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Chloride Bound to Oxidized Cytochrome *c* Oxidase Controls the Reaction with Nitric Oxide*

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The reaction of nitric oxide (NO) with oxidized *fast* cytochrome *c* oxidase was investigated by stopped-flow, amperometry, and EPR, using the enzyme *as prepared* or after "pulsing." A rapid reduction of cytochrome *a* is observed with the *pulsed*, but not with the enzyme as prepared. The reactive species (λ_{max} = 424 nm) reacts with NO at $k = 2.2 \times 10^5$ M^{-1} s^{-1} at 20 °C and is stable for hours unless Cl^- is added, in which case it decays slowly ($t_{1/2} \sim 70$ min) to an unreactive state (λ_{max} = 423 nm) similar to the enzyme as prepared. Thus, Cl^- binding prevents a rapid reaction of NO with the oxidized binuclear center. EPR experiments show no new signals within 15 s after addition of NO to the enzyme as prepared. Amperometric measurements show that the pulsed NO-reactive enzyme reacts with high affinity and a stoichiometry of 1 NO/aa_3, whereas the enzyme as prepared reacts to a very small extent (<20%). In both cases, the reactivity is abolished by pre-incubation with cyanide. These experiments suggest that the effect of "pulsing" the enzyme, which leads to enhanced NO reactivity, arises from removing Cl^- bound at the oxidized cytochrome a_3-Cu_B site.

The explosion of interest in the biological effects of NO has led to the discovery that this remarkable gas is a reversible inhibitor of COX[1] (1–3) and may play an important role in the regulation of cellular respiration *in vivo* (4). Kinetic studies aimed at understanding the mechanism of this inhibition (3, 5) or those involving NO as a trapping ligand for reduced cytochrome a_3 (6) failed to reveal a fast reaction between NO and the oxidized enzyme *as prepared.* This is in agreement with previous studies (7–10) which showed that a reaction with oxidized COX was only observed following prolonged incubation with NO, inducing uncoupling of cytochrome a_3 and Cu_B. In contrast, recent data (11–13) have shown that on mixing *pulsed* COX with excess NO, a fraction of cytochrome *a* (up to 50%) is rapidly reduced on a millisecond time scale. In this paper, we provide an explanation for these conflicting observations.

On the basis of extended x-ray absorption fine structure measurements, it has been suggested that Cl^- may be bridging oxidized cytochrome a_3 and Cu_B when the enzyme is purified in the presence of this anion (14, 15). Consistently, Moody *et al.* (16, 17) reported that addition of Cl^- to a Cl^--free enzyme preparation is associated with slow changes in the Soret and in the reactivity toward cyanide. In addition it has been suggested that when the enzyme undergoes a cycle of reduction and reoxidation (*i.e.* is pulsed), Cl^- dissociates (15).

To clarify if Cl^- has an effect on the reaction of oxidized COX with NO, we have carried out a series of stopped-flow and amperometric measurements in the presence and absence of Cl^-. Nitric oxide was mixed with bovine heart oxidized COX in either of two states, *i.e.* (i) the enzyme as prepared following the procedure of Soulimane and Buse (18), which yields a highly active ($TN > 200$ s^{-1}), fast cyanide binding and presumably Cl^--bound protein; or (ii) the same preparation having undergone a cycle of reduction and reoxidation to yield the pulsed, Cl^--free enzyme (13, 19–21). The experiments reported below resolve the apparent discrepancies in the literature, showing that the same preparation of oxidized enzyme may or may not react rapidly with NO depending on Cl^- removal/addition.

MATERIALS AND METHODS

Cytochrome *c* oxidase from beef heart, prepared according to Soulimane and Buse (18), yields the so-called *fast* enzyme as defined by Baker *et al.* (22). The enzyme concentration is expressed in terms of functional units (aa_3). NO was from Air Liquide (Paris, France).

To remove Cl^- and generate the pulsed state, we followed an unpublished protocol kindly provided by Prof. F. Malatesta (University of L'Aquila, Italy). Briefly, the enzyme (in 100 mM Hepes, pH 7.3, containing 0.1% lauryl maltoside) was reduced with dithionite and passed through a G25 column equilibrated with the above buffer containing 2 mM dithionite and catalytic amounts of catalase. After elution, the enzyme was oxidized by addition of 0.5 mM ferricyanide in air and then passed through a second G25 column and immediately used for the experiments with NO. The whole procedure typically takes ~10 min.

Stopped flow experiments were carried out using either a Durrum-Gibson apparatus equipped with a photodiode array (TN6500; Tracor Northern, Madison, WI), which acquires a 1024-elements spectrum in 10 ms, or using a single wavelength apparatus (DX.17MV; Applied Photophysics, Leatherhead, UK). Noise filtering of experimental spectra was performed by singular value decomposition (23). Absorption spectra were collected at room temperature by using a V-550 Jasco spectrophotometer, 1 cm light-path. Amperometric NO measurements were performed at room temperature using a Clark-type NO electrode (ISO-NO, World Precision Instruments, UK) following the procedure given by Stubauer *et al.* (24). For the EPR measurements, 25–40 μM

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[1] The abbreviation used is: COX: cytochrome *c* oxidase.

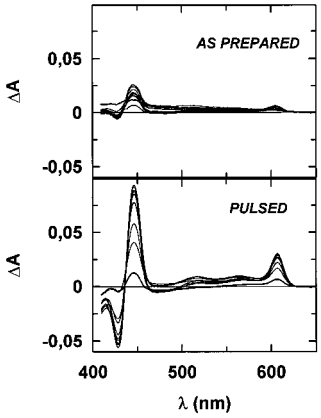


FIG. 1. **Reaction of nitric oxide with oxidized cytochrome *c* oxidase as prepared and after "pulsing."** Difference spectra observed within 100 ms after mixing 0.85 mM NO with 2.8 μM air-equilibrated oxidized COX either as prepared (*top*) or after "pulsing" (*bottom*). Baseline: oxidized spectrum; T = 20 °C; light path = 2 cm. The spectra are displayed at 10-ms intervals

COX was mixed with NO at molar ratios of 1:1, 12:1, and 30:1 NO/aa_3, and the mixture was frozen in ~15 s. EPR spectra were measured on a Bruker EMX spectrometer with an ER 041XG microwave bridge (X-band).

RESULTS

When NO is mixed with oxidized COX, a large absorbance change is seen with the pulsed enzyme, whereas with the enzyme as prepared only a small signal is detected (Fig. 1). The reaction corresponds to the reduction of 50–60% of cytochrome *a*, with no spectral changes assigned to cytochrome a_3 showing, in agreement with earlier work (11–13), that the reaction with NO is only observed when the enzyme is subjected to a "pulsing" protocol.

The small optical change seen with the oxidized enzyme as prepared may be due either to heterogeneity or to binding of NO to Cu_B, as reported by Brudvig *et al.* (8). EPR experiments (not shown) in which oxidized COX as prepared was frozen in the EPR tube within 15 s after mixing with NO showed no new signals, suggesting essentially no binding of NO to the enzyme as prepared.

The reaction of oxidized COX with NO was also followed amperometrically (Fig. 2). Addition of oxidized oxidase, as prepared, to NO yields a very small signal corresponding to ~20% of the total aa_3 (*trace a*); treatment of the enzyme as prepared with cyanide (3 mM for ~1 h at 25 °C) completely abolished this reaction (*trace b*). The reactivity of COX toward NO increased after "pulsing," yielding a 1:1 stoichiometry (*trace c*); also this reaction was abolished by incubation with cyanide (10 mM). Interestingly, when Cl^- was added to the pulsed enzyme, the reactivity toward NO decayed as a function of time and reached a level similar to that of the enzyme as prepared with a half-time of ~70 min (*bottom panel* of Fig. 2).

Because Cl^- binds to the binuclear site and affects its reactivity (14–17), these experiments have been repeated in the absence of Cl^-. To remove Cl^- bound to the enzyme, the 2-column "pulsing" procedure, described under "Materials and Methods," was carried out using Cl^--free buffers. Amperometric and stopped-flow experiments show that, after "pulsing" in the absence of Cl^-, the extent of the NO reaction (Fig. 3, *left*) and cytochrome *a* reduction (Fig. 3, *right*) are both time-independent (over several hours). Only upon addition of KCl does NO reactivity decay, with a half-time of ~40–50 min (Fig. 3); this time course is similar to that of the spectral shift of the absorption maximum (from 424 to 423 nm, see Fig. 4) observed upon Cl^- addition and previously assigned to Cl^- binding to

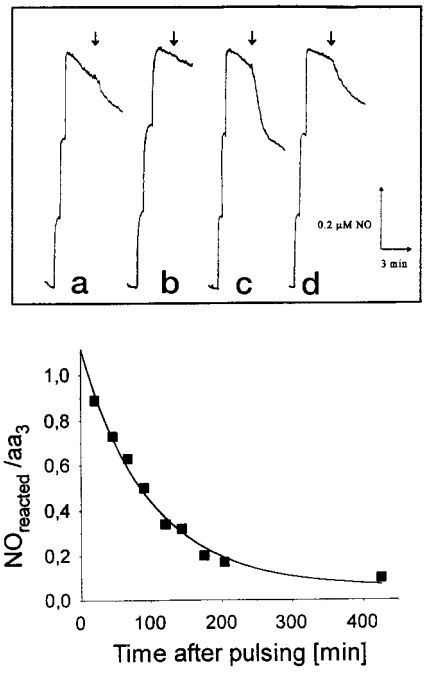


FIG. 2. **Amperometric experiments of the reaction of NO with oxidized cytochrome *c* oxidase.** NO (three additions of 250 nM each) was added at room temperature to degassed 50 mM Hepes, pH 7.3, containing 0.1% lauryl maltoside, 100 mM KCl, and 0.5 mM diethylenetriaminepentaacetic acid. *Top panel*, NO decay after addition of 200 nM COX as indicated by the *arrows*: enzyme as prepared (*a*); after 1 h of incubation with 3 mM NaCN (*b*); 20 min after "pulsing" (*c*); and 143 min after "pulsing" (*d*). *Bottom Panel*, Time dependence of the NO reacted with COX after "pulsing"; continuous line is the best fit for an exponential decay with a $t_{½}$ = 70 min.

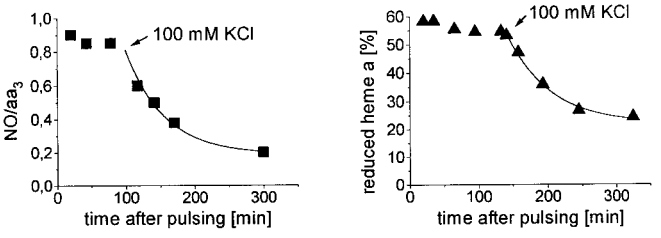


FIG. 3. **Effect of chloride on the NO reactivity of oxidized pulsed oxidase.** NO reactivity of the enzyme, following the "pulsing" procedure and the addition of KCl, was probed as a function of time either by amperometry (*left*) or by stopped-flow spectroscopy (*right*). Buffer contents were: 100 mM K/Hepes, pH = 7.3 + 0.1% lauryl maltoside. Experimental conditions were as in Fig. 2 (*top panel*) or Fig. 1 (*bottom* panel). In the *right panel*, we report the fraction of reduced cytochrome *a* at 100 ms after mixing.

the enzyme (17). This all-or-none effect on NO reactivity is because of Cl^- binding and not because of an increase in ionic strength since it was not observed by adding 33 mM K_2SO_4 (data not shown).

The bimolecular rate constant of the reaction of NO with the Cl^--free enzyme was measured by stopped-flow following the reduction of cytochrome *a* with pulsed oxidase (Fig. 5). Amplitude analysis shows independence on [NO] down to equimolar concentration, suggesting high affinity, as already indicated by amperometric measurements (Fig. 2). Up to 200 μM NO, the observed rate constant increases linearly with ligand concentration, yielding a bimolecular rate constant $k = 2.2 \times 10^5$ M^{-1} s^{-1}; at higher concentrations, a deviation from linearity was observed, with a limiting rate extrapolated at approximately 150–300 s^{-1}.

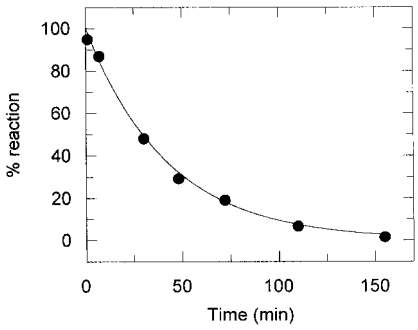


FIG. 4. **Kinetics of chloride binding to oxidized cytochrome *c* oxidase.** Time course of Cl^- binding measured spectroscopically after addition of 100 mM KCl to 2.2 μM pulsed COX. Data shown were obtained by singular value decomposition analysis of the absorption changes observed over 160 min, corresponding to a shift of the Soret peak from 424 to 423 nm.

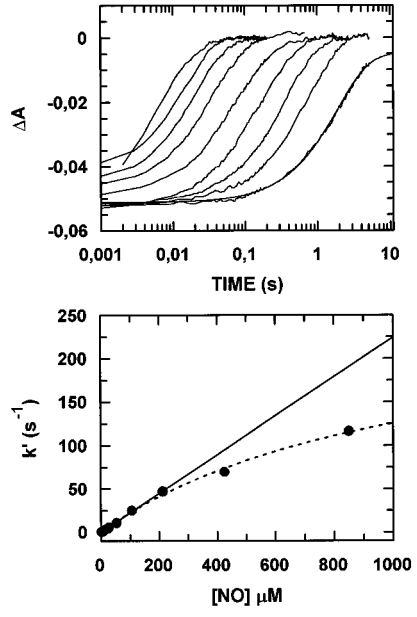
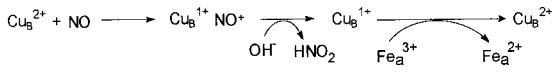


FIG. 5. **Kinetics of the NO reaction with oxidized pulsed oxidase.** *Top panel*, absorption changes recorded at 444 nm upon anaerobically mixing, in the absence of Cl^-, oxidized pulsed oxidase (4 μM aa_3) with NO at the following concentrations: 1700, 850, 425, 210, 105, 50, 26, 13, and 4 μM before mixing (from *left* to *right*). T = 20 °C; light path = 1 cm. Time courses were fitted to single exponentials. *Bottom panel*, observed rate constant as a function of NO concentration. Linear regression of the data at [NO] $\leq$ 200 μM yields a bimolecular rate constant of $k = 2.2 \times 10^5$ M^{-1} s^{-1}.

DISCUSSION

Torres *et al.* (13) reported that, upon mixing NO with oxidized pulsed COX, a rapid (ms) reduction of cytochrome *a* is observed. This process was suggested to proceed via reaction of NO with the cytochrome a_3/Cu_B binuclear center and was presumed to occur through initial reduction of Cu_B and migration of the electron to the cytochrome a/Cu_A site.



SCHEME 1

Using NO concentrations >100 μM and the pulsed enzyme, as much as 50% of cytochrome *a* and 20% of Cu_A became reduced. It was argued that one electron equivalent per functional unit of the enzyme (aa_3) is donated by one NO molecule (13).

Because this redox reaction was not observed in seemingly similar spectroscopic experiments (5, 6), we have attempted to clarify the origin of this variability in behavior carrying out experiments by time-resolved optical spectroscopy, amperometry, and EPR. As shown in Fig. 1, we have been able to induce two different responses to NO for the same preparation of oxidized bovine heart COX (18): (i) consistent with previous results (13), rapid reduction of cytochrome *a* was seen if the enzyme was subjected to a "pulsing" protocol before exposure to NO (Fig. 1, *bottom*); whereas in agreement with Giuffrè *et al.* (5), only a small reduction was observed when the enzyme as prepared was used (Fig. 1, *top*).

Brudvig *et al.* (8) showed that, despite some sample heterogeneity, NO reacts slowly with Cu_B^{2+} and breaks the coupling between this metal and the heme of cytochrome a_3, permitting the latter to exhibit its inherent $g = 6$ high-spin ferric signal. The reaction was shown to be slow and with low affinity (K_d ~200 μM), to be reversible, and to yield no optical change; partial reduction of the binuclear center was only observed on a long time scale (hours) (8). Palmer *et al.* (10), using a fast enzyme preparation, observed that over a 10-min period approximately 20% of the enzyme developed the $g = 6$ signal. Consistent with these reports (see Baker *et al.* (22)), addition of NO to the fast enzyme as prepared does not generate new EPR signals within the first 15 s. Had binding to Cu_B^{2+} taken place, we would have expected to see an EPR signal arise, but we did not. Therefore, even with the fast cyanide-binding oxidized enzyme prepared by the method of Soulimane and Buse (18), NO (at 100 μM) does not bind within seconds. This behavior is strikingly different from the same preparation after "pulsing", in which electron migration to cytochrome *a* occurs within 100 ms at 0.85 mM NO. Incidentally, it was previously observed that the sluggish reactivity of the binuclear center of this preparation toward CO and H_2O_2 is dramatically increased following the "pulsing" procedure (13).

To determine the stoichiometry of the reaction of NO with the oxidized enzyme (before and after "pulsing") and to test if the binuclear site is the unique target for NO, we carried out amperometric measurements. The results clearly show, consistent with our stopped-flow and EPR data, that the enzyme as prepared can only react with NO to a small extent (~20%), whereas after the "pulsing" procedure NO reaction occurs with a 1:1 stoichiometry (Fig. 2). Because these amperometric experiments were performed at very low NO and enzyme concentrations (<1 μM), we conclude that the pulsed enzyme has a high affinity for NO. In addition, since this reaction is prevented by pre-incubation of the enzyme (either as prepared or pulsed) with cyanide, this demonstrates that the binuclear site is the unique target for NO on the time scale explored.

We have reconciled, therefore, experiments reported in the literature regarding the reactivity of oxidized COX with NO by showing (Fig. 1) that the NO-unreactive preparation becomes reactive after "pulsing." However the NO reactivity of the pulsed enzyme is totally different in the presence or absence of Cl^-. After "pulsing" the enzyme, the NO reactivity decreases in the presence of Cl^- (Fig. 2), but is time-independent in its absence (Fig. 3) unless Cl^- is added (in which case, the reactivity of the enzyme as prepared is restored). These results suggest that the enzyme as prepared according to Soulimane and Buse (18) contains Cl^- bound, as expected from the presence of Cl^- in the buffers used in the purification. During the "pulsing" procedure, reduction of the binuclear center is associated to loss of Cl^-, allowing fast reaction of NO with the oxidized site, unless Cl^- is newly added. In agreement with this interpretation, a spectral shift (from 424 to 423 nm) synchronous with the decay of NO reactivity was observed upon addition of Cl^- to the pulsed enzyme (Fig. 4); this spectral

perturbation was previously assigned to the binding of Cl^- to the binuclear center of the oxidized enzyme (17).

The results depicted in Fig. 5 show that upon Cl^- removal, the oxidized cytochrome a_3-Cu_B site is highly reactive toward NO, with a bimolecular rate constant of $k = 2.2 \times 10^5\ M^{-1}\ s^{-1}$ at 20 °C. An interesting observation is that at high NO concentrations (>200 μM), the rate at which cytochrome *a* is reduced is no longer linear with ligand concentration. This behavior is consistent with a bimolecular reaction between NO and Cu_B^{2+}, followed by an intramolecular reverse electron transfer to cytochrome *a* (see Scheme I). Because the latter process is monomolecular, at high NO concentrations it may become rate-limiting for the reduction of cytochrome *a* at a rate of k = 150–300 s^{-1}.

In conclusion, our results show that the reactivity toward NO of bovine oxidized COX depends on Cl^-. Our observation is in general agreement with the results on the reaction of NO with oxidized *bo* from *Escherichia coli*, recently reported by Butler *et al.* (25). On the basis of optical and EPR titrations, these authors argue that, in this bacterial oxidase, two molecules of NO can bind to Cu_B^{2+} with different affinities ($K_d \approx 2.3$ and 33 μM) in the absence of Cl^-, whereas in the Cl^--bound enzyme only one NO molecule seems to have access to the metal. Therefore, in both terminal oxidases, Cl^- seems to have a similar effect, controlling the accessibility of NO to the oxidized a_3-Cu_B binuclear site. However, the 2:1 stoichiometry reported for NO binding to Cl^--free *bo* oxidase (25), although inferred by indirect spectroscopic evidence and not measured directly by NO amperometry, seems inconsistent with the 1:1 stoichiometry measured in the case of the mitochondrial enzyme. This difference may underlie a distinct property of the two enzymes, but it cannot be excluded that the mitochondrial enzyme may bind a second NO, though with much lower affinity; our amperometric measurements may have missed detecting the second reaction, having been performed at very low enzyme and NO concentrations (<1 μM) to assess *in vivo* events (4).

The point of general interest is whether this effect of Cl^- might be relevant to our understanding of the reaction of NO with mitochondrial COX *in vivo*. Because Cl^- is removed from the binuclear center by a redox cycle, and re-binding to the oxidized enzyme is extremely slow (Fig. 4), it is very likely that the enzyme during turnover is Cl^--free. One may therefore expect that *in vivo* the oxidized cytochrome a_3-Cu_B center of COX is reactive toward NO, as expected from our *in vitro* experiments in the absence of Cl^-. On the other hand, the reaction of NO with oxidized Cl^--free oxidase is much slower ($k = 2.2 \times 10^5\ M^{-1}\ s^{-1}$) than binding to reduced cytochrome a_3 ($k = 1 \times 10^8\ M^{-1}\ s^{-1}$, (26)); moreover, at the NO concentrations prevailing in different tissues (≤ 1 μM, (4)), the reaction of NO with the oxidized cytochrome a_3-Cu_B site is expected to be much slower (≤ 0.2 s^{-1}) than the rate of reduction of cytochrome a_3 (20–25 s^{-1}), independent of the physical interpretation of this rate that has been assigned either to a proton gate (27) or an electron gate (6). In conclusion, it is clear that several intermediates of COX (including the O_2 intermediates) can bind NO, and some of them can accept an electron from NO. The preferential target for NO based on kinetics is expected to be reduced cytochrome a_3; however, because during turnover the different intermediates are differently populated, a more complete assessment of the mechanism of inhibition by NO demands an estimate of the relative populations of the various species as well as their reactivity toward NO, a job in progress.

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