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Nitric oxide, cytochrome *c* oxidase and myoglobin: Competition and reaction pathways

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Abstract **It is relevant to cell physiology that nitric oxide (NO) reacts with both cytochrome oxidase (CcOX) and oxygenated myoglobin (MbO_2). In this respect, it has been proposed [Pearce, L.L., et al. (2002) J. Biol. Chem. 277, 13556–13562] that (i) CcOX in turnover out-competes MbO_2 for NO, and (ii) NO bound to reduced CcOX is "metabolized" in the active site to nitrite by reacting with O_2. In contrast, rapid kinetics experiments reported in this study show that (i) upon mixing NO with MbO_2 and CcOX in turnover, MbO_2 out-competes the oxidase for NO and (ii) after mixing nitrosylated CcOX with O_2 in the presence of MbO_2, NO (and not nitrite) dissociates from the enzyme causing myoglobin oxidation.**

Keywords: Respiration; NO inhibition; Time-resolved spectroscopy; NO catabolism; Hemeproteins

1. Introduction

Myoglobin (Mb) is a monomeric hemeprotein, expressed in myocytes of the cardiac and striated muscles, where it facilitates O_2 diffusion to mitochondria [1]. Mb reacts with NO both in the oxygenated (MbO_2) and in the deoxygenated (deoxy-Mb) states, according to the following reactions:

$$MbO_2 + NO \rightarrow \text{met-Mb} + NO_3^- \quad (1.0)$$

$k = 3\text{–}4 \times 10^7\ M^{-1}\ s^{-1}$, at 20 °C [2,3]

$$\text{deoxy-Mb} + NO \rightleftarrows \text{Mb–NO} \quad (1.1)$$

$k_{on} = 1.7 \times 10^7\ M^{-1}\ s^{-1}$, $k_{off} = 1.2 \times 10^{-4}\ s^{-1}$ at 20 °C [4]

Similarly to O_2, NO promptly reacts also with mitochondrial cytochrome *c* oxidase (CcOX), the terminal acceptor of the respiratory chain, causing enzyme inhibition [5–9]. The O_2 chemistry occurring at the heme a_3-Cu_B active site of the enzyme is a complex reaction (see reviews [10–13] and references therein) involving the rapid (μs) formation of several intermediates, which differ in the redox and ligation state of the metals in the active site. [2] NO reacts at the active site both with the fully reduced enzyme (R) and the so-called intermediates O, P and F [14,15], according to the following reactions:

$$Cu_B^+a_3^{2+} + NO \rightleftarrows Cu_B^+a_3^{2+}\text{–}NO \quad (2.1)$$

$k_{on} = 0.4\text{–}1.0 \times 10^8\ M^{-1}\ s^{-1}$ [16,17], $k_{off} = 4 \times 10^{-3}\ s^{-1}$ [18] at 20 °C

$$Cu_B^{2+}a_3^{3+} + NO \xrightarrow{k_1} Cu_B^+NO^+a_3^{3+} \underset{OH^-}{\overset{k_2}{\nearrow}} Cu_B^+a_3^{3+}NO_2^- + H^+ \quad (2.2)$$

$k_1 = 10^4\text{–}10^5\ M^{-1}\ s^{-1}$, $k_2 \gg k_1$, at 20 °C [19–22]

These reactions account for the prompt, reversible inhibition of respiration always observed when NO is added to a respiring system, no matter whether CcOX is solubilized [18] or present in intact cells [23]. Depending on the CcOX intermediates prevailing at steady-state under a given set of experimental conditions, one or the other of these two different reaction pathways prevails, leading to accumulation of either a ferrous NO-bound derivative or to a CcOX-mediated degradation of NO to NO_2^-.

By measuring the accumulation of nitrite and nitrate originating from the reaction of NO with purified CcOX and MbO_2, respectively, it has been advocated [24] that in vitro CcOX in turnover with cytochrome *c* and O_2 can efficiently compete with MbO_2 for NO, despite the high rate and the essentially irreversible character of the reaction of NO with MbO_2. It has been also proposed [24,25] that upon reaction of nitrosylated fully reduced CcOX with O_2, NO bound to the enzyme is "metabolized" to nitrite within the active site prior to dissociation into the bulk.

Given that the interplay among NO, CcOX and Mb is of patho-physiological relevance, both the aforementioned hypotheses have been addressed in the present work and experimentally tested by performing time-resolved stopped-flow experiments with purified beef heart CcOX, Mb and NO gas in solution. Data herein presented do not support the proposals by Pearce et al. [24,25].

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Abbreviations: CcOX, cytochrome *c* oxidase; CcOX-NO, fully reduced NO-bound cytochrome *c* oxidase; Mb, myoglobin; MbO_2, oxygenated myoglobin; met-Mb, oxidized myoglobin; Mb-NO, nitrosylated myoglobin; deoxy-Mb, deoxygenated myoglobin

[2] The catalytic cycle can be divided into a reductive and an oxidative part. In the reductive part, the oxidised active site **O** accepts two electrons sequentially from Cu_A via heme *a*. This is an intra-molecular electron transfer that yields the fully reduced site **R**, through the formation of single electron reduced intermediates **E** ($\mathbf{E_1}$ and $\mathbf{E_2}$). The rate-limiting step in the overall cycle is the reduction of the binuclear site prior to the reaction with O_2 [33,34]. The oxidative part of the cycle is much faster (μs vs. ms) and restores the initial **O** state via formation of intermediates, so-called peroxy, **P**, and ferryl, **F**. The occupancy of each intermediate during turnover is crucial to the understanding of the overall mechanism of the reaction between CcOX and NO [22].

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2. Materials and methods

2.1. Materials

Ascorbate, ruthenium (III) hexamine, ascorbic oxidase, oxidized myoglobin (met-Mb) from horse heart were from Sigma (St. Louis, MO). Dodecyl-β-D-maltoside was from Biomol (Hamburg, Germany). Stock solutions of NO (Air Liquide, Paris, France) were prepared equilibrating degassed water with the pure gas at 1 atm; NO concentration in stock solutions was measured by spectrophotometric titration of bovine CcOX [26].

CcOX was purified from beef heart according to the protocol of Soulimane and Buse [27]. Enzyme concentration was determined using $\Delta\varepsilon_{444(red-ox)} = 156\ mM^{-1}\ cm^{-1}$ and is expressed as functional units (aa_3). Mb in the oxygenated state (MbO_2) was prepared by dithionite-reduction of met-Mb, followed by removal of excess dithionite by chromatography on a G25 column and subsequent oxygenation. The MbO_2 concentration was determined spectrophotometrically using $\varepsilon_{580} = 14.4\ mM^{-1}\ cm^{-1}$ and $\varepsilon_{542} = 13.9\ mM^{-1}\ cm^{-1}$ and the oxidation of MbO_2 to met-Mb using $\Delta\varepsilon_{423} = 48.8\ mM^{-1}\ cm^{-1}$ and $\Delta\varepsilon_{405} = -51.7\ mM^{-1}\ cm^{-1}$.

Experiments were carried out in 20 mM phosphate, pH 7.4, 1 mM EDTA and 0.1% dodecyl-β-D-maltoside. Anaerobic conditions were obtained by extensive N_2-equilibration of the buffer; when necessary, contaminant oxygen was further scavenged by addition of ascorbate and ascorbic oxidase used at the specified concentrations.

2.2. Absorption spectroscopy

Static spectra were recorded with a double-beam spectrophotometer (Jasco V-550). Stopped-flow experiments were carried out at 20 °C with an instrument (DX.17MV, Applied Photophysics, Leatherhead, UK), equipped with a monochromator or with a diode array. Light path = 1 cm. The instrument can work in a sequential mixing mode, which allows two mixing events (both in a 1:1 ratio) in sequence with a preset delay time in between. Spectra were collected over the 190–730 nm range (with a resolution of 2.1 nm), but analyzed only in the visible region. As previously described [18], when using the photodiode array mode, there is a contribution of the incident light to the NO dissociation from reduced heme a_3; thus, the reaction proceeds at a rate ($k' \sim 0.01\ s^{-1}$) faster than the thermal dissociation of NO in the dark ($k = 4 \times 10^{-3}\ s^{-1}$).

2.3. Data analysis

Data were analyzed using the software MATLAB (MathWorks, Natick, MA).

In the stopped-flow experiments in which fully reduced NO-bound CcOX (CcOX-NO) was mixed with MbO_2, the optical contribution of the two proteins was separated by analysis of the absorption changes at 599 and 577 nm; these wavelengths were found to be close to isosbestic in our diode-array stopped-flow instrument for MbO_2 and CcOX-NO oxidation, respectively. Time courses at these two selected wavelengths were then normalized for the following extinction coefficients independently estimated in our instrument: $\Delta\varepsilon_{577} = 8.7\ mM^{-1}\ cm^{-1}$ (for MbO_2 oxidation) and $\Delta\varepsilon_{599} = 14.7\ mM^{-1}\ cm^{-1}$ (for CcOX-NO oxidation). Alternatively, the optical contributions of CcOX and Mb were separated by using the "left division" command. The two analyses converged to essentially identical outputs.

The time-resolved absorption changes collected upon mixing CcOX-NO with deoxy-Mb were analyzed by using the singular value decomposition (SVD) algorithm [28]. SVD analysis yielded just one significant basis spectrum (first U column), whose time course (first V column) was fitted to an exponential decay; from the fitted amplitude, the overall (100%) optical species was reconstructed.

3. Results

3.1. Competition for NO between MbO_2 and CcOX in turnover

In these experiments, the stopped-flow apparatus was used in the sequential mixing mode to test whether CcOX in turnover competes with MbO_2 for NO. CcOX, reduced by anaerobic incubation with excess reductant (ascorbate and Ru–hexamine), is pre-mixed with equimolar MbO_2 in air-equilibrated buffer; as expected, after exposing CcOX to O_2 turnover begins. After a fixed delay time of 10 s, the mixture of MbO_2 and CcOX in turnover is further mixed with an anaerobic NO solution, and absorption spectra collected as a function of time in the stopped-flow apparatus. Concentrations of reactants were chosen in order to have in the final reaction mixture (i.e., after the second mixing) 1 μM NO in the presence of 1.2 μM MbO_2 and 1.2 μM CcOX in turnover.

As shown in Fig. 1, the absorption changes occurring within 200 ms after mixing the MbO_2/CcOX solution with NO are identical to those observed in the absence of CcOX. Thus, they are solely attributable to Mb oxidation, showing that all NO present in solution (1 μM) is very rapidly trapped by MbO_2 leading to formation of met-Mb, and does not react with CcOX while turning over. Identical results were obtained at two different (low and high) Ru–hexamine concentrations (Fig. 1), showing that all NO reacts with MbO_2 independently of the concentration of reductants, thus of the turnover rate of CcOX. The steady-state spectra of CcOX collected at the two Ru–hexamine concentrations are shown in the inset of Fig. 1: the small difference detected between the two spectra does not provide information on the relative occupancy of the different enzyme intermediates at steady-state, being compatible with a slight increase in heme a reduction. Overall, these data show that in solution MbO_2 outcompetes CcOX in turnover for NO.

3.2. Reaction of nitrosylated CcOX with O_2

This experiment aims at investigating the reaction of reduced nitrosylated cytochrome oxidase (CcOX-NO) with O_2 and specifically at verifying whether, upon exposure to O_2, NO bound to CcOX is "metabolized" to nitrite in situ at the active site of

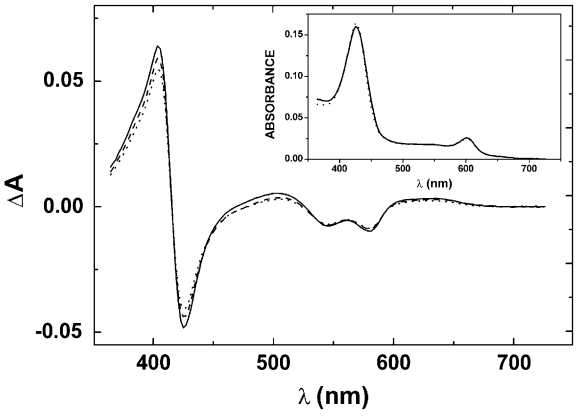

Fig. 1. On the competition between MbO_2 and CcOX in turnover for NO. Absorption changes occurring within 200 ms after exposing to NO (1 μM) a solution containing MbO_2 (1.2 μM) and CcOX (1.2 μM aa_3) in turnover, the steady-state of CcOX being sustained by 2.5 mM ascorbate and either 5 μM (dotted spectrum) or 125 μM (solid spectrum) Ru–hexamine. $T = 20$ °C. Description of this sequential mixing stopped-flow experiment is given in the text. The observed difference spectra (dotted and solid) correspond solely to 1 μM MbO_2 being oxidized to met-Mb; an identical signal is observed when omitting CcOX from the experiment (dashed spectrum). This shows that all the NO is consumed to oxidize MbO_2 to met-Mb, and thus that MbO_2 outcompetes CcOX in turnover for NO. Inset: steady-state absolute spectra of CcOX collected at 5 μM (dotted) or 125 μM (solid) Ru–hexamine.

the enzyme, prior to dissociation into the bulk. The rationale of the experiment was to follow the reaction of CcOX-NO with O_2 in the presence of MbO_2 used to probe the product released from the enzyme (whether NO or NO_2^-).

The absorption spectra collected over 200 s after mixing CcOX-NO with an air-equilibrated solution containing excess MbO_2 are depicted in Fig. 2 (top panel). As indicated by the absorption decrease at ~605 nm, NO slowly dissociated from reduced heme a_3 in the presence of O_2 and CcOX was oxidized; concomitantly, MbO_2 was oxidized to met-Mb as indicated by the absorption decrease at the α and β bands of MbO_2 (Fig. 2, top panel).

The relative optical contributions of Mb and CcOX were estimated at 577 nm (middle panel, trace 1) and 599 nm (bottom panel, trace a), which are isosbestic for CcOX-NO and MbO_2 oxidation, respectively. As shown in the middle panel of Fig. 2 (trace 1), met-Mb was accumulated at a rate ($k' = 0.012\ s^{-1}$) compatible with NO dissociation from reduced heme a_3 (slow phase of trace a in the same figure) and to an extent that approaches the amount of NO initially bound to CcOX (8.5 μM). Under otherwise identical experimental conditions, upon mixing MbO_2 with NO, the rapid oxidation of MbO_2 (see Section 1) is lost in the dead time of the stopped-flow (trace 4). Moreover, in line with the literature [29], met-Mb accumulation was not observed upon mixing MbO_2 with excess (50 μM) nitrite or nitrate (Fig. 2, middle panel, traces 2 and 3). This finding rules out the possibility that nitrite putatively produced in the reaction of CcOX-NO with O_2 [24] is responsible for the oxidation of MbO_2. Rather, these data support the original proposal [9] that, in the presence of excess O_2, NO is displaced from reduced heme a_3 and released as such into the bulk, where it reacts with MbO_2. The latter

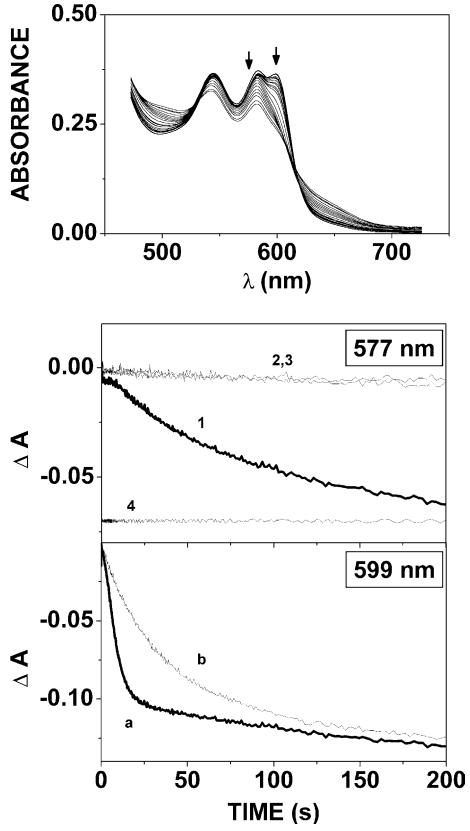

Fig. 2. Reaction of reduced nitrosylated CcOX with O_2 in the presence of MbO_2. Reduced nitrosylated CcOX was mixed with an air-equilibrated solution of MbO_2. Nitrosylated CcOX was prepared by anaerobic incubation with 2 mM ascorbate and 50 nM Ru–hexamine, followed by addition of stoichiometric amounts of NO immediately before the experiment. Concentrations after mixing: [CcOX] = 8.5 μM aa_3; [MbO_2] = 17.4 μM. $T = 20$ °C. *Top panel.* Absorption spectra collected over 200 s after mixing. The oxidation of MbO_2 and CcOX were monitored at 577 and 599 nm (see arrows and Section 2) and the relative time courses are depicted in the middle (trace 1) and in the bottom panel (trace a). *Middle panel.* The oxidation of MbO_2 (trace 1) proceeds at $k' = 0.012\ s^{-1}$ and the amount of accumulated met-Mb (~8.0 μM) matches the concentration of NO bound to CcOX. Over the same time range, no significant oxidation of MbO_2 is observed after mixing with either nitrite or nitrate at a final concentration of 50 μM (traces 2 and 3). As expected, MbO_2 is oxidized within the dead-time of the instrument upon mixing with free (not CcOX-bound) NO in solution (trace 4). *Bottom panel.* The oxidation of CcOX is biphasic and faster in the presence of MbO_2 (trace a) than in its absence (trace b).

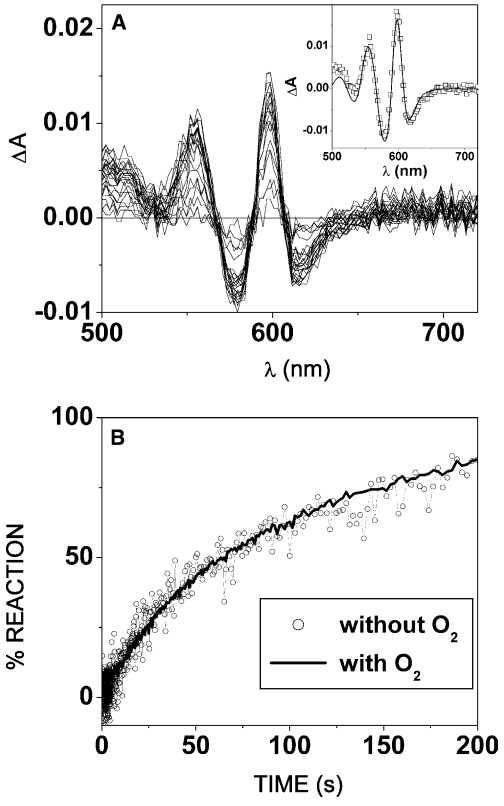

Fig. 3. NO transfer from reduced CcOX to deoxy-Mb. (A) Absorption changes collected over 200 s after anaerobically mixing reduced nitrosylated CcOX with deoxy-Mb. Baseline: final spectrum. Nitrosylated CcOX was prepared by anaerobic incubation with 2 mM ascorbate and 1 μM Ru–hexamine followed by addition of stoichiometric NO; MbO_2 was deoxygenated by anaerobic incubation with 2 mM ascorbate + 90 ng/ml ascorbic oxidase. Concentrations after mixing: [CcOX] ~ 7.0 μM aa_3; [deoxy-Mb] ~ 15 μM. $T = 20$ °C. Inset: SVD analysis of the time-resolved spectra shown in (A) yields the difference spectrum indicated by open squares (see Section 2); this corresponds to at least 6.0 μM NO being transferred from reduced CcOX to deoxy-Mb (solid spectrum). (B) Time course of NO transfer from nitrosylated CcOX to either deoxy-Mb (open circles, as obtained by SVD analysis of the spectra in A) or MbO_2 (solid curve, from the data in Fig. 2, middle panel, after normalization to 100%). The presence/absence of O_2 makes no difference to the rate and extent of NO transfer arising through dissociation from CcOX.

conclusion is also consistent with the finding that addition of MbO_2 accelerates the apparent oxidation of CcOX-NO by scavenging dissociated NO and preventing its recombination to reduced CcOX (Fig. 2, bottom panel). Consistent with previous data on the dissociation of CO from the fully reduced CO-derivative of CcOX [30,31], the oxidation of CcOX observed in the presence of MbO_2 was biphasic (Fig. 2, bottom panel, trace a) with the fast oxidation of heme *a* accounting for approximately 80% of the reaction amplitude, followed by the slower oxidation of heme a_3, at a rate that matches the oxidation of MbO_2 ($k' = 0.01\ s^{-1}$). Thus MbO_2 oxidation is synchronous to NO dissociation from reduced CcOX. Interestingly, the oxidation of heme *a* is faster than the rate of met-Mb accumulation, i.e., of heme a_3 oxidation. A very similar behavior was originally observed with the CO derivative of the enzyme [30,31], and was interpreted in terms of electron transfer between heme *a* moieties in different CcOX molecules, the oxidation of heme *a* being enzyme concentration dependent.

3.3. Anaerobic reaction of nitrosylated CcOX with Mb

To reinforce the conclusion that the slow accumulation of met-Mb observed after mixing CcOX-NO with O_2 is indeed caused by NO dissociation from reduced heme a_3, the kinetics of NO transfer from CcOX to Mb has been also investigated under strict anaerobic conditions. Upon mixing CcOX-NO with a twofold excess deoxy-Mb, we observed an essentially quantitative and synchronous transfer of NO from reduced heme a_3 to deoxy-Mb, proceeding at $k' = 0.012\ s^{-1}$ (Fig. 3, see Section 2). The time course of NO binding to Mb under anaerobic conditions was identical to that of met-Mb formation measured under aerobic conditions, i.e., after exposing CcOX-NO to O_2 (Fig. 3B).

4. Discussion

NO reacts with MbO_2 quickly and irreversibly, forming inert nitrate [2,3]. CcOX also reacts with NO very rapidly, although the kinetics of the reaction are quite different with different CcOX intermediates (see [15] for a review). Recently, based on steady-state experiments carried out in vitro with the purified MbO_2 and CcOX in solution, it has been suggested that CcOX in turnover with substrates could efficiently compete with MbO_2 for NO [24].

In the present work, we re-address this issue making use of a sequential rapid mixing apparatus that allows a tight control of the experimental timing in the millisecond time regime, and also a safe control of the concentration of the highly reactive and diffusible gaseous components (NO, O_2). We found that at equimolar concentration of CcOX and MbO_2, and regardless of the turnover rate of CcOX, added NO solely and quantitatively reacted with MbO_2 (Fig. 1) leading to formation of met-Mb (stoichiometric with NO). This result is fully consistent with expectations since among all the CcOX intermediates (see footnote 2), only the fully reduced species (R) was shown to bind NO as rapidly as MbO_2; the occupancy of R, however, is almost negligible at steady-state. On the contrary, the intermediates O, P, F, which are predominantly populated during turnover, react with NO much more slowly then MbO_2 ($k \sim 10^4$–$10^5\ M^{-1}\ s^{-1}$ [19–22] vs. 3–$4 \times 10^7\ M^{-1}\ s^{-1}$ [2,3]). The data reported in this work rules out the possibility that purified CcOX in turnover with reductants and O_2, can outcompete in vitro MbO_2 for NO even when the two proteins are at comparable concentration.

By using MbO_2 as an optical probe, we have also experimentally tested the proposal [24,25] that NO bound to reduced CcOX, upon exposure to O_2, is "metabolized" to nitrite in situ, i.e., within the active site of the enzyme. The spectral changes clearly show that upon mixing nitrosylated CcOX with MbO_2, both proteins become oxidized with met-Mb accumulating at the rate of NO dissociation from reduced heme a_3 (Fig. 2). Moreover, no reaction was observed over the time scale of hundreds of seconds upon mixing MbO_2 with high concentrations (50 μM) of nitrite or nitrate. These results show that NO bound to CcOX, in the presence of O_2, is released into the bulk as such (not as nitrite, see Fig. 2) and oxidizes MbO_2. This is further suggested by experiments carried out anaerobically whereby nitrosylated myoglobin (Mb-NO) was formed stoichiometrically with the oxidase, and at a rate equal to NO dissociation from CcOX as measured in air (Fig. 3). Taken together our results raise some doubts that in the presence of O_2 peroxynitrite is formed at the active site [24,25] and is released in the bulk, where it would oxidize MbO_2 [32], although this possibility is not yet excluded.

In summary, the data herein presented suggest that, working in vitro with purified proteins, (i) MbO_2 outcompetes CcOX in turnover for NO and (ii) upon exposing CcOX-NO to O_2, NO is not "metabolized" to nitrite within the active site of CcOX, but released as such into the bulk aqueous phase.

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