Dynamics of Nitric Oxide in the Active Site of Reduced Cytochrome *c* Oxidase aa_3

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ABSTRACT: Nitric oxide (NO) is involved in the regulation of respiration by acting as a competitive ligand for molecular oxygen at the binuclear active site of cytochrome *c* oxidase. The dynamics of NO in and near this site are not well understood. We performed flash photolysis studies of NO from heme a_3 in cytochrome *c* oxidase from *Paracoccus denitrificans*, using femtosecond transient absorption spectroscopy. The formation of the product state—the unliganded heme a_3 ground state—occurs in a similar stepwise manner (period ∼700 fs) as previously observed for carbon monoxide photolysis from this enzyme and interpreted in terms of ballistic ligand motions in the active site on the subpicosecond time scale [Liebl, U., Lipowski, G., Négrerie, M., Lambry, J.-C., Martin, J.-L., and Vos, M. H. (1999) *Nature 401*, 181−184]. A fraction (∼35% at very low NO concentrations) of the dissociated NO recombines with heme a_3 in 200−300 ps. The presence of this recombination phase indicates that a transient bond to the second ligand-binding site, a copper atom (CuB), has a short lifetime or may not be formed. Increasing the NO concentration increases the recombination yield on the hundreds of picoseconds time scale. This effect, unprecedented for heme proteins, implies that, apart from the one NO molecule bound to heme a_3, a second NO molecule can be accommodated in the active site, even at relatively low (submicromolar) concentrations. Models for NO accommodation in the active site, based on molecular dynamics energy minimizations are presented. Pathways for NO motion and their relevance for the regulation of respiration are discussed.

The respiratory enzyme cytochrome *c* oxidase (CcO)[1] catalyzes the reduction of molecular oxygen to water. Among the cofactors of this membrane protein, an A-type heme, heme a_3, and a nearby (∼5 Å) copper atom, CuB, coordinated with three histidines via electrostatic interactions, are comprised in the "active site": they are directly involved in the binding of, and the delivery of electrons to, oxygen and the reaction intermediates (*1*). Apart from oxygen, the small inhibitor and signaling molecules nitric oxide (NO) and carbon monoxide (CO), as well as cyanide and azide, can be bound to the active site with high affinity. Recently, evidence has been produced that NO is directly involved in the regulation of the respiration rate via its reversible binding to the enzyme, in competition with oxygen (*2*−*4*). On a molecular level, the dynamics of NO into, within and out of the active site, characterizing this competitive binding, are not well established, but they appear to be different from the relatively well-studied dynamics of CO traffic (ref *5*, see below).

During the reaction cycle, binding of molecular oxygen occurs when the catalytic site of the enzyme is in the reduced state ($a^{2+}a_3{}^{2+}CuB^{+}$, heme *a* being a second A-type heme not involved in ligand binding) (*5*−*7*). Whereas O_2 and competitive ligands will eventually bind to heme a_3, CuB is thought to act as a "doorstep" for ligand traffic into or out of the active site. For CO and O_2, the maximum rate of binding to heme a_3 is limited by transfer from CuB to heme a_3 (*8*−*11*). In contrast, for NO such a limitation has not been observed (*8*), indicating that NO does not or only very briefly transiently bind to CuB. Also, whereas virtually all photolyzed CO—after being transferred to CuB in a ballistic way (*12*)—leaves the protein on the microsecond time scale (*13*), flash photolysis leads to significant geminate recombination of the heme a_3−NO pair on the submicrosecond time scale (*8*). It is unknown whether NO transiently binds to CuB after dissociation from heme a_3.

An important issue with respect to the regulatory competitive binding of different ligands is whether more than one ligand can simultaneously be present in or near the active site. For CO no evidence for more than one ligand has been reported [ref *14*, also the crystal structure of the CO-bound form of the mitochondrial enzyme shows only one CO molecule accommodated (*15*)]. For NO, binding both to heme a_3 and to CuB has been reported to occur at high ligand concentrations (*14*, *16*). At low concentrations however, NO uptake from the solvent has been reported to be stoichiometric (*17*).

In this work, we study the femtosecond and picosecond dynamics of NO in and near the active site following flash photolysis, using ultrafast spectroscopy. In heme proteins, the dynamics of NO rebinding to heme ("geminate recombination") have often been used as a probe for protein relaxation and dynamics of the heme environment (*18*, *19*).

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[1] Abbreviations: CcO, Cytochrome *c* oxidase.

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In the present work on CcO, where NO can act as a physiological ligand, we show that alterations in the heme environment induced by *additional* NO molecules can be sensed by the dynamics of the NO dissociated from the heme. The functional relevance of these findings will be discussed.

MATERIALS AND METHODS

Cytochrome *c* oxidase aa_3 from *Paracoccus denitrificans* was purified as described (*12*). Samples were prepared to an enzyme concentration of ~35 μM, extensively degassed in a gastight vessel, reduced with 10 mM sodium ascorbate and 5 mM *p*-phenylenediamine (or 20 mM sodium dithionite for CO-liganded samples) and transferred to a degassed gastight optical cell (Hellma, 117.007 QS, optical path length, 1 mm) sealed with a rubber septum. NO-liganded samples were obtained by equilibration with gaseous NO at a partial pressure of 0.1 bar (and 0.9 bar Ar). For experiments with different NO:enzyme stoichiometries, specific quantities of gaseous NO were added using a gastight Hamilton syringe, and the sample was equilibrated during 15 min at 4 °C prior to the measurements. For the lowest NO concentrations (1:1 and 5:1), only partial occupancy of the a_3 site by NO occurred as judged from the steady-state spectra (~70% for the equimolar concentration) and the NO binding diminished significantly during signal averaging (~1 h). The amplitude of the dissociation signal at $t > 30$ ps diminished concomitantly, but its kinetic shape did not significantly alter during this time.

Steady-state spectra were recorded with a Shimadzu 1601 UV−vis spectrophotometer. Femtosecond spectroscopy using 55-fs pump pulses centered at 590 nm and white-light continuum probe pulses was performed as described (*18*). For the 300-ps time scale experiments, small variations in pump−probe overlap as a function of delay time were corrected for using the photodissociation signal of CO-myoglobin, where no recombination occurs on the time scale less than 300 ps. All experiments were performed at room temperature.

Structural modeling of NO-liganded CcO based on molecular dynamics energy minimizations were performed using version 24 of CHARMM (*20*) as described previously for CO-bound CcO (*12, 21*). The initial Fe−N and Cu−N bond lengths (1.74 and 1.76 Å, respectively) and Fe−N−O and Cu−N−O angles (145 and 163°, respectively) were taken from model ironporphyrin−NO complexes (*22*) (similar values are found in structures from other NO-liganded heme proteins, with the notable exception of NO−myoglobin (*23*)) and a model Cu−NO complex (*24*).

RESULTS

Figure 1A shows the steady-state absorption spectrum of the enzyme in its reduced and NO-liganded form. The NO-bound spectrum, showing well separated Soret bands for the *a* (442 nm) and a_3−NO (432 nm) bands is very similar to that previously reported for the NO-bound mitochondrial enzyme (*16*).

We have previously demonstrated (*12*) that CO dissociation from heme a_3 leads to the coherent formation of the product state—the ground state of unliganded heme a_3—which has a maximum at 442 nm. Figure 2 shows that at this wavelength the oscillatory modulations (frequency ≈ 47 cm^{-1}) on the time scale of ~1 ps, reflecting the coherent nature of this reaction, are very similar for CO dissociation and for NO dissociation. In addition, as with CO dissociation, the amplitude of this modulation follows the band shape of unliganded heme a_3 and its phase is approximately constant over the band (not shown). As outlined in detail in ref *12*, the ensemble of these characteristics shows that the product state is populated in a coherent, stepwise, manner, indicating

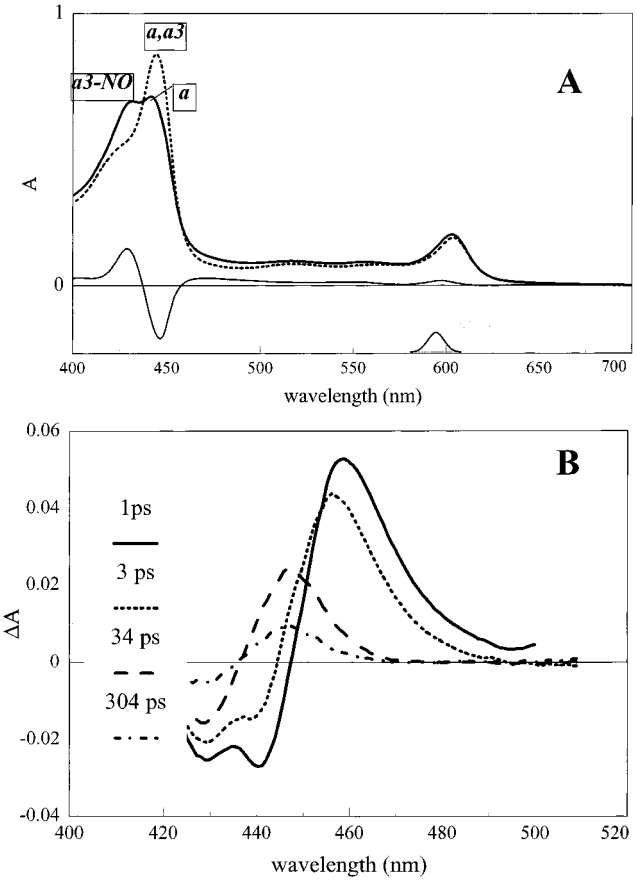

FIGURE 1: (A) Steady-state absorption spectra of fully reduced cytochrome *c* oxidase in the unliganded state (dashed) and NO-liganded state (solid). Lower solid curve: NO-liganded minus unliganded difference spectrum. The hatched curve represents the spectral profile of the pump pulse. (B) Transient absorption spectra in the Soret region at various pump−probe delay times.

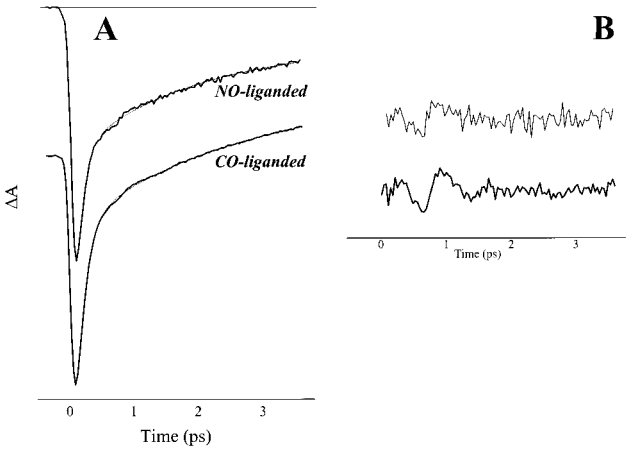

FIGURE 2: (A) Transient kinetics (solid) at 442 nm for fully reduced cytochrome *c* oxidase liganded with NO (upper) and CO (lower, taken from ref *12*) and fits to a multiexponential function (thin dashed lines). (B) Fit residuals (expanded ×10).

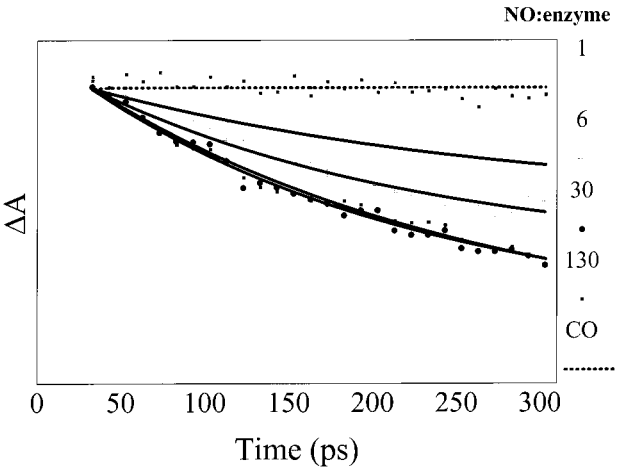

FIGURE 3: Geminate recombination of heme a_3−NO after photodissociation as a function of NO concentration. The kinetics at 447 nm are shown after complete decay of the excited states and normalized at 34 ps. The dashed line represents a similar curve (442 nm) for CO photodissociation.

that the motions of NO in the active site are ballistic (see Discussion).

On the hundreds of picoseconds time scale, however, the heme-ligand interactions are different for NO and CO. CO remains bound to CuB up to the microsecond time range. For NO, the intensity of the photoinduced band of the unliganded heme a_3 decreases on this time scale (Figure 1B), demonstrating significant geminate recombination of the a_3−NO pair. This indicates that, if at all formed, a CuB−NO bond is less stable than the CuB−CO bond. Alternatively, dissociated NO may only, in part, bind to CuB and, in part, occupy another site from which fast recombination with heme a_3 is possible.

To investigate the possibility that more than one NO molecule can be accommodated in or near the active site, the kinetics of NO rebinding to heme a_3 as a function of NO:enzyme stoichiometry were measured (Figure 3). As the transient spectral features reflecting the photophysics of the excited unliganded hemes are completed within 30 ps (*25*), the kinetics of Figure 3, at $t > 30$ ps, reflect only a_3−NO recombination and can be directly compared. The kinetics of recombination are clearly dependent on the NO concentration in the NO:enzyme stoichiometry range of 1−30. Samples equilibrated with up to 1 bar of NO (corresponding to a NO:enzyme ratio of 13 000) gave similar results as the highest concentrations in Figure 3 (not shown). On the picosecond time scale, binding of NO from the solution cannot occur [bimolecular NO:CcO binding occurs in microseconds (*8*)], and therefore the kinetics must reflect geminate recombination or recombination with NO present within the protein at the time of the photodissociation. The observed concentration dependence thus implies that, in addition to the NO bound to heme a_3, at least one more NO can be accommodated in or near the active site. To our knowledge, this constitutes the first observation of a ligand concentration dependence on intra-protein heme-ligand recombination.

On the 30−300 ps time scale, the kinetics can be fit with a single-exponential decay component with time constant in the range of 200−300 ps and a nondecaying component at all NO concentrations. The relative amplitude of the decaying phases increases with increasing NO concentration and saturates at a NO:enzyme ratio of the order of 10, a value that, under our experimental conditions, corresponds to [NO] ≈ 1 μM in solution. Thus, assuming there are two possible sites for NO accommodation, the second site has an affinity that is roughly 10 times lower than the a_3 site.

Single-exponential fits were sufficient for describing the kinetics in the time window 30−300 ps. Using this approach, the relative amplitude of the decaying 200−300 ps phase varied from ~35% at equimolar concentrations to ~80% at the highest concentrations. We note that the overall recombination kinetics are likely to be highly multiphasic, as at least two additional exponential phases were resolved on the nanosecond time scale (*8*).

Structures of NO-bound forms of CcO have not been reported. To get some insight in possible conformations of the active site in the presence of NO, we performed molecular dynamics energy minimizations. Starting from a model based on the unliganded 2-subunit X-ray structure from the *P. denitrificans* CcO enzyme (*26*), minimizations were carried out with NO bound to the heme a_3 Fe (Figure 4A), to CuB (Figure 4B), and to both (Figure 4C). Of particular interest is that a configuration with two NO ligands, one bound to heme a_3 and one to CuB, appears possible (Figure 4C). In the minimized structure of Figure 4C, the oxygen atoms of the two NO molecules are at 3.5 Å. In this case, in comparison to the two one-NO-containing structures, the volume between CuB and heme a_3 is increased by a rearrangement which consists mainly of a displacement of CuB along with two of its coordinating histidines (His325 and His326) parallel to the heme a_3 plane. The third coordinating histidine (His276) which is covalently linked to Tyr280 (*15*, *26*, *27*), alters its position with respect to the heme only very slightly, and the distance between CuB and the His276 Nδ_1 increases from 1.76 Å (a_3−NO structure) to 3.04 Å. It is interesting to note that in recent X-ray absorption studies of quinol oxidase bo_3 the coordination of CuB by one of the three histidines was reported to be sensitive to the environment (*28*, *29*).

DISCUSSION

Coherent Motions within the Active Site. Earlier work from our laboratory has shown that dissociation of CO from heme a_3 sets in motion low-frequency concerted vibrations which are directly coupled to the reaction leading to the formation of the heme a_3 ground state, as witnessed by the stepwise formation (first and major step at ~350 fs; subsequent steps at ~700 fs interval) of this state (*12*). Apart from heme motions, the reaction coordinate of this coherent process presumably involves dynamics of the ensemble of the active site, as such dynamics are specific for CcO and for instance are not observed in myoglobin. In particular we proposed that transfer of CO to CuB is involved. This proposition was supported by molecular dynamics simulations (*21*), which also indicated a role of the unusual covalent bond between His 276 and Tyr 280 (*15*, *26*, *27*) in the concerted dynamics in different parts of the active site. We have now shown that similar reaction dynamics occur with NO as a ligand. This finding of similar dynamics upon photodissociation of different ligands strengthens our proposal that the "guided" motions involve different parts of the active site. In analogy

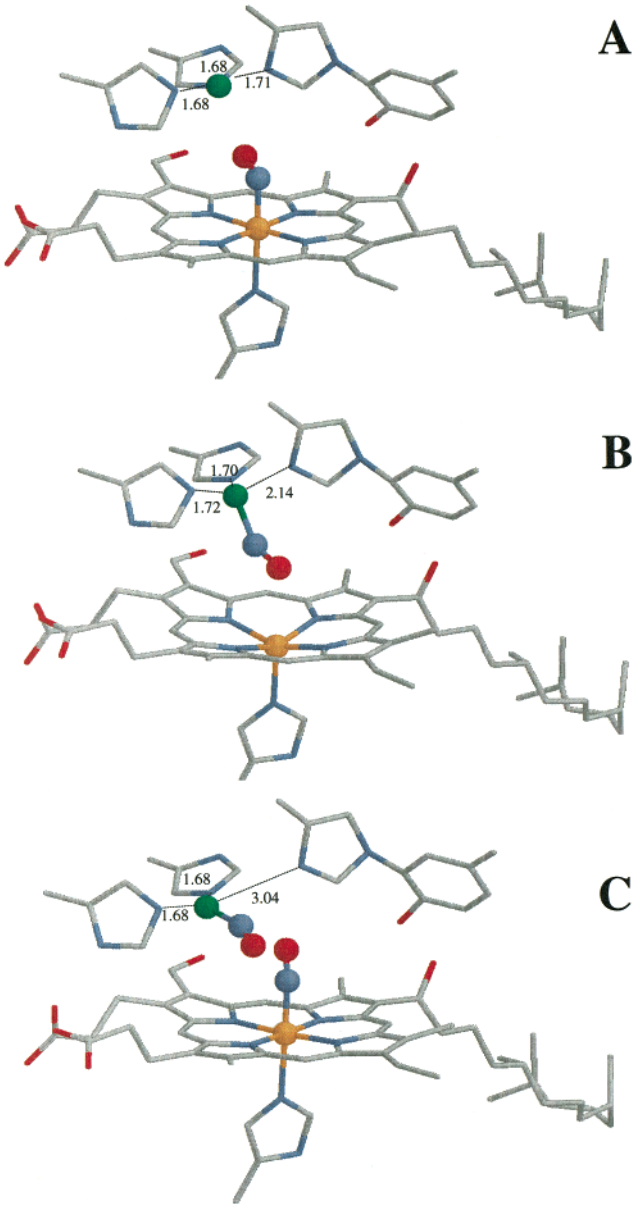

FIGURE 4: Simulated structures of the active site of CcO with NO bound to heme a_3 (A), CuB (B), and both (C). The distances between CuB and its coordinating histidines are indicated in angstroms. The program RASMOL (*38*) was used to prepare the figure.

to our reasoning for CO, it appears also possible that NO binding to CuB is part of the reaction coordinate. The possibility of formation of a NO−CuB bond will be discussed below.

Recombination of NO with Heme a_3. Generally in heme proteins, kinetics of geminate recombination of NO with heme are a useful probe for protein dynamics in the vicinity of the heme (*18*). Here, the low electronic barrier for NO (as compared to CO) rebinding to heme allows relatively fast recombination, on the same, picosecond time scale as relaxation processes in the protein. The rebinding kinetics reflect the competition between rebinding to the heme and pathways out of the "heme pocket" (the volume in which a released ligand can freely move without energetic barriers). For example, the highly multiphasic kinetics in NO-myoglobin have been used to study the influence of specific residues on the heme pocket dynamics (*30*−*32*). Recently, recombination of NO dissociated from the local NO-producing enzyme NO-synthase has also been used to study factors influencing the efficiency of NO release (*33*).

For CcO, a priori the presence of a second ligand binding site (CuB) near the heme creates a different situation: efficient trapping by this site could prevent NO from exploring the heme environment on the picosecond time scale and from rebinding. Our results clearly show that even at the lowest NO concentrations (see below) significant rebinding occurs on the time scale of hundreds of picoseconds. Thus, if all NO is transferred to CuB upon photolysis from heme a_3, the CuB−NO bond appears to be weak and (presumably thermally) cleavable on the time scale of picoseconds. In principle, it is also possible that the picosecond decay phase originates from a fraction of NO that does not transiently bind to CuB, and that occupies a different site from where it can rebind to heme a_3. In both cases, this contrasts with the situation for CO, where virtually all ligands remain bound to CuB for several microseconds (*13*). This result for NO is in general agreement with the observation that NO binding to heme a_3 is not rate limited by the presence of CuB (*8*) and with the observation that very low temperatures are required to achieve virtually irreversible NO photodissociation from heme a_3 (*34*,*35*).

Multiple Ligands. Our results demonstrate a ligand concentration effect on the *intra*-protein rebinding kinetics. As no such observation has been reported previously in a heme protein, we will shortly discuss its possible origin in a more general context. The dependence of the picosecond recombination kinetics on the NO concentration must reflect a change in the heme environment in the presence of more than one ligand. The accommodation of a second NO near the heme may reduce the volume in which the released NO can move (the heme pocket) and hence increase the probability of colliding with the heme iron, so as to rebind. At the same time, if the second NO can also move in the heme pocket (i.e., it does not initially occupy a "bound" site) both NO molecules can rebind with the heme, increasing the rate of rebinding. In the latter case, the recombination is not strictly "geminate".

For the case of cytochrome oxidase, however, the latter concept is not likely to apply directly. As it appears from our previous simulations of the CcO−CO system (*21*), the "free volume" for ligand motion is very limited in the active site, allowing mainly for rotation rather than translation of the ligand. It is therefore more likely that the second NO is present at a restricted site. The most obvious candidate for such a site is CuB, but other docking sites cannot be excluded from our data. Indeed, the simultaneous presence of a_3^{2+}-NO and CuB^{+}−NO forms under *high NO* concentration has been reported previously from EPR (*16*) and steady-state infrared (*14*) data. Our simulations (Figure 4) also indicate that such an arrangement is possible. The affinity of NO for the CuB site was reported to be weaker (*14*, *16*), in general agreement with our data. Our results indicate that already at very low NO concentrations a second NO can be accommodated by CcO.

Giuffrè and co-workers (*36*), measuring NO uptake by mammalian CcO, reported a stoichiometry of 1 NO:enzyme for low (∼5-fold excess) NO concentrations, within a few minutes. It is possible that the lower affinity of the second NO molecule leads to a slower binding (under our conditions,

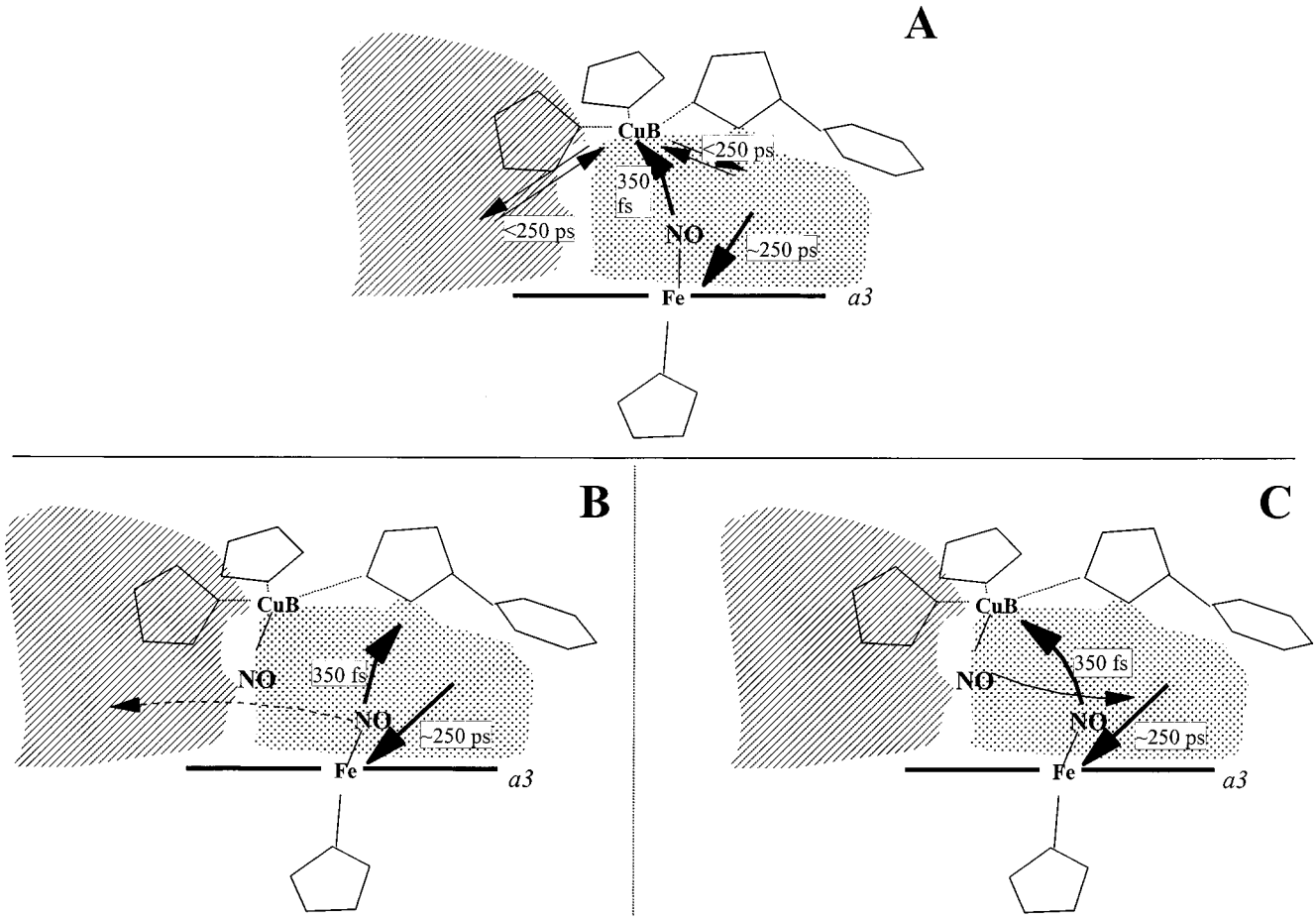

FIGURE 5: Schematic models of NO motions after dissociation from heme a_3 in the absence (A) or presence (B and C) of a second NO. The dotted and dashed zones indicate volumes from which NO is or and is not, respectively, accessible to heme a_3. The shape and separation of these zones is largely arbitrary. See text for further details.

upon addition of small amounts of gaseous NO, ~15 min were needed for maximal binding). In addition, we noted that the observed NO concentration effect on recombination in beef heart CcO is also apparent, but is less pronounced than in the bacterial enzyme (unpublished results).

Possible scenarios to understand the ensemble of results are schematically drawn in Figure 5. If NO is initially bound to heme a_3 only (Figure 5A), it can be transferred to CuB in a ballistic fashion on the subpicosecond time scale. There it resides only briefly (it is also possible that a bond is not really formed, see above). Subsequently, NO is released either into the pathway eventually leading out of the protein that is not directly exposed to heme a_3 (dashed zone) or, with a relatively low probability, into the active site area (dotted zone). From the active site it can recombine with heme a_3 in 200−300 ps.

If a second NO is present and bound to CuB, it is reasonable to assume that the dissociated NO is released into the protein moiety (Figure 5B). As the pathway out of the protein may be hindered or blocked by the presence of the CuB-bound NO, the probability for the dissociated NO to be released directly into the active site is high, with a concomitant high yield of 200−300 ps recombination. As noted above, a nonbound docking site (or equilibrium between CuB-bound and nonbound) for the second NO is also possible. In this case, it can be proposed that dissociated NO be transferred to CuB, but that release to the pathway out of the protein is hindered. We are developing parallel time-resolved optical kinetics and steady-state EPR experiments to study the question whether NO bound to CuB is at the origin of the NO concentration dependence.

Figure 5C depicts an alternative, and possibly more speculative, scheme for the situation where a second NO is initially bound to CuB. Here, we suggest that NO released from heme a_3 is transferred to CuB, in a ballistic manner, concomitantly breaking the existing CuB−NO bond. This proposal would be consistent with our previous proposition (*12*, *21*) of involvement of ligand transfer to CuB in the reaction coordinate of the coherent, stepwise reaction, that we observe with NO in high concentrations in a similar manner as with CO (Figure 2). In this way, the second NO would be released in the active site and have a high chance of combining with heme a_3. At present, we cannot discriminate between the different scenarios. Molecular dynamics simulations and extension of our experiments to a longer time range will be needed for further evaluation.

Relevance for NO Reductase Activity. The catalytic site of bacterial NO-reductase (*37*) is structurally related to that of the heme-copper oxidases, but bears a non-heme iron at the equivalent of the CuB position. This enzyme catalyzes the reduction of NO to N_2O ($2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O$), a reaction which requires two NO molecules. The co-presence of two NO molecules in the active site of CcO, as inferred from the present work, is therefore generally consistent with heme-copper oxidases being able to exert, albeit low, NO-reductase activity (*14*, *16*, *36*).

Significance for NO-Mediated Regulation of Cytochrome Oxidase Activity. A general conclusion from our work is that, even at relatively low concentrations, two ligands can be accommodated in or near the active site of CcO and influence each others kinetics. This can be relevant for the mechanism of competitive binding of NO and O_2. In particular, it can be envisaged that while NO is bound to heme a_3, thus inhibiting the enzyme, O_2 binds to CuB. It has been shown that during reversal of the inhibition, NO does not react with O_2 (*17*). A mechanism of concerted ligand swap, analogous to the one outlined in Figure 5C for two NO molecules, could avoid formation of potentially toxic [NO:O_2] complexes.

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