of NO by reduction of bound nitrite would be associated to appearance of nitrosyl-cytochrome a_3^{2+}. This was not the case since the endpoint species was the fully reduced ligand-free COX, as shown in Figure 6 (top panel): fast reduction of cytochrome *a* (within 50 ms) was followed by the biphasic reduction of cytochrome a_3 (Figure 6, bottom panel). It is therefore likely that the rate of dissociation of nitrite, like other anions (Cl^-, CN^-), increases upon reduction of COX.

Is There a Physiological Role for the Reaction of COX Intermediates with NO? An important issue is whether the reactions described above do or do not have a finite probability to occur during turnover. This is a crucial question since a positive answer (i) may contribute to account for the inhibition of COX by NO, and perhaps more important, (ii) may support a role of COX in the oxidative catabolism of NO to the less toxic NO_2^- under physiological conditions. These two problems are interconnected, since both require to know the probability of each state of the enzyme to react with NO, at steady-state. To address this point we have carried out kinetic simulations according to Scheme 3. We are aware that our knowledge of the system "COX in turnover + NO" is still insufficient to support a conclusive mechanism of inhibition, largely because we ignore if and how quickly NO reacts with the one-electron reduced cytochrome a_3-Cu_B site (so-called species **E**).

Scheme 3



In Scheme 3, only the changes of state occurring to the cytochrome a_3-Cu_B binuclear site during catalysis are shown. We emphasize that this is an oversimplified view in many respects, since in the scheme (i) **P** stands for all the intermediates at the redox level of the so-called $\mathbf{P_M}$ species, thus including also the recently postulated $\mathbf{F^\bullet}$ species;[3] these intermediates should be oxo-ferryl species stabilized by a nearby protein radical, (ii) **F** stands for all the intermediates which differ by one electron equivalent from $\mathbf{P_M}$ (and $\mathbf{F^\bullet}$), namely the (currently) called **F** and the $\mathbf{P_R}$ intermediates; according to Michel (*7*), these should be oxo-ferryl species without the protein radical, (iii) **E** groups the two possible intermediates with half-reduced binuclear site, i.e., without discriminating whether the electron resides on cytochrome a_3 or Cu_B, and (iv) the first and the second electrons transferred to binuclear site, starting from **O**, were assumed to be transferred at the same rate, because the rate differences reported before (*40*), though measurable, are quite small; the value of k_5 and $k_6 = 150\ s^{-1}$ was chosen to be close to the value estimated in the membrane-integrated enzyme (*41*), and (v) most of the steps were assumed to be irreversible. Despite all these approximations, the model is already very complex involving about 80 different species, including the substrates (cytochrome *c* and O_2) and the reductants (ascorbate and TMPD) and taking into account that each of the

[3] This refers to the **F′** intermediate as meant by Michel (*7*).

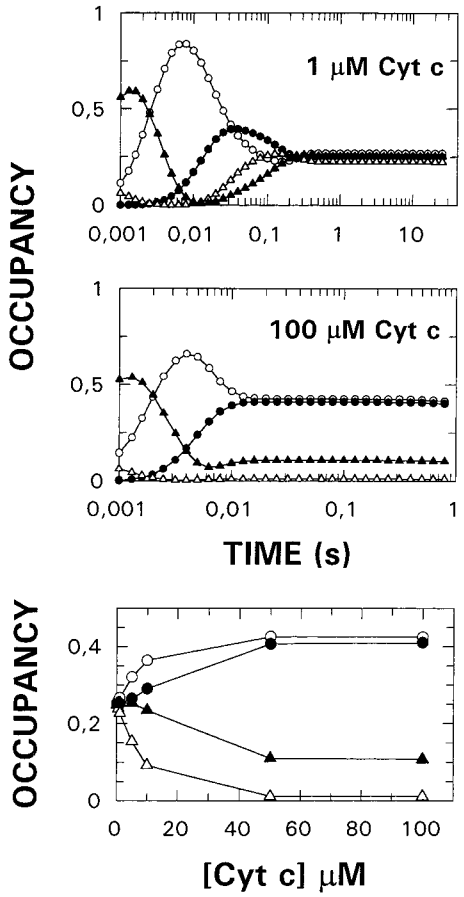


FIGURE 7: Steady-state simulations. Simulations have been carried out according to Scheme 3 and the resulting time dependence (from 1 ms to 100 s) of the occupancy of the intermediates **O** (○), **E** (●), **P** (△), and **F** (▲) is depicted: traces have been truncated at the end of the steady-state phase. (Top) Low [cytochrome *c*]. (Middle) High [cytochrome *c*]. (Bottom) Dependence of the occupancy of the intermediates at steady state on [cytochrome *c*]. Notice that at low rate of electron supply to COX (i.e., low [cytochrome *c*]), the steady-state occupancy of **P** and **F** intermediates raises and approaches the occupancy of the states **O** and **E** (~25% each).

intermediates in Scheme 3 might have bound-cytochrome *c*, Cu_A, and cytochrome *a* in different redox states. The time dependence of each of these species has been simulated from 1 ms to 100 s and all the parameters, except cytochrome *c* concentration, were kept constant from one simulation to the other: a selected output of these simulations is reported in Figure 7.

At steady-state, the **O**, **E**, **P**, and **F** species are more populated than **R** and the so-called compound **A** (not shown). As anticipated, simulations indicate that **E** is, under all conditions (low and high reductant), highly populated; unfortunately, the reactivity of **E** with NO is unknown. Moreover, while the partition of the electron between cytochrome a_3 and Cu_B strongly favors Cu_B, at present, the value of this equilibrium constant is uncertain. And, more important, we still do not know whether NO can combine with reduced cytochrome a_3 in the **E** state with the high rate characteristic of the **R** state [$k = 1 \times 10^8$ M^{-1} s^{-1} (*24*)]. Of course, if this was not the case, owing to the high occupancy of **O**, **P**, and **F**, the probability that they react with NO under turnover conditions would be significant, even taking into account their intrinsically low reactivity [$k = (0.1-1) \times 10^5$ M^{-1} s^{-1}].

The other crucial information obtained from the simulations is that the steady-state occupancy of **O**, **E**, **P**, and **F** clearly depends on reductant pressure, i.e., on cytochrome *c* concentration. This is shown in Figure 7, where we compare the simulated time dependencies of these intermediates at low (top panel, 1 μM) and high cytochrome *c* concentration (middle panel, 100 μM): decreasing the rate of electron entry into COX, **P** and **F** become more populated at the expenses of **O** and **E**. This is also shown in Figure 7 (bottom panel), where the predicted steady-state occupancy of the four intermediates on varying [cytochrome *c*] is reported. This result is not surprising, since **O**, **E**, **P**, and **F** demand transfer of an electron from cytochrome *a* to convert into the following intermediate in the catalytic cycle: it is therefore expected that they display longer lifetimes and higher steady-state occupancies at lower rate of electron entry into COX. Perhaps an important consequence of this behavior is that the highest probability for **P** and **F** to react with NO to produce NO_2^- could occur at low electron flux through COX, which would account for an oxidative COX-mediated NO catabolism. On the contrary, at higher reducing pressure, the inhibited a_3^{2+}-NO adduct builds up. This hypothesis is fully consistent with the very recent observation that COX inhibition by NO under turnover conditions (O_2 consumption assay), when sustained by high cytochrome c^{2+}, is promptly removed on illumination of the sample, and the (photosensitive) a_3^{2+}-NO is spectrophotometrically detected (*28*). If this was confirmed to be the case also in vivo, then the NO-degrading activity of COX would acquire patho-physiological meaning. A pathway to dispose of NO overproduction, under conditions of low electron flux through the electron transport chain may indeed be welcome by the cell: this would prevent the reaction of NO with radical oxygen species, leading to production of the very toxic peroxynitrite.

CONCLUDING REMARKS

The novel finding reported above is that the reaction of NO *stoichiometric* with oxidase is associated to formation of the nitrite adduct of oxidized cytochrome a_3, by reacting not only with the **O** state of the enzyme (*25*−*27*), but also with **P** and **F**. To rationalize this finding, we propose (see Scheme 2) a modified version of the mechanism postulated by Torres et al. (*27*), where NO, by reacting with **P** and **F**, is responsible for the one-electron reduction of the oxo-ferryl cytochrome a_3 leading to ferric cytochrome a_3 with nitrite bound. This reaction pathway is consistent with the recently proposed identity of the **P** intermediate as an oxo-ferryl species (*11*−*15*).

Although the reactions of NO with **P**, **F**, and **O** are much slower [$k = (0.1-1) \times 10^5$ M^{-1} s^{-1}] than NO combination to cytochrome a_3^{2+} ($k = 1 \times 10^8$ M^{-1} s^{-1}), they may provide the oxidative degradation pathway of NO into nitrite. We have presented experimental evidence that the nitrite-inhibited enzyme, produced by reaction of the oxidized states of COX with stoichiometric NO, completely recovers its activity during turnover with physiological substrates, presumably via more rapid dissociation of nitrite from the binuclear site. Interestingly, kinetic simulations suggest that this COX-mediated degradation of NO is more likely to occur at low reductant pressure because of the higher occupancies of **P** and **F**; under these conditions, radical oxygen species can react with NO leading to very toxic compounds for the

cell. On the contrary, at high reductant pressure, the same simulations predict accumulation of the cytochrome a_3^{2+}-NO species; since this is the only species which is photosensitive (like all complexes of ferrous hemeproteins), this prediction provides an explanation for the dramatic effect of illumination on the recovery of oxidase activity after inhibition by NO (*28*).

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NOTE ADDED IN PROOF

After this manuscript had been submitted for publication, a report by Torres, J., et al. [*FEBS Lett.* (2000) *475*, 263−266], showing that COX may metabolize NO to NO_2^-, has been published.

REFERENCES

1. Moncada, S., Palmer, R. M. J., and Higgs, E. A. (1991) *Pharmacol. Rev. 43*, 109−142.
2. Giulivi, C., Poderoso, J. J., and Boveris, A. (1998) *J. Biol. Chem. 273*, 11038−11043.
3. Brown, G. (1999) *Biochim. Biophys. Acta 1411*, 351−369.
4. Brunori, M., Giuffrè, A., Sarti, P., Stubauer, G., and Wilson, M. T. (1999) *Cell. Mol. Life Sci. 56*, 549−557.
5. Sarti, P., Lendaro, E., Ippoliti, R., Bellelli, A., Benedetti, P. A., and Brunori, M. (1999) *FASEB J. 13*, 191−197.
6. Babcock, G. T., and Wikström, M. (1992) *Nature 356*, 301−309.
7. Michel, H. (1999) *Biochemistry 38*, 15129−15140.
8. Han, S., Ching, Y. C., and Rousseau, D. L. (1990) *Nature 348*, 89−90.
9. Varotsis, C., Zhang, Y., Appelman, E. H., and Babcock, G. T. (1993) *Proc. Natl. Acad. Sci U.S.A. 90*, 237−241.
10. Kitagawa, T., and Ogura, T. (1998) *J. Bioenerg. Biomembr. 30*, 71−79.
11. Proshlyakov, D. A., Ogura, T., Shinzawa-Itoh, K., Yoshikawa, S., Appelman, E. H., and Kitagawa, T. (1994) *J. Biol. Chem. 269*, 29385−29388.
12. Proshlyakov, D. A., Ogura, T., Shinzawa-Itoh, K., Yoshikawa, S., and Kitagawa, T. (1996) *Biochemistry 35*, 8580−8586.
13. Proshlyakov, D. A., Pressler, M. A., and Babcock, G. T. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 8020−8025.
14. Fabian, M., Wong, W. W., Gennis, R. B., and Palmer, G. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 13114−13117.
15. MacMillan, F., Kannt, A., Behr, J., Prisner, T., and Michel, H. (1999) *Biochemistry 38*, 9179−9184.
16. Das, T. K., Pecoraro, C., Tomson, F. L., Gennis, R. B., and Rousseau, D. L. (1998) *Biochemistry 37*, 14471−14476.
17. Ostermaier, C., Harrenga, A., Ermler, U., and Michel, H. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 10547−10553.
18. Yoshikawa, S., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., Yamashita, E., Inoue, N., Yao, M., Fei, M. J., Libeu, C. P., Mizushima, T., Yamaguchi, H., Tomizaki, T., and Tsukihara, T. (1998) *Science 280*, 1723−1729.
19. Buse, G., Soulimane, T., Dewor, M., Meyer, H. E., and Blüggel, M. (1999) *Protein Sci. 8*, 985−990.
20. Torres, J., Darley-Usmar, V. M., and Wilson, M. T. (1995) *Biochem. J. 312*, 169−173.
21. Giuffrè, A., Sarti, E., D'Itri, E., Buse, G., Soulimane, T., and Brunori, M. (1996) *J. Biol. Chem. 271*, 33404−33408.
22. Stubauer, G., Giuffrè, A., Brunori, M., and Sarti, P. (1998) *Biochem. Biophys. Res. Commun. 245*, 459−465.
23. Giuffrè, A., Stubauer, G., Sarti, P., Brunori, M., Zumft, W. G., Buse, G., and Soulimane, T. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 14718−14723.
24. Blackmore, R. S., Greenwood, C., and Gibson, Q. H. (1991) *J. Biol. Chem. 266*, 19245−19249.
25. Cooper, C. E., Torres, J., Sharpe, M., and Wilson, M. T. (1997) *FEBS Lett. 414*, 281−284.
26. Giuffrè, A., Stubauer, G., Brunori, M., Sarti, P., Torres, J., and Wilson, M. T. (1998) *J. Biol. Chem. 273*, 32475−32478.
27. Torres, J., Cooper, C. E., and Wilson, M. T. (1998) *J. Biol. Chem. 273*, 8756−8766.
28. Sarti, P., Giuffrè, A., Forte, E., Mastronicola, D., Barone, M. C., and Brunori, M. (2000) *Biochem. Biophys. Res. Commun. 274*, 183−187.
29. Soulimane, T., and Buse, G. (1995) *Eur. J. Biochem. 227*, 588−595.
30. Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1961) *J. Biol. Chem. 236*, 165−168.
31. Greenwood, C., Wilson, M. T., and Brunori, M. (1974) *Biochem. J. 137*, 205−215.
32. Wikström, M., and Morgan, J. E. (1992) *J. Biol. Chem. 267*, 10266−10273.
33. Vygodina, T. V., and Konstantinov, A. (1988) *Ann. N. Y. Acad. Sci 550*, 124−138.
34. Henry, E., and Hofrichter, J. (1992) *Methods Enzymol. 210*, 129−192.
35. Mitchell, R., and Rich, P. R. (1994), *Biochim. Biophys. Acta 1186*, 19−26.
36. Zaslavsky, D., Smirnova, I. A., Ådelroth, P., Brzezinski, P., and Gennis, R. B. (1999) *Biochemistry 38*, 2307−2311.
37. Verkhovsky, M., Morgan, J. E., and Wikström, M. (1996) *Proc. Natl Acad. Sci U.S.A. 93*, 12235−12239.
38. Fabian, M., and Palmer, G. (1999) *Biochemistry 38*, 6270−6275.
39. Chen, Y. R., Gunther, M. R., and Mason, R. P. (1999) *J. Biol. Chem. 274*, 3308−3314.
40. Malatesta, F., Sarti, P., Antonini, G., Vallone, B., and Brunori, M. (1990) *Proc. Natl. Acad. Sci U.S.A. 87*, 7410−7413.
41. Brunori, M., Antonini, G., Giuffrè, A., Malatesta, F., Nicoletti, F., Sarti, P., and Wilson, M. T. (1994) *FEBS Lett. 350*, 164−168.

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