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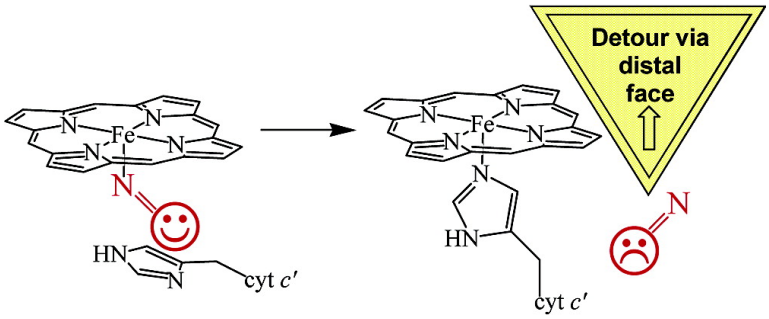

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A Novel Kinetic Trap for NO Release from Cytochrome *c*′: A Possible Mechanism for NO Release from Activated Soluble Guanylate Cyclase

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Nitric oxide (NO) exerts many of its physiological effects through interaction with the heme-containing enzyme, soluble guanylate cyclase (sGC). Activation of sGC is thought to be triggered allosterically by the formation of a thermodynamically stable five-coordinate ferrous heme-nitrosyl (5c-NO) and the accompanying scission of the endogenous Fe−His bond.[1] However, explaining how NO is released from sGC presents a puzzle due to the high formation constants of 5c-NO complexes.[2] Although the heme binding face (proximal or distal) of the sGC 5c-NO complex is not known, the recent crystallographic discovery of proximal heme-NO coordination in *Alcaligenes xylosoxidans* cytochrome *c*′ (AXCP), a protein with NO-binding chemistry that is mechanistically similar to that of sGC, has led to the suggestion that sGC might also bind NO proximally.[3−6] Herein, we report a flash photolysis study on 5c-NO AXCP. Significantly, we observe 5c-ferrous heme as a photolysis product, implying that reattachment of the His ligand is fast enough to compete with second-order rebinding of the proximal NO. We propose that this trapping of 5c-ferrous heme by the His ligand facilitates the unloading of NO at low ambient [NO] and that a similar mechanism, featuring a proximal heme-NO, could be the key to how NO is released from activated sGC.

Photolysis of 5c-NO AXCP, using 3-ns pulses of 532-nm light, yielded transient delta absorbance (ΔA) spectra, which were recorded in the heme-Soret region at delays from 5 ms to 2 s. The 5-ms ΔA spectrum (Figure 1 A) shows a 432-nm peak and 392-nm trough.[4] Similarity between the 392-nm feature and the λ_{max} of 5c-ferrous AXCP, which is known to have a proximal Fe−His bond,[3,4] suggests that photolysis of NO is followed by reattachment of the proximal His ligand. Further evidence is found in the kinetic similarities between this intermediate and equilibrium 5c-ferrous AXCP, which is discussed below. The absence of four-coordinate (4c) heme (predicted $\lambda_{max} \approx 426$ nm)[7,8] implies that His rebinding is complete within 5 ms. On the basis of the ΔA amplitude, we estimate that 5% of the photolyzed 5c-NO species converts to the 5c-ferrous state, with the remaining 95% undergoing geminate recombination within the detection time. At longer delay times, the peak at 432 nm is replaced by one at 417 nm (Figure 1, traces B and C), which we ascribe to a six-coordinate heme-nitrosyl (6c-NO).[6] The 417-nm and 392-nm features subsequently decay to the baseline (data not shown).

Single-wavelength ΔA time courses, recorded from 0.4 ms to 2 s after the laser flash, reveal three relaxation phases, clearly evident at 415 nm (Figure 2A) where sequential phases have ΔA of opposite sign. Analysis of data from 10 wavelengths yields time constants of 5.7(0.1), 14.6(0.1), and 151(0.3) ms for phases 1, 2, and 3,

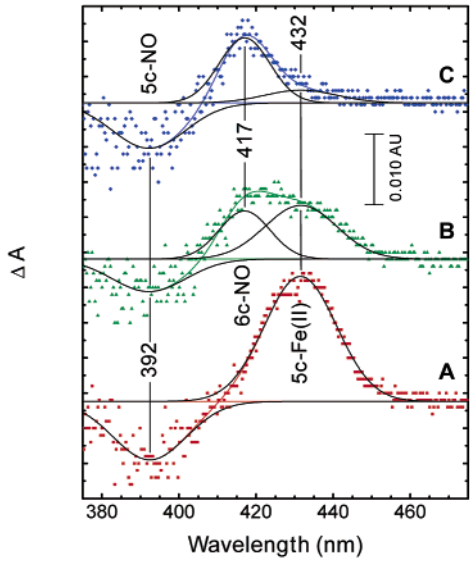

Figure 1. Transient ΔA spectra of photolyzed 5c-NO AXCP solutions recorded (A) 5 ms, (B) 15 ms, and (C) 50 ms after a 3-ns (37 mJ) pulse of 532-nm light. Each spectrum is the average of 30 repetitions. Solid lines show absorbance bands derived from peak fitting analyses. S/N ratios below 400 nm preclude conclusions about relative concentrations of 5c-NO. Samples contained in a 0.3-mm path length cell; 0.1 mM heme, 2 mM NO, 100 mM NaCl, and 50 mM Tris, pH 8.9.

respectively, for samples containing 2 mM NO. Decreasing [NO] to 1 mM slows all three phases (Figure 2A). Assuming a linear [NO]-dependence, we estimated second-order rate constants for phases 1, 2, and 3 to be 8.7×10^4, 3.4×10^4, and 3.3×10^3 M^{-1} s^{-1}, respectively. We assign phase 2, involving the appearance of the 417-nm species, to NO binding on the distal face of the 5c-ferrous heme to yield a 6c-NO intermediate, similar to the k_{6on} reaction between 5c-ferrous AXCP and NO (4.4×10^4 M^{-1} s^{-1}) measured in a recent stopped-flow study.[6] Phase 3, involving the concerted decay of the 417- and 392-nm features, is assigned to an NO-catalyzed 6c-NO → 5c-NO conversion, analogous to k_{6-5} from stopped-flow studies (8.1×10^3 M^{-1} s^{-1}), in which a presumed second NO substitutes for the His ligand, thereby expelling the distal NO to yield the proximal 5c-NO complex.[6] On the basis of the ΔA_{430} decay and ΔA_{400} increase prior to 5 ms (Figure 2B), we ascribe phase 1 to formation of 5c-NO by attack of NO on the proximal face of a strained 5c ferrous intermediate (vide infra). Our model for the relaxation of the photolyzed 5c-NO adduct is summarized in Scheme 1. One scenario is that reattachment of the His ligand (k_{His}) is fast enough to compete directly with ultrafast recombination of the geminate pair (k_{gem}).[8] This is not beyond reason, as previous photolysis studies on 6c-ferrous cytochromes b_5 and *c* reported k_{His} to be $\sim 1.4 \times 10^{11}$ s^{-1},[9] although k_{His} for

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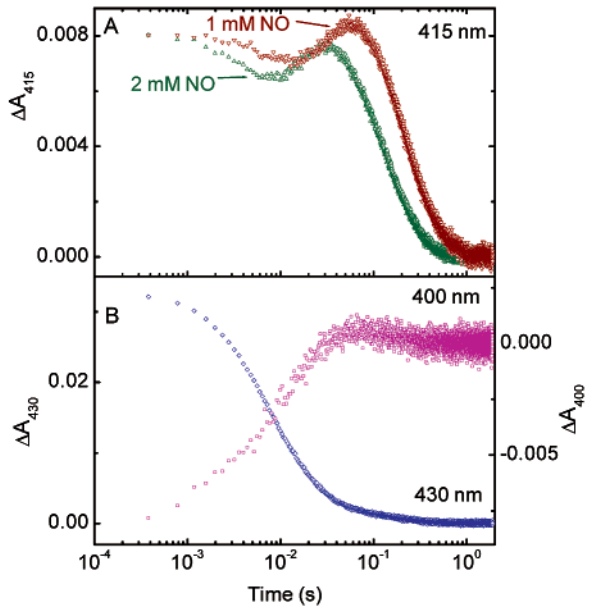
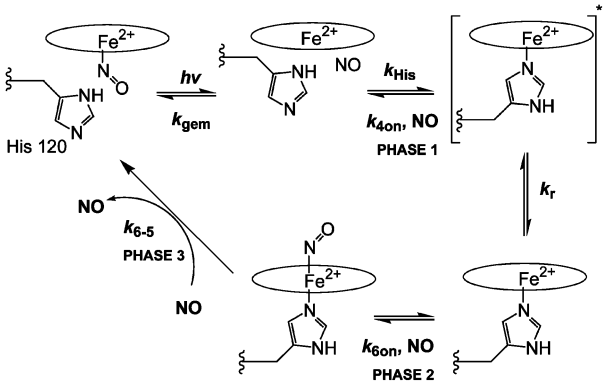


Figure 2. Single wavelength Δ*A* time courses following photolysis of 5c-NO AXCP solutions at (A) 415 nm and (B) 400 and 430 nm. $T = 25$ °C, [NO] as indicated. Variations in [AXCP heme] between 0.1 and 0.2 mM did not affect the kinetics. Time courses are averages of 500−1000 repetitions. Other experimental conditions are as described in Figure 1.

Scheme 1. Relaxation of Photolyzed 5c-NO AXCP



5c-NO AXCP may be much lower due to the displaced H-bonded His conformation.[3] Alternatively, the extensive solvent exposure of the AXCP proximal pocket[3] likely facilitates escape of some heme-dissociated NO into solution, which in turn allows k_{His} to compete with the relatively slow bimolecular NO recombination (k_{4on}). In both scenarios, it is the proximal nature of the heme-NO adduct that facilitates NO release from the heme pocket because rapid reattachment of the proximal His blocks direct access of a single NO to the same coordination site.

An additional feature of Scheme 1 is that His reattachment initially generates a strained ferrous heme (5c-ferrous*), which then relaxes in an [NO]-independent step (k_r). While the relaxed ferrous state reacts with NO via a distal intermediate (phases 2 and 3), the 5c-ferrous* state appears to react with NO directly on the proximal heme face (phase 1), which we propose is due to the significantly labilized Fe−His bond. It is possible that the 5c-ferrous* state does not exhibit the His 120/Arg 124 electrostatic interaction implicated in imparting imidazolate character to the Fe−His bond.[10] Despite a lack of detectable Δ*A* associated with k_r, we conclude that it is [NO]-independent, as lower [NO] increases the 6c-NO:5c-ferrous* ratio, which is governed by the relative rates of [NO]-independent and [NO]-dependent relaxations of 5c-ferrous*. Support for this can be seen in Figure 2A, wherein the rise in absorbance at 415 nm (due to 6c-NO) is seen to increase upon lowering [NO].

Evidence that His reattachment traps NO-dissociated heme in AXCP is the key finding of this study and points to a novel mechanism for NO release, which we term the "kinetic trap". The kinetic trap facilitates NO release from 5c-NO AXCP by disfavoring direct second-order recombination of NO to the proximal heme face following photochemical or thermal NO dissociation. Moreover, by forcing the 5c-NO adduct to reform via two [NO]-dependent steps (k_{6on} and k_{6-5}), the kinetic trap amplifies the effect of decreased [NO] on the depopulation of 5c-NO AXCP. In the case of sGC, 5c-NO formation also occurs via two [NO]-dependent steps.[1c,d] A previous flash photolysis study on 5c-NO sGC revealed only geminate NO recombination to 4c heme with no sign of 5c-ferrous heme.[8] However, the reported measurements were confined to the hundreds of picoseconds time scale, at the end of which ~3% of the transient Δ*A* had not decayed. If this fraction of Δ*A* corresponds to 4c heme for which the proximal geminate pair has dissociated, His reattachment could be competing with bimolecular NO recombination to the proximal heme face in this fraction of the sGC hemes. Hence, it is reasonable to hypothesize that a kinetic trap NO release mechanism analogous to that reported here for AXCP could be operating in NO-activated sGC.

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