Molecular Basis for Nitric Oxide Dynamics and Affinity with *Alcaligenes xylosoxidans* Cytochrome c’**§**

Sergei G. Kruglik, Jean-Christophe Lamby, Simona Cianetti, Jean-Louis Martin, Robert R. Eady, Colin R. Andrew, and Michel Neggieri

The bacterial heme protein cytochrome c’ from *Alcaligenes xylosoxidans* (AXCP) reacts with nitric oxide (NO) to form a 5-coordinate ferrous nitrosyl heme complex. The crystal structure of ferrous nitrosyl AXCP has previously revealed that NO is bound in an unprecedented manner on the proximal side of the heme. To understand how the protein structure of AXCP controls NO dynamics, we performed absorption and Raman time-resolved studies at the heme level as well as a molecular computational dynamics study at the entire protein structure level. We found that after NO dissociation from the heme iron, the structure of the proximal heme pocket of AXCP confines NO close to the iron so that an ultrafast (7 ps) and complete (99%) geminate rebinding occurs, whereas the proximal histidine does not rebind to the heme iron on the timescale of NO geminate rebinding. The distal side controls the initial NO binding, whereas the proximal heme pocket controls its release. These dynamic properties allow the trapping of NO within the protein core and represent an extreme behavior observed among heme proteins.

Nitric oxide (NO) acts as a second messenger in several physiological systems, involved in the production of several cytotoxic chemical species and nitrosative stress, and appears as an intermediate in the denitrification process. Cells have, thus, developed numerous heme proteins whose diverse functions involve nitric oxide binding and/or release. In some bacteria heme sensors were recently discovered with a femtomolar affinity for NO, whereas cytochromes c’ able to bind NO are found in many nitrogen-fixing, denitrifying, and photosynthetic bacteria. Although the physiological role the bacterial cytochrome c’ from *Alcaligenes xylosoxidans* (AXCP) is not yet established, the homologous cytochrome c’ from *Rhodobacter capsulatus* has been shown to increase the resistance of this bacteria against NO toxicity. Thus, the bacterial cytochromes c’ are inferred to disclose a particular behavior regarding NO dynamics and affinity. How heme proteins interact with NO and control its reactivity is ultimately related to their structure and the heme surroundings. A wide range of affinity and kinetic constants can be observed that correlates with the molecular dynamics of NO within the protein core. A striking example of this diversity is offered when comparing the NO dynamics governed by the enzyme NO synthase, the endogenous cellular source, and the NO receptor-soluble guanylate cyclase (sGC) whose heme cofactor has a somewhat opposite role.

Nitrosylated heme proteins usually form 6-coordinate complexes having histidine and NO as axial ligands at the proximal and distal sides of the heme, respectively, but some become 5-coordinate (5c) with NO (5c-NO) like sGC (11, 12) and AXCP (13, 14). These latter proteins have an overall structure that presumably controls the strain upon the proximal histidine. Although sGC and AXCP possess different sequences and tertiary structures, they share the property that the binding of NO at the distal position of the heme leads to the cleavage of the proximal Fe²⁺-His bond and to the formation of 5c-NO complex. This so-called trans effect is due to a weakening of the axial Fe-His bond caused by the trans-Coordination of NO and is governed by protein structure. In 5c-NO species, the probability of fast NO rebinding after dissociation (either thermal or photo-induced) is increased and tends to a single phase as observed in sGC (10). In sGC, the breaking of the proximal Fe-His bond in the resting state of the enzyme triggers the catalytic activity for cGMP production, whereas in AXCP the role of the proximal bond breaking and formation of a 5c-NO complex in a second order reaction is not known.

Unexpectedly, the crystal structure of AXCP (Fig. 1) has revealed that NO is bound to the proximal side of the ferrous heme replacing the endogenous His-120 ligand (14, 19). The relation between this property not observed for other proteins and the function of AXCP should be crucial and remains to be established. In the present study we investigated how this unusual property of the novel proximal 5c-NO AXCP complex from Rhodobacter capsulatus was inferred to disclose a particular behavior regarding NO dynamics and affinity. How heme proteins interact with NO and control its reactivity is ultimately related to their structure and the heme surroundings. A wide range of affinity and kinetic constants can be observed that correlates with the molecular dynamics of NO within the protein core. A striking example of this diversity is offered when comparing the NO dynamics governed by the enzyme NO synthase, the endogenous cellular source, and the NO receptor-soluble guanylate cyclase (sGC) whose heme cofactor has a somewhat opposite role.

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controls NO dynamics through its heme structural environment and how this can influence activity. We used jointly absorption and resonance Raman time-resolved spectroscopies to follow the dynamics of the dissociated 5c-NO transient complex on the picosecond timescale and have extended the spectroscopic results with molecular dynamics calculations on the entire protein level using the AXCP crystal structure. In these time-resolved spectroscopies, a femtosecond excitation pulse allows us to simulate the thermal dissociation of NO from the heme binding site, then to synchronously probe its interaction with the protein as a function of time. Overall, our results reveal that upon NO dissociation from the heme iron, the side chains within the proximal heme pocket of AXCP confine NO close to the heme so that immediate rebinding occurs. A very high proportion of NO (≈99%) is trapped, whereas the very minor population escaping does not rebind, likely because of the proximal His reattachment. This represents an extreme behavior observed among heme proteins.

EXPERIMENTAL PROCEDURES

Preparation of the Samples—AXCP was purified as previously described (20, 21). The protein was concentrated to 0.47 mM in heme in MES buffer (pH 6). Aliquots were kept frozen (−80 °C), thawed immediately before the spectroscopic measurements, and diluted to the working concentration. The solution of ferric AXCP (100 μl, 75 μM) was put in a 1-mm optical path length quartz cell sealed with a rubber stopper and degassed by means of four successive cycles of vacuum and purging with pure argon. The heme iron was reduced by the addition of 10 μl of degassed sodium ascorbate solution (5 mM final concentration). For preparing the NO-liganded AXCP, gas phase 10% NO diluted in N2 was directly introduced into the spectroscopic cell (~200 μM NO in the aqueous phase). Equilibrium spectra were recorded at each step for monitoring the evolution of ligation. The absorbance of the sample was in the range 0.7–1 at the Soret maximum for 1-mm path length.

Time-resolved Absorption Spectroscopy—Transient spectra were recorded simultaneously to kinetics as a time-wavelength matrix data using the pump-probe laser system previously described (22) for generating the broad spectral band probe pulse. The photodissociation of NO was achieved with an excitation pulse at 564 nm whose duration was ~40 fs with a repetition rate of 30 Hz. The transient absorption spectrum after a variable delay between pump and probe pulses was recorded by means of a CCD detector. The same sample quartz cell (1-mm optical path length) was used for recording the equilibrium spectra and the transient absorption. The sample temperature was 18 °C during the measurements. The 20-ps time window was scanned with a 400-fs delay between two transient spectra and a dwell time of 2 s at each time point. Up to 40 scans were averaged. Global analysis of the data was performed by singular value decomposition (SVD) of the time-wavelength matrix (23). The SVD component having the highest contribution corresponded to the geminate rebinding process, and its kinetics was fit to a minimum number of exponential components.

Time-resolved Raman Spectroscopy—for subpicosecond Raman spectroscopy, we used a femtosecond Ti:sapphire laser producing 50-fs pulses (λ = 810 nm) and amplified with a 1-kHz repetition rate (23, 24). The output beam was frequency-doubled at 405 nm and then used to generate tunable pump and probe pulses by means of optical parametric generator, amplifiers, and frequency doubler, all based on barium borate crystals. The photodissociating pump pulse energy was about 2 μJ in the sample cell with a 100-fs duration and had a wavelength centered at 560 nm. Narrowband interference filters allowed us to obtain probe pulses at 420 nm and to achieve the best compromise between spectral (30 cm⁻¹) and temporal (0.7 ps) resolutions in time-resolved Raman measurements. The probe pulse energy was 20–30 nJ in the sample cell. The pump and probe beams were collinearly superimposed and focused on the sample by a 10-cm lens, and the optical time delay between both pulses was controlled by a motorized translation stage. Raman scattering light was collected through a spectograph (Jobin-Yvon) by a CCD detector (Roper Scientific).

The sample (100 μl, 150 μM) was placed in a cylindrical spinning cell with a rubber stopper, vacuumed, and reduced with
sodium ascorbate. The cell was filled with 10% NO gas diluted in nitrogen. The integrity and the coordination state of the sample were verified before and after the experiments by recording its absorption spectrum in anaerobic conditions.

**Structural Dynamics Simulation**—To build the initial model we have used the structure of AXCP (Protein Data Bank code 1E85) in its reduced form bound to NO determined at 100 K and at 1.35 Å (resolution 14). In this structure file of the monomer, NO appears liganded to the iron at the proximal side and at 1.35 Å of resolution (14). In this structure file of the AXCP and with the transient absorption spectrum (5c-NO AXCP) reveals the maximum of the Soret band located at 425 and 396 nm (Fig. 2A) characteristic of a 5-coordinate heme with histidine and nitric oxide, respectively, as axial ligands (18, 19). Fig. 2B shows the raw difference transient absorption spectra at various time delays after NO photodissociation from 5c-NO AXCP (40-fs time resolution). A bleaching centered at 392 nm, due to the disappearance of the bond between iron and NO. For dissociated NO, a three-charge model was used to calculate its interactions (26). The calculation was performed with an integration step of 1 fs, and all the structural and energy parameters were stored every ps.

**RESULTS**

**Transient Absorption Changes and Kinetics after NO Dissociation**—The steady-state absorption spectra of unliganded and NO-ligated ferrous AXCP reveal the maximum of the Soret band located at 425 and 396 nm (Fig. 2A) characteristic of a 5-coordinate heme with histidine and nitric oxide, respectively, as axial ligands (18, 19). Fig. 2B shows the raw difference transient absorption spectra at various time delays after NO photodissociation from 5c-NO AXCP (40-fs time resolution). A bleaching centered at 392 nm, due to the disappearance of the 5c-NO AXCP, is seen immediately and simultaneously with an induced absorption centered at 416 nm. The intensity of the difference transient spectrum decayed without any shift of the maximum and minimum or of the isosbestic point, showing that only one process takes place until the spectra vanishes after 25 ps. The absorbance decay associated with geminate rebinding was fitted by global analysis to a monoexponential function with a time constant of 7 ± 0.5 ps and an amplitude of 99 ± 1% (Fig. 2C). The introduction of a base line in the fit gave a value in the range 0.0–0.8% of the total amplitude and did not improve the fit.

In Fig. 2D the pure transient spectral contribution decaying with the 7-ps time constant (trace c) was compared with the steady-state difference spectrum (5c-His minus 5c-NO AXCP; trace e) and also with the transient absorption spectrum after NO dissociation from myoglobin (trace m), which is in the

All the energy and dynamics calculations were performed by using the software CHARMM (25) using a HP J5600 work station. We started a dynamic trajectory on the built model structure by minimizing the total energy, and we obtained −28,181 kcal/mol. Then the structure was heated up to 300 K in 30 steps of 10 K for 30 ps (calculation and assignment of the speed of each atom every 1-ps step), and we allowed the equilibration of the kinetic energy over the entire structure at 300 K for 300 ps. Several 50-ps free dynamics steps were performed to obtain different initial structures for NO dissociation and are represented by the trajectory at negative time in Fig. 5. Then NO was dissociated by suppression of the bond between iron and NO. For dissociated NO, a three-charge model was used to calculate its interactions (26). The calculation was performed with an integration step of 1 fs, and all the structural and energy parameters were stored every ps.

**FIGURE 2. Absorption evolution for 5c-NO ferrous AXCP.** A, equilibrium absorption spectra of AXCP in unliganded (5c-His) and NO-liganded (5c-NO) states. The wavelength range probed by transient absorption is indicated by the shaded area, and that of the photodissociation pulse is indicated by an arrow. B, raw transient absorption spectra at different time delays after photodissociation of NO from 5c-NO ferrous AXCP. The wavelength of the resonance Raman probe is indicated by an arrow. C, kinetics of NO geminate rebinding obtained by SVD analysis of the data. In the inset the kinetics of NO geminate rebinding are compared for four different proteins up to 2 ns, endothelial NO synthase (s), myoglobin (m), guanylate cyclase (g), and AXCP (c). Cyt, cytochrome. D, transient spectral component from SVD analysis (c) associated with the kinetics of NO geminate rebinding to 5c-His myoglobin.
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5c-His transient state. The pronounced difference between spectra in Fig. 2 indicates that the transient photodissociated AXCP species cannot have a 5-coordinate heme with the proximal His replacing NO since this latter species has maxima at 426–434 nm, whereas the transient AXCP species has a broad induced absorption centered at 416 nm (Fig. 2D). This position of the Soret-band maximum is apparently similar to that of 6c-NO-His AXCP in which NO binds at the distal side, in “trans” position to the endogenous His proximal ligand (18). However, the global analysis of the absorption evolution reveals only one kinetic process. Thus, the transient species immediately formed and decaying mono-exponentially cannot be the 6c-NO-His AXCP because it would require the prior rebinding of His followed by the re-binding of NO and would lead to the presence of two kinetic phases that we did not observe. Therefore, the transient difference spectrum (trace c in Fig. 2D) associated with the 7-ps kinetics is assigned to the NO geminate rebinding process. The other SVD spectral components, accounting for ≈1% of the total amplitude, are within the noise level and cannot be assigned with certainty.

Identification of the Transient Species by Time-resolved Raman Spectroscopy—To unambiguously identify the transient species, we recorded transient resonance Raman spectra at different time delays after NO photodissociation with 0.6-ps time resolution and a Raman probe (420 nm) close to the absorption maximum of the transient species induced by the dissociating pulse (560 nm). Fig. 3 presents the time-resolved resonance Raman (TR3) spectra of ferrous AXCP in the low-frequency spectral region. Spectrum a is a ground-state spectrum of the 5c-His species measured in a probe-only configuration. Spectrum b is the ground-state spectrum of 5c-NO AXCP, measured in a pump-and-probe configuration where the probe pulse arrived before the dissociating pump pulse (time delay $\Delta t = -5$ ps). Besides the decreased spectral resolution (30 cm$^{-1}$) due to subpicosecond probe pulse duration, Raman spectra a and b in Fig. 3 closely correspond to the reported steady-state spectra recorded with continuous-wave probe (19). Spectra c–f in Fig. 3 are those of the pure transient photoproduction species at various time delays. They were obtained by weighted subtraction of the spectrum before dissociation (spectrum b) from the experimentally measured spectra. The details of such a subtraction procedure have been described elsewhere (23, 27).

The intense stretching vibration $\nu$(Fe-His) at $\sim 233$ cm$^{-1}$ is active in 5c-His ferrous AXCP (spectrum a), whereas the stretching vibration $\nu$(Fe-NO) at $\sim 525$ cm$^{-1}$, identified by isotopic substitution (19), is active only in the 5c-NO AXCP (spectrum b). Because none of these bands is present in the photoproduction spectra (Fig. 3, c–f) and there is also no $\nu$(Fe-NO) vibration at $\sim 579$ cm$^{-1}$ ascribed to 6c-NO-His AXCP (18), we readily identify the transient species as a four-coordinate AXCP formed after NO photodissociation. These are the first Raman spectra reported for a four-coordinate photodissociated heme protein. Both absorption and Raman data indicate that the Fe-NO bond is photocleaved immediately, i.e. within the excitation pulse duration (<100 fs). The overall intensity of the transient spectra decreases so that the photoproduction Raman spectrum is rather weak at $\Delta t = +10$ ps, showing that the transient species rapidly reaches the initial 5c-NO state. The pattern of vibrational modes in the 300–500 cm$^{-1}$ range is different for the transient form (c–f) compared with the spectra of the ground-state forms (a and b). This pattern contains heme skeletal vibrational modes and especially the $\delta$(C$\beta$-$\delta$C$_{\alpha}$-$\delta$C$_{\alpha}$) and $\delta$(C$\beta$-$\delta$C$_{\alpha}$-$\delta$S) bending modes of thioether substituents participating in specific interactions of the c-type heme with the cysteine side chains. The evolution of their frequency and shift with time after NO detachment is indicative of a structural relaxation limited to the heme that is the direct consequence of the iron motion within the heme plane but does not lead to an overall structural rearrangement of the entire protein (Fig. 1B).

The high frequency region of TR3 spectra of ferrous AXCP is presented in Fig. 4, in the same manner as in Fig. 3. The photoproduct spectra is neither that of equilibrium 5c-His AXCP (spectrum a) nor that of its 5c-NO adduct (spectrum b), therefore confirming its assignment to the four-coordinate transient species. The frequencies of the structure-sensitive (28, 29) porphyrin marker bands $\nu_1$ (1482–1483 cm$^{-1}$), $\nu_2$ ($\sim 1574$ cm$^{-1}$), and $\nu_{10}$ ($\sim 1624$ cm$^{-1}$) are shifted to lower frequency with respect to the ground-state 5c-NO form. On the basis of these frequencies, we have calculated (29, 30) the heme core size D,
which corresponds to the distance between the center of the heme and the N₂ nitrogen of the four pyrroles coordinating the iron and constituting the heme macrocycle (Table 1). We have also observed a change in intensity ratio of the oxidation state markers \( v_4 \) (1352–1373 cm\(^{-1}\)) and \( v_2 \) (1574–1591 cm\(^{-1}\)) bands after photodissociation, characteristic of a change in the Fe\(^{2+}\) spin state. For the high spin 5c-His AXCP, the intensity of \( v_4 \) is much stronger than that of \( v_2 \) (Fig. 4, spectrum a), whereas for the low-spin 5c-NO adduct the intensity of \( v_4 \) appears slightly smaller than that of \( v_2 \) (Fig. 4, spectrum b). In the case of the 4c photoproduct, the band \( v_4 \) is stronger than \( v_2 \) (spectra c–f), consistent with a high spin species. The \( v_3 \), \( v_4 \), and \( v_{10} \) frequencies of 4c-AXCP are different from the respective values measured at steady state for a ferrous b-type heme of the heme-oxygenase mutant lacking the proximal His coordination but having a very weak ligand (31).

Molecular Dynamics Simulation—To visualize possible trajectories for NO within the heme pocket and possibly in the protein core after dissociation, we performed molecular dynamics simulations on the entire protein structure over a 50-ps time range after the cleavage of the Fe-NO bond. These calculations were performed without simulation of NO rebinding to the heme-iron to focus on NO motions and its possible escape. The 50-ps time range is long enough since the absorption kinetics revealed a \( \approx 99\% \) recombination after 50 ps. Eight independent calculations were performed on the AXCP dimer and are compared, not averaged, resulting in trajectories for 16 NO molecules (Fig. 5 and supplemental Fig. 1 available online). There was no difference in behavior between both monomers. The calculated trajectories are represented as the distances between Fe-N atoms and Fe-O atoms as a function of time after NO dissociation from the heme. Before NO dissociation, the structure equilibrated at 300 K has an average Fe-NO bond length of 1.9 Å and a distance of 3.0 Å between the iron and the oxygen atom of NO. The fluctuations of Fe-NO bond length at negative times correspond only to thermal vibrations (Fig. 5, A–B).

Conspicuously, after breaking of the Fe-NO bond, the nitrogen atom moves immediately by 1 Å from iron and keeps this average position for the entire 50-ps range, whereas the oxygen atom keeps an average position at 3 Å from the iron, similar to its bound distance. This results in an overall tilt of the NO with respect to its equilibrium bound position, with a fluctuating motion around a stabilized position corresponding to NO having (on average) a more parallel orientation with respect to the heme plane. The Fe-N-O angle fluctuations are shown in Fig. 5B, with a mean value of 80–85°. The distance d(Fe, NO) = 3 Å must be compared with that calculated in the case of myoglobin (32), which is 4.4 Å and has much larger fluctuations (up to 6 Å) than in the case of AXCP. For only 1 trajectory among 16 (number 2, chain 1 in Fig. 5A) the NO molecule moved transiently up to 5.5 Å from iron, exploring the heme pocket and retrieving its previous position at 3 Å in less than 10 ps.

Fig. 6 compares three calculated structures of 5c-NO AXCP, showing the heme pocket conformation and the NO position before dissociation and 1 and 50 ps after the disso-

**TABLE 1**

Porphyrin core size \( D \) for the 5c species of ferrous AXCP and its 4c photoproduct

| Vibrational mode\(^{a}\) | 5c-His | | 5c-NO | | 4c-transient |
|-------------------------|-------|-------|-------|-------|
| \( v \) cm\(^{-1}\) | Frequency | Core size \( D \) | Frequency | Core size \( D \) | Frequency | Core size \( D \) |
| \( \nu_3 \) | 1467 | 2.07 | 1506 | 1.98 | 1483 | 2.03 |
| \( \nu_2 \) | 1577 | 2.00 | 1591 | 1.95 | 1574 | 2.00 |
| \( \nu_{10} \) | 1603 | 2.05 | 1642 | 1.98 | 1624 | 2.02 |

\(^{a}\) The mode numbering is according to Abe et al. (28).

\(^{b}\) The porphyrin core size values \( D \) are calculated from the porphyrin marker band frequencies \( v \) according to Spiro and Li (29) and Parthasarathi et al. (30).

\(^{c}\) For the steady-state 5c-NO AXCP, the values are from Andrew et al. (19).
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During all the trajectories NO remained within the proximal heme pocket. At 1 ps, NO had already adopted the position that it will keep during the entire dynamic trajectories, whereas His-120 had not, still being close to that of the equilibrium NO-ligated form. After relaxation, the His-120 side chain was tilted compared with the structure at 1 ps. During its trajectory within the heme pocket, NO came into close contact with the N atom of NO has contacts with the hydrogen atoms of Cys-119 and Arg-124 hydrogens also occurred. As for the O atom, it also experiences contacts with iron but less frequently than the N atom and contacts with hydrogen atoms of His-120, Cys-119, and Arg-124 as frequently as the N atom.

From the distribution of partial charges over groups of atoms and their distance, the energy of interaction was calculated between NO and iron, Arg-124, and His-120 at each position of NO during the dynamics (Fig. 6B). The energy of interaction is the highest with Arg-124 (6.78 kcal/mol) and almost 0 with His-120 (−0.35 kcal/mol), whereas it reaches an intermediate value with iron (3.2 kcal/mol) with very little variation over the 50-ps time range. For His-120, the average value of the interaction energy is not exactly the same for both monomers. This should reflect the large flexibility of His-120 and, consequently, a higher variability of NO motion with respect to this side chain.

a higher variability of NO motion with respect to this side chain. NO has similarly frequent contacts but different energies of interaction with both Arg-124 and His-120 during its motion, likely due to the fact that these side chains, respectively, impose an electrostatic barrier and a steric barrier to NO escape.

DISCUSSION

Connection with Previous Photolysis Experiments—A previous study reported transient absorption measurements of photodissociated 5c-NO AXCP after a 3-ns excitation pulse (33). Because of the nanosecond time resolution and data collection on a much slower time scale (>0.5 ms), no 4-coordinate AXCP species or geminate rebinding process could be observed. This previous study indicated that ~5% of the photodissociated NO was able to escape to the solvent, allowing His rebinding to trap the 5c-His ferrous heme, with a NO re-binding amplitude of 95%. By contrast, the present study reveals that 99% of photodissociated NO recombines to the heme on the ps time-scale, preventing the 5c-His ferrous heme formation. This difference in rebinding probability observed between these two studies most likely originates from the different light pulse durations and time scales. When the duration of the photodissociating pulse is 1 to several orders of magnitude longer than the geminate rebinding time constant, the dissociation of NO can occur many times for the same molecule during the same pulse, therefore increasing the probability of ligand escape out of the heme pocket, a phenomenon already observed in the case of the oxygen sensor DOS (34). Consequently, the processes probed in these two experiments are not the same; the present study detects geminate recombination on the picosecond timescale, whereas the previous study (33) monitored much slower bimolecular NO recombination process on the millisecond timescale after the rebinding of the internal proximal His.

Dynamics of Nitric Oxide within the Heme Pocket—The rebinding of NO after dissociation occurs readily with a single ultrafast phase (7 ps) in a very efficient manner (99 ± 1%). Before and simultaneously to NO rebinding leading to the recovery of the initial 5c-NO species, no other coordination process was detected, showing that the proximal Fe-His bond does not reform before 7 ps. The reattachment of the His-120, thus, occurs on a longer time scale than NO geminate recombination. Moreover, the ultrafast rebinding does not allow the diffusion of NO toward the distal side of the heme, and therefore, the 5-coordinate species that is reformed cannot be
nitrosylated with NO bound on the distal side. Indeed, neither absorption nor Raman spectroscopy detects a transient 6-coordinate species, and the molecular dynamics calculation does not show the possibility of NO moving out of the proximal heme pocket.

The dynamics of NO from and to the heme is governed by internal energy barriers within the protein core. Therefore, the geminate rebinding process appears bi-exponential for the 6c-NO-His heme proteins myoglobin and hemoglobin (35–37) and occurs with three exponential phases for the 6c-NO-His complex of NO-synthase (9) and the O2-sensor FixL (38), whereas it is mono-exponential for the mammalian 5c-NO sGC (10). Their kinetics are compared in Fig. 2C with the purpose of showing the influence of the protein structures. These energy barriers are provided by amino acid side chains of the heme pocket, as evidenced by variations in the amplitude and time constant of the rebinding phases after mutations in myoglobin (39–44) and in soybean leghemoglobin (45).

Similarly to AXCP, the 5c-NO complex of sGC exhibits a 4-coordinate iron after photodissociation together with a strictly mono-exponential geminate NO recombination with $\tau = 7.5$ ps (10). Such a fast rate corresponds to a process occurring without any energy barrier for NO binding to the heme. Because rapid mono-exponential recombination is observed for both AXCP and sGC 5c-NO complexes, this mono-exponential behavior could only be due to the 4-coordinate state of the heme iron. However, we recently showed that the internal energy barrier of the heme pocket protein structure, not the 4-coordinate state of the iron, is at the origin of the multi- or monophasic character of the geminate rebinding (16).

The energy barriers exist not only for NO entering within the heme pocket but also for NO escaping it, and we may define the heme pocket as the volume from which NO can bind to the heme without any energy barrier due to the protein side chains.

It is informative to compare the NO dynamics in AXCP to that of bare 4-coordinate heme systems. Remarkably, the rebinding of NO to the heme alone in solution is also mono-exponential (7.5 ps) but with a constant base line (8%) that corresponds to NO escape into the solvent (46, 47). Thus, a time constant of $\sim 7$ ps for NO rebinding to the 4-coordinate heme after dissociation (either thermal or photoinduced) seems to be the lower limit for this barrierless process when no protein structure is involved, in agreement with calculations (16, 48) and with temperature-dependent rebinding measurements (42).
Fe–NO distance of 2.0 Å in the static crystal structure of NO-bound AXCP (14). This clearly shows that the heme pocket structure of AXCP imposes high energy barriers to NO motion out of the heme pocket and confines NO within a volume such that the distance Fe–NO is minimized, and the transition to bound state has a high probability of occurring without an enthalpic barrier, in agreement with the fast rate of rebinding. The barriers for escape are provided by side chains of the proximal environment, the cleaved proximal His-120 and the Arg-124, as shown by the calculated dynamics. Arg-124 and His-120, thus, appear as key side chains involved in NO dynamics and affinity. On the other hand, the presence of NO in the heme pocket may preclude the motion of His-120 toward the heme plane in AXCP, the dissociation of NO results in the motion of the iron toward NO. In the calculated 4-coordinate transient AXCP, the dissociation of NO results in the motion of the iron by ~0.6 Å, from a proximally domed to a distally domed configuration (Fig. 6A). We conclude that the position of the iron with respect to the heme plane in AXCP is not a determinant in imposing a fast monophasic rebinding, whereas the heme pocket side chains are.

Properties of the Four-coordinate Heme in Ferrous AXCP—The low frequency shift of the porphyrin marker bands for the transient photoproduct with respect to the ground-state 5c-NO form (Fig. 4) provides evidence of the porphyrin size expansion by 0.04–0.05 Å in the 4-coordinate species (Table 1). This difference in core sizes is comparable with that (0.06 Å) between 6c-HS and 6c-LS hemes (29). Accordingly, we suggest that the four-coordinate AXCP transient species is a high spin form (4c-HS) with single electron occupation of both d(\(\varepsilon_2\)) and d(\(\chi^2\)) orbitals. The intensity ratio of the modes \(\nu_4\) and \(\nu_2\) (Fig. 4), which depends upon the spin state, further supports this assignment. The lower core size of 4c-HS AXCP (2.02 Å) relative to that of 6c-HS heme complexes (2.06 Å; Ref. 29) is explained by the absence of axial ligands (49). In contrast to the 4c-HS AXCP, the frequency of the porphyrin bands implies that 5c-NO AXCP has a low spin heme and a decreased core size (1.98 Å) that can be explained by the out-of-plane displacement of the central metal toward NO axial ligand with subsequent contraction of the porphyrin macrocycle.

The evolution of band shapes and band shifts of transient Raman spectra within the low frequency range pattern located at 300–500 cm\(^{-1}\) reveals that a conformational relaxation of the heme takes place after NO detachment. This is supported by the change of geometry of the 4c heme obtained by dynamic calculations (Fig. 6A). Thus, both experimental data and calculations indicate that the 4c heme does not yet adopt a relaxed geometry 1 ps after the release of NO and that the full relaxation takes a few ps and occurs simultaneously with NO recombination. In the equilibrium AXCP-nitrosoylated structure, the heme is

![FIGURE 7. Model for the binding and release of NO to and from the different transient forms and coordination states of AXCP. a, 5c-NO AXCP with NO at the proximal side, which is the starting species of our experiments. b, transient 4c-AXCP with NO dissociated and located in the heme pocket. c, transient 4c-AXCP with NO dissociated and located in the solvent. d, 5c-His resting state AXCP. e, 6c-His-NO AXCP. The rate constants of conversion between these species are the following: \(k_1\), rate for NO rebinding; \(k_2\), rate for NO release from 5c-NO AXCP; \(k_{5off}\), overall rate of NO release from 5c-NO AXCP to the solvent. \(k_{5on}\), rate of NO release from 6c-His-NO AXCP; \(k_{6off}\), rate of NO release from 6c-His-NO AXCP. The two last processes correspond to several elementary steps. For the completeness of the model, we indicated within brackets transient species leading to 5c-NO and 6c-NO AXCP without further detailing the corresponding rates.](image-url)
and efficient geminate recombination rate, $k_{\text{gem}}$. The absence of a second slower geminate rebinding component implies the existence of a high energy barrier for NO access to the heme pocket of AXCP so that $k_{-2}$ is very small. The very minor population of NO escaping from the heme pocket cannot rebind directly to the proximal heme face but dissipates into solution. Thus, the release of NO from 5c-NO AXCP appears virtually unidirectional.

How do the Fe–NO dissociation rate $k_1$ and the escape rate $k_2$ influence the overall rate of NO release from 5c-NO complex of AXCP $k_{\text{off}}$? According to Fig. 7, $k_{\text{off}}$ can be expressed by Equation 1, where $k_1$ is the intrinsic rate constant for Fe–NO bond dissociation (whose probability is set to 1 by photodissociation), and $Y_{\text{off}}$ is the fractional efficiency of NO escape from the proximal heme pocket to the solvent. The value of $Y_{\text{off}}$ is also given by Equation 2 assuming that the fate of dissociated NO within the proximal pocket is governed only by $k_{-1}$ and $k_2$ because $k_{-3}$ is very small (see above). We observe $k_{\text{gem}} = k_{-1} + k_2$ since the geminate yield is $\sim 99\%$, we assume that $k_{-1} = k_{\text{gem}}$.

$$k_{\text{off}} = Y_{\text{off}} k_1$$

$$Y_{\text{off}} = k_2/(k_{-1} + k_2) = k_2/(k_{\text{gem}} + k_2)$$

On the basis of the geminate recombination efficiency ($99\%$) we measured $Y_{\text{off}} \sim 10^{-2}$. Together with the value $k_{\text{gem}} = 1.4 \times 10^{-4}$ s$^{-1}$ and the recently determined value of $k_{\text{off}} (4.1 \times 10^{-4}$ s$^{-1})$ this gives $k_1$ in the range $0.02$–$0.05$ s$^{-1}$ (error range of $k_{\text{gem}}$) and $k_2 \sim 10^9$ s$^{-1}$.

The kinetic model in Fig. 7 assumes that His rebinding to 4c-heme ($k_{\text{His}}$) occurs after NO release in solvent ($k_2$). In this case, $k_{\text{His}}$ does not influence $k_{\text{off}}$ since the probability to reform the 5c-NO state after NO exit competes only with the relatively slow bimolecular NO rebinding from solvent ($k_{-2}$). However, if $k_{\text{His}}$ does not depend upon the exit of NO ($k_2$), His rebinding could compete directly with $k_{\text{gem}}$ to influence $k_{\text{off}}$ (i.e. $k_{\text{His}}$ would appear in Equation 2). Although it is known that His-120 reattachment to the heme is complete within 1 ms (33), an actual $k_{\text{His}}$ value has yet to be determined. In the absence of NO, the photodissociated endogenous ligand rebinding has been shown to occur with time constants of 5–35 ps in the cases of mitochondrial cytochrome c (23) and oxygen sensor DOS (34), although His rebinding in AXCP is likely to be slower due to its displaced conformation. In this study, 5c-His AXCP formation was not observed within 30 ps of Fe–NO dissociation either because His-120 does not rebind on the same timescale or because of its associated absorbance change (resulting from only $\leq 1\%$ escape of NO) is too small to be detected. Our kinetic data (single exponential) and structural dynamics calculation (NO staying in the heme pocket and between iron and His-120) suggest that His rebinding cannot occur when NO is still located within the proximal heme pocket but may occur once NO overcame the energy barrier to solvent. Then it involves no structural rearrangement of the entire protein between both states but only the rotation of the His-120 side chain followed by reorientation of the Arg-124 side chain (Fig. 1B; Ref. 14).

According to the previously proposed “kinetic trap” mechanism (33), the small fraction of NO that escapes the heme pocket is prevented from direct bimolecular recombination to the proximal heme face ($k_{-2}$) by the reattached His ligand, a mechanism that forces NO to bind through two NO concentration dependent steps ($k_{\text{non}}$ and $k_{a\rightarrow s}$) via a distal 6c-NO intermediate.

It is noteworthy that, despite the efficient and fast geminate recombination, the $k_{\text{off}}$ value for 5c-NO AXCP ($4.1 \times 10^{-4}$ s$^{-1}$) is of the same order of magnitude as 5c-NO heme protein complexes such as sGC ($8.2 \times 10^{-4}$ s$^{-1}$; Ref. 50) and H93G-Mb ($1.1 \times 10^{-4}$ s$^{-1}$; Ref. 51) even though the latter shows a biphase, slower recombination rate with lower amplitude than AXCP (16). The high geminate recombination efficiency imposed by the proximal heme environment of AXCP implies that the typical $k_{\text{off}}$ value is due to an elevated value of $k_1$. A consequence of the high geminate yield together with a high $k_1$ value is to increase the number of events during which NO is transiently unbound within the heme pocket, a property that influences the heme–NO reactivity.

Although the exact function of AXCP has yet to be identified, the unique ability of NO to bind to AXCP on the proximal heme face (52) provides a means of discriminating against other diatomics to achieve a selective response to NO. Recent studies have shown that cytochrome c$'$ from R. capsulatus (RCCP) helps protect this organism against NO toxicity (7, 8). Specifically, the resistance of the bacteria to NO is increased when RCCP is expressed, but buffering alone cannot account for this effect (8). The wild type RC strain was further shown to produce N$_2$O when grown in the presence of NO, leading to the suggestion that RCCP is involved in reductase activity (8). Particularly since NO-reductase activity has not been demonstrated for purified RCCP or any other cytochrome c$'$, it is possible that cytochrome c$'$ could be a transporter coupled to a NO reductase, as recently proposed for the species Chromatium vinosum (53) and Rhodobacter sphaeroides (54). In the context of a physiological NO binding role for AXCP, the overall picture is that the distal side controls the initial NO binding, whereas the proximal heme pocket controls the release of NO with the ability of trapping and gating NO despite its close proximity to solvent with a virtually unidirectional release of NO. Because buffering does not explain the bacterial resistance toward NO (8), the trapping and kinetic mechanisms developed by AXCP should be connected to a catalytic turnover, maybe provided by a partner protein.

Future spectroscopic studies will focus on the 6c-NO AXCP intermediate as well as on the His rebinding process, which is difficult to detect due to its small amplitude with respect to the NO geminate rebinding. This will provide a more complete picture of the implications of proximal versus distal heme-NO coordination.

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