Nitric Oxide Reacts with the Single-electron Reduced Active Site of Cytochrome c Oxidase*

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The reduction kinetics of the mutants K354M and D124N of the Paracoccus denitrificans cytochrome oxidase (heme aa₃) by ruthenium hexamine was investigated by stopped-flow spectrophotometry in the absence/presence of NO. Quick heme reduction precedes the biphase heme a₃ reduction, which is extremely slow in the K354M mutant (kₐ = 0.09 ± 0.01 s⁻¹; kₖ = 0.005 ± 0.001 s⁻¹) but much faster in the D124N aa₃ (kₐ = 21 ± 6 s⁻¹; kₖ = 2.2 ± 0.5 s⁻¹). NO causes a very large increase (>100-fold) in the rate constant of heme a₃ reduction in the K354M mutant but only a ~5-fold increase in the D124N mutant. The K354M enzyme reacts rapidly with O₂ when fully reduced but is essentially inactive in turnover; thus, it was proposed that impaired reduction of the active site is the cause of activity loss. Since at saturating [NO], heme a₉ reduction is ~100-fold faster than the extremely low turnover rate, we conclude that, contrary to O₂, NO can react not only with the two-electron but also with the single-electron reduced active site. This mechanism would account for the efficient inhibition of cytochrome oxidase activity by NO in the wild-type enzyme, both from P. denitrificans and from beef heart. Results also suggest that the H⁺-conducing K pathway, but not the D pathway, controls the kinetics of the single-electron reduction of the active site (heme a₇-Cu₉) center, a prerequisite for the reaction with O₂, occurs via intramolecular electron transfer from heme a, which in turn is reduced by Cu₉, the metal center accepting electrons from cytochrome c. Protons (both scalar and vectorial) are made available in situ via two putative H⁺-conducting pathways, identified in the crystallographic structure (3, 4). These pathways, called K and D from the residues Lys-354 and Asp-124 of subunit I, play different roles in the mechanism, as extensively investigated by site-directed mutagenesis (see Refs. 1, 2, and 5 for reviews).

The catalytic cycle of cytochrome c oxidase can be divided into a reductive and an oxidative part. In the reductive part, two electrons are sequentially transferred to the fully oxidized heme a₇-Cu₉ center called O, yielding the two-electron reduced site R via a single-electron reduced intermediate E. In the oxidative part, upon reaction with O₂, R restores the fully oxidized enzyme O, by populating the O₂ intermediates P and F (depending on the redox state of heme a, two different P intermediates are formed, called P₉ and P₈). The idea that the O → R process is the rate-determining step in the overall catalytic cycle is gaining further support (6–8).

Mutation of Lys-354 to M within the K pathway yields a virtually inactive enzyme, as shown for the Rhodobacter sphaeroides aa₃ (9–11), the Escherichia coli bo₃ (12), and the Paracoccus denitrificans aa₃ (5). This mutation affects primarily, but not exclusively (see Ref. 13), the reductive part of the catalytic cycle (9, 11, 14); in the absence of O₂ and with a large excess of reductant, heme a₉ is reduced at an extremely low rate (time scale of several minutes) as compared with the wild type (time scale of tens of milliseconds). This reduction block is presumably due to an impaired H⁺ transfer in the K354M mutant, consistent with the loss of the millisecond phase in laser-triggered reverse electron transfer experiments observed with the analogous mutant of the R. sphaeroides enzyme (15).

Recentely, two groups (16–18) reported time-resolved electrometric measurements on liposome-reconstituted mutants of the P. denitrificansCcOX by laser excitation of ruthenium(II) bispyridyl. According to Ruitenberg et al. (16), injection of a single electron into the oxidized enzyme is coupled to an H⁺ transfer through the K pathway, linked to reduction of heme a. In contrast, Verkhovskiy et al. (18) proposed that an H⁺ uptake through the K pathway controls the single-electron reduction of heme a₇-Cu₉ (O → E), impaired in the K354M mutant, whereas the formation of the two-electron reduced active site (E → R) would be coupled to an H⁺ uptake through the D pathway, as deduced from data on the inactive D124N mutant.

The abbreviations used are: CcOX, cytochrome c oxidase; SVD, singular value decomposition; O, enzyme with oxidized heme a₇-Cu₉ site; E, enzyme with a single-electron reduced heme a₇-Cu₉; R, enzyme with a two-electron reduced heme a₇-Cu₉. The amino acid numbering is based on the P. denitrificans cytochrome c oxidase sequence.

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(17). The first of the two protons taken upon reduction of the active site has been proposed to charge-compensate the reduction of Cu in the single-electron reduced active site via protonation of a putative OH– bound to this metal in the oxidized state (1, 17).

The effect of the K354M mutation is drastic in the reductive part of the catalytic cycle but much smaller in the oxidative part. As assessed by the flow-flash technique using the R. sphaeroides CcOX analogous to the K354M mutant, the fully reduced enzyme exposed to O2 becomes fully oxidized within ~5 ms (15), although without the formation of P4 (13). The loss of oxidase activity associated to the K354M mutation has been therefore assigned to the extremely slow formation of R (9, 11, 14), which is a prerequisite for the reaction with O2.

Differently from O2, NO has been suggested to bind not only to R (19) but also to a single-electron reduced intermediate E (20, 21). This hypothesis, raised to account for the very low apparent K for NO inhibition, although consistent with computer simulations (20, 21), is not yet supported by direct experimental evidence. In this report, we provide evidence for the reaction of NO with E by studying the kinetics of reduction of the K354M and D124N mutants of P. denitrificans CcOX in the presence of NO.

**EXPERIMENTAL PROCEDURES**

Dodecyl-β-p-maltoside was purchased from Biomol (Hamburg, Germany); ascorbate, glucose oxidase, and catalase were purchased from Sigma; and ruthenium(III) hexamine was purchased from Aldrich. Stock solutions of NO (Air Liquide, Paris, France) were prepared by equilibrating degassed water with the pure gas at 1 atm ([NO]~2 m M at 20 °C).

The K354M and D124N mutants of cytochrome c oxidase from P. denitrificans were purified according to Ref. 22 and stored at –80 °C. Before use, the enzymes were equilibrated by dialysis (at 4 °C for at least 5 h) with the buffer used in the experiments (100 m M K/phosphate, pH 7.0, + 0.1% dodecyl maltoside or 35 m M K/phosphate, pH 7.0, + 50 m M KCl + 0.1% dodecyl maltoside). Cytochrome oxidase is expressed in terms of functional units (units) using the extinction coefficient Δεredox = 156 m M cm−1 in the visible range (22).

Stopped-flow experiments were carried out with a DX.17MV Applied Photophysics instrument equipped with a diode array (Leatherhead, UK). The mixing apparatus allows rapid mixing of equal volumes of solutions either in a simple or a sequential mode; in the latter mode, two solutions are premixed, and after a preset delay, they are mixed again with another solution. The instrument has a 1-cm light path and can equilibrate degassed water with the pure gas at 1 atm (50% total amplitude) and k = 0.005 s−1 (70% total amplitude). In the presence of NO, heme a3 reduction is much faster with rate constants of k = 8.9 s−1 (50% total amplitude) and k = 0.58 s−1 (50% total amplitude).

**RESULTS**

In the present investigation, we studied by stopped-flow spectrophotometry the kinetics of reduction of the K354M and the D124N mutants of the P. denitrificans CcOX both in the presence and in the absence of NO. As shown in Fig. 1, the K354M mutation yields a dramatic decrease in the rate of heme a3 reduction, consistent with the literature (9, 11, 14, 17). Upon mixing anaerobically oxidized K354M CcOX with a large excess of ascorbate and ruthenium hexamine, heme a reduction is very fast (~7 ms), whereas reduction of heme a3 is extremely slow (>500 s, Fig. 1A). SVD analysis of the latter process shows a single significant optical component (corresponding to the reduced minus oxidized heme a3 spectrum), displaying a biphasic time course (Fig. 1C). Best fit of the time course yields k = 0.09 ± 0.01 s−1 and k = 0.005 ± 0.001 s−1 for the two phases, accounting for ~30 and 70% of the total amplitude, respectively. We cannot exclude that the intrinsic reduction rate might be even slower than observed given that over the very long time scale explored (500 s), the high intensity light beam of the diode array instrument causes partial enzyme reduction even in the absence of reductants (data not shown). In agreement with others (5, 9, 11), we conclude that the very slow heme a3 reduction may account for the extremely slow turnover observed with O2 (~0.02 mol of O2/mol of CcOX × s at 170μm cytochrome c).

A different scenario is observed when the reduction of the K354M CcOX is carried out in the presence of NO (Fig. 1B). In these experiments, the stopped-flow apparatus was used in the sequential mixing mode to prevent the prolonged incubation of NO with the reductants in the stopped-flow syringe to avoid NO loss (see “Experimental Procedures”). At 500 μM NO (concentration after mixing), heme a reduction is again complete within a few milliseconds. In this case, however, the end point species (i.e. the fully reduced enzyme with NO bound to heme a3) is already populated after about 10 s, indicating a much
Fig. 2. Reduction of the D124N aa₃ and the effect of NO. The oxidized D124N mutant of the *P. denitrificans* cytochrome oxidase is anaerobically mixed with reductants in the absence (A) and in the presence of NO (B). Experimental conditions are as described in the legend for Fig. 1. A and B, similar to the K354M aa₃, heme a reduction is complete within a few milliseconds, independently of NO. Afterward, complete reduction of the enzyme is achieved within a few seconds in the absence of NO (A) and on an even shorter time scale in the presence of NO (B). C, time courses of heme a₃ reduction as obtained by the pseudoinverse analysis. In the absence of NO, heme a₃ is reduced with rate constants of \( k₁ = 15 \text{ s}^{-1} \) (60% total amplitude) and \( k₂ = 2.7 \text{ s}^{-1} \) (40% total amplitude). At 500 \( \mu \text{M} \) NO, heme a₃ reduction is faster and proceeds with rate constants of \( k₁ = 78 \text{ s}^{-1} \) (60% total amplitude) and \( k₂ = 5.1 \text{ s}^{-1} \) (40% total amplitude).

Faster internal electron transfer in the presence of NO. SVD analysis of the absorption spectra collected from 7 ms up to 10 s reveals only a single optical component corresponding to the [heme a₃²⁻]-NO]-[heme a₃] difference spectrum, indicating that NO binding is rate-limited by (and thus, apparently synchronous with) heme a₃ reduction. Analysis of the time course in Fig. 1C shows that the time course of heme a₃²⁻-NO formation is biphasic with rate constants of \( k₁ = -8.9 \text{ s}^{-1} \) and \( k₂ = -0.6 \text{ s}^{-1} \), the two phases having similar amplitude. Thus, NO seems not to interfere with heme a₃ reduction (very fast both with and without NO) but clearly drives heme a₃ reduction, which occurs in the presence of NO at least 100-fold faster than in its absence (Fig. 1C).

The experiments reported above were extended to the D124N mutant (Fig. 2). In the latter mutant, similarly to the K354M mutant, the reduction of heme a is fast both in the presence and in the absence of NO, being complete within a few milliseconds after mixing with reductant (Fig. 2, A and B). In agreement with the literature (17), the enzyme is completely reduced within a few seconds even in the absence of NO (compare Figs. 1A and 2A), and thus, heme a₃ reduction is much faster than in the case of the K354M mutant. Also, for the D124N mutant, heme a₃ reduction is biphasic with \( k₁ = 21 \pm 6 \text{ s}^{-1} \) and \( k₂ = 2.2 \pm 0.5 \text{ s}^{-1} \) (relative amplitudes −70 and 30%, respectively). Complete reduction of the D124N mutant is accelerated in the presence of NO (Fig. 2B) and, for instance, at 500 \( \mu \text{M} \) NO, the formation of the heme a₃²⁻-NO complex proceeds at \( k₁ = 74 \pm 11 \text{ s}^{-1} \) and \( k₂ = 3.6 \pm 1.6 \text{ s}^{-1} \) (relative amplitudes −60 and 40%, respectively). Thus, we conclude that in both mutants, the addition of NO increases the rate of internal electron transfer; however, this increase corresponds to a factor of −5 for the D124N mutant and to >100-fold in the K354M mutant. This result is better visualized in Fig. 3, in which the two observed rate constants of heme a₃ reduction for both mutants are reported at different NO concentrations (from 0 to 500 \( \mu \text{M} \)). The data show that internal electron transfer in the D124N mutant is faster than in the K354M mutant. Moreover, in both mutants, the two rate constants (relative to the fast and the slow kinetic phases) depend on the NO concentration. Although the dependence is much less pronounced in the D124N mutant, all rate constants become essentially independent of [NO], reaching plateau values at \( k₁ = 13 \pm 6 \text{ s}^{-1} \) and \( k₂ = 0.7 \pm 0.3 \text{ s}^{-1} \) in the K354M mutant and \( k₁ = 110 \pm 16 \text{ s}^{-1} \) and \( k₂ = 3.5 \pm 1.5 \text{ s}^{-1} \) in the D124N mutant. All asymptotic values are independent of reductant concentration, as assessed by experiments at 500 \( \mu \text{M} \) NO and variable ruthenium hexamine concentration; at ruthenium hexamine concentrations above 1 mM (after mixing), the observed rate constants were independent of reductant concentration.

It is worth noticing that the faster heme a₃ reduction observed with both mutants in the presence of NO is not due to direct reduction of the oxidized binuclear center O by NO. As demonstrated with beef heart CeOX and confirmed with the *P. denitrificans* wild-type CeOX, the reaction of NO with O occurs rapidly with the chloride-free enzyme, yielding nitrite-bound heme a₃ and reduced heme a (25), but it is prevented with the chloride-bound oxidase (26). Spectrophotometrically, we did not detect a reaction after mixing both oxidized mutants with NO (1 mM after mixing, not shown). Further in this respect, the reactivity of NO with the oxidized K354M oxidase was probed by amperometry by using a NO-selective Clark-type electrode (24). If the oxidized enzyme is anaerobically added to a degassed NO-containing solution, a reaction would be detected as a decrease in the NO concentration. Upon the addition of oxidized K354M CeOX (0.4 \( \mu \text{M} \)) to a solution containing NO (1 \( \mu \text{M} \)), only a small decrease in the NO concentration (−0.2 mol of NO/mol of oxidase) was detected; in contrast, a stoichiometric (1:1) NO binding was observed after the addition of the fully reduced enzyme, as shown for the mammalian CeOX (24). We therefore conclude that both the *P. denitrificans* mutants
tested in this study are in the chloride-bound state, due to the presence of chloride in the buffers used during both the purification and the experiments.

**DISCUSSION**

NO is a very efficient, yet reversible, inhibitor of cytochrome c oxidase activity (27, 28), leading to the proposal that it may act as a physiological modulator of cell respiration (29). Since both NO and O2 react with the fully reduced heme a3-CuB center R with high affinity and similar rates (19), the small inhibition constant Kd determined with mitochondrial CcOX in turnover (Kd = 270 nm NO at [O2] = 140 μM, (27)) was somewhat puzzling. To account for this observation, it was proposed that NO can react with a single-electron reduced active site E (20–21), which is known to be unreactive toward O2. Such a hypothesis is consistent with computer simulations (20–21) but has never been demonstrated experimentally. The kinetics of reduction of the K354M mutant of *P. denitrificans* in the presence of NO, reported above, provides evidence that this hypothesis is correct.

The K354M mutation is associated with the loss of oxidase activity (5, 9, 11), although this mutant in the fully reduced state (R) is very quickly (Δt = 5 ms) oxidized by O2, as reported for the *R. sphaeroides* CcOX (15, but see also Ref. 13). The same mutation has a dramatic effect on the reductive part of the catalytic cycle, and the extremely low rate of reduction of heme a3 was correlated to the marginal turnover rate (9, 11, 14). It was therefore assumed that in this mutant, the turnover with O2 is rate-limited by the extremely slow formation of R, which is an obligatory intermediate in the catalytic cycle. This is consistent with the widely accepted idea that O2 can react exclusively with the two-electron reduced heme a3-CuB site, like CO.

The novel result reported in this study on the K354M mutant of *P. denitrificans* CcOX is that, in the presence of NO, the reduction of heme a3 occurs at a rate much faster (>100-fold) than in its absence and much faster than the extremely low turnover rate of this mutant with O2 (Fig. 1). This effect depends on NO concentration and is maximal at [NO] > 100 μM (Fig. 3), at which the reduction of heme a3 proceeds at rates (k1 = 13 ± 6 s–1; k2 = 0.7 ± 0.3 s–1) both remarkably larger than the turnover rate (Δt = 0.02 mol of O2/mol of CcOX x s at [O2] > 250 μM). This finding is diagnostic of a different reactivity of O2 and NO with CcOX. It is indeed difficult to account for this result, assuming that NO, similarly to O2, can react exclusively with R, which combines with very high affinity and second order rate constants with both ligands. If this were the case, the formation of the reduced NO-bound heme a3 would be much slower, being rate-limited by the formation of R, which in turn accounts for the extremely low oxidase activity. Therefore this result implies that NO can react not only with the two-electron reduced heme a3-CuB site R (19) but also with the single-electron reduced site E, whose occurrence in this mutant was already documented (11). We do not have a valid explanation for the observed heterogeneity in the reduction of heme a3, but we notice that a similar biphasic behavior was reported also for the beef heart enzyme (7, 8).

Working with beef CcOX, it was shown that the reaction of NO with CuB in the oxidized binuclear site (O) occurs rapidly only with the chloride-free enzyme, leading to reduced heme a and nitrite-bound oxidized heme a3 (25) but is prevented by the binding of chloride (26). This behavior was reproduced with the *P. denitrificans* wild-type enzyme. On mixing either of the mutants in the oxidized state with a large excess of NO, we did not observe spectrophotometrically either heme a reduction or nitrite formation. Moreover, we observed by amperometry only a small reaction between NO and oxidized K354M (similar to that generally detected even with the beef heart enzyme in the chloride-bound form, see Ref. 26). We therefore conclude that the two mutants employed in this study are in the Cl–bound form since chloride is present in the buffers used during the purification and the experiments. This further implies that the enhanced reduction of heme a3 observed in the presence of NO cannot be assigned to the direct reaction of NO with O2.

It is noteworthy that the dependence on [NO] reported in Fig. 3 is consistent with the idea that the K354M mutation impairs the O → E electron transfer step (17, 18). Assuming that NO binds to heme a3 in the E and the R states with very high affinity and second order rate constants, one may expect that NO should efficiently drive thermodynamically the reduction of heme a3 in the O → E step. On this basis, the NO concentration dependence of the apparent rate constants measured for the K354M mutant (Fig. 3) seems diagnostic of a relatively slow forward electron transfer (maximal value of 13 ± 6 s–1) as compared with an unusually fast reverse electron transfer (heme a3/CuB → heme a) caused by the mutation. In this context, it is interesting to notice that the D124N mutant shows a kinetic behavior remarkably different from the one displayed by the K354M mutant. In the D124N mutant, (i) heme a3 reduction in the absence of NO is much faster than in the K354M mutant, in agreement with Wikström *et al.* (17), and (ii) NO increases this rate at most by a factor of −5 (ratio of the rate constant at saturating [NO] over the value measured in the absence of NO), i.e., much less than the over 100-fold increase observed with the K354M mutant. These results are fully consistent with the hypothesis that the K pathway, but not the D pathway, controls the first electron transferred to the oxidized heme a3-CuB site (16–18).

The analogous K354M mutant of the *R. sphaeroides* CcOX has been reported to display cytochrome c-peroxidase activity with a Km value of ~50 μM H2O2 and a Vmax value of ~25 s–1 (14, 30). The maximal turnover with H2O2 was much faster than the turnover with O2, and this result was interpreted as an evidence that H2O2 reacts with O, yielding the intermediate P, i.e., bypassing the whole reductive part of the catalytic cycle (14). Later on, it was proposed that H2O2 might react with E in the K354M mutant, yielding directly the F intermediate, thus bypassing the formation of both R and P (2, 31). We wish to point out that according to our results, the latter interpretation has to be favored, and in this respect, H2O2 and NO behave similarly since they are both assumed to react with E. We notice that the maximal turnover number for the peroxidase activity (25 s–1, see Ref. 14) is not inconsistent with the faster rate constant for the O → E process that we estimate from our data on the K354M mutant at saturating [NO] (13 ± 6 s–1; Fig. 3, top panel). In other words, we propose that in the K354M mutant, the rate constant of heme a3 reduction measured at saturating concentrations of NO and ruthenium hexamine is the forward rate constant for the single-electron reduction of the heme a3-CuB site, which is also rate-limiting the cytochrome c-peroxidase activity in saturating [H2O2].

In conclusion, our results provide direct evidence that NO, differently from O2, can react with a single-electron reduced heme a3-CuB in CcOX. This finding validates the original hypothesis (20, 21) raised to account for the finding that NO is a potent inhibitor of CcOX (27, 28).

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