Inorg. Chem. **2003**, *42*, 270–272

Selective One-Electron Reduction of *Nitrosomonas europaea* Hydroxylamine Oxidoreductase with Nitric Oxide

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Received June 7, 2002

Hydroxylamine oxidoreductase (HAO) from the autotrophic bacterium *Nitrosomonas europaea* catalyzes the 4-e$^-$ oxidation of NH_2OH to NO_2^-. The e$^-$ are transferred from NH_2OH to an unusual 5-coordinate heme known as P460, which is the active site of HAO, and from there to an array of seven *c*-type hemes. NO•, generated by laser flash photolysis of *N,N′*-bis(carboxymethyl)-*N,N′*-dinitroso-1,4-phenylenediamine, is found to act as a 1-e$^-$ donor to HAO. Most likely NO• binds P460 to yield a $\{Fe(NO)\}^6$ moiety, which then hydrolyzes to give the reduced enzyme and NO_2^-. The $\{Fe(NO)\}^6$ moiety is also a plausible final intermediate in the oxidation of NH_2OH.

The enzyme hydroxylamine oxidoreductase (HAO) from the autotrophic bacterium *Nitrosomonas europaea* catalyzes the 4-e$^-$ oxidation of NH_2OH to NO_2^-.[1−3] This is the second of two steps by which NH_4^+ is oxidized to NO_2^- in *Nm. europaea*; the first step, oxidation of NH_4^+ to NH_2OH, is catalyzed by the enzyme ammonia monooxygenase.[3,4] The net oxidation of NH_4^+ to NO_2^- is critical to *Nm. europaea*, which derives all its energy for growth from the process.[3,5] As part of the biological nitrogen cycle, the oxidation of NH_4^+ to NO_2^- also has broad economic and ecological importance.[5,6] HAO is a large and very complex homotrimer with a molecular weight of 68 kDa per monomer.[7] Each monomer contains seven *c*-type hemes and a novel eighth heme known as P460.[7,8] The Fe center of P460 has a vacant coordination site at which NH_2OH binds and is then oxidized.[9−12] The role of the *c*-hemes appears to be the transfer of e$^-$ from the P460 active site to one or more e$^-$ acceptors. Cytochrome c_{554}, an abundant periplasmic protein in *Nm. europaea*, is considered the most likely candidate as the e$^-$ acceptor.[4,13]

With a total of 24 hemes, *Nm. europaea* HAO is one of the most complex heme-containing enzymes known.[5] By contrast, heterotrophic bacteria that can also oxidize NH_4^+ to NO_2^-, but do not use NH_4^+ oxidation as a source of energy, contain a much simpler form of HAO.[14,15] An important question, then, is what role the added complexity of the *Nm. europaea* HAO plays. This interesting mechanistic question inspired the investigation described here. Schemes 1 and 2 suggest a plausible formal sequence by which NH_2OH oxidation could take place,[4] that was chosen as a starting point for this investigation. Scheme 1 implies that NH_2OH oxidation to NO_2^- takes place via two sequential 2-e$^-$ steps, in which fragments formally designated as HNO and NO^+ are P460-bound intermediates. As shown on the right-hand side of Scheme 2, after consideration of the corresponding formal oxidation state of the P460 iron, the NO^+ intermediate of Scheme 1 must properly be written in terms of two resonance structures, often collectively represented as $\{Fe(NO)\}^6$.[16,17] With this point in mind, Scheme 2 also suggests that NO• could act as a 1-e$^-$ reductant of HAO, selective

Scheme 1

$$NH_2OH \longrightarrow HNO + 2H^+ + 2e^-$$
$$HNO \longrightarrow NO^+ + H^+ + 2e^-$$
$$NO^+ + H_2O \longrightarrow NO_2^- + 2H^+$$

Scheme 2

$$\left.\begin{array}{l} \bullet NO + Fe^{III}_{P460} \rightleftharpoons \{Fe_{P460}(NO)\}^6 \\ \{Fe_{P460}(NO)\}^6 + H_2O \rightleftharpoons Fe^{II}_{P460} + NO_2^- + 2H^+ \\ Fe^{II}_{P460} \longrightarrow Fe^{III}_{P460} + e^- \end{array}\right\} \left[\begin{array}{c} Fe^{III}(\bullet NO) \\ \updownarrow \\ Fe^{II}(NO^+) \end{array}\right] \equiv \{Fe(NO)\}^6$$

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(1) Hooper, A. B.; Nason, A. *J. Biol. Chem.* **1965**, *240*, 4044−4057.
(2) Andersson, K. K.; Hooper, A. B. *FEBS Lett.* **1983**, *164*, 236−240.
(3) Ferguson, S. J. *Curr. Opin. Chem. Biol.* **1998**, *2*, 182−193.
(4) Hooper, A. B. In *Autotrophic Bacteria*; Schlegel, H. G., Bowien, B., Eds.; Science Tech Publishers: Madison, WI, 1989; pp 239−265.
(5) Prince, R. C.; George, G. N. *Nat. Struct. Biol.* **1997**, *4*, 247−250.
(6) *Biological Nitrogen Fixation*; National Academy Press: Washington, DC, 1994; pp 6−32.
(7) Igarashi, N.; Moriyama, H.; Fujiwara, T.; Fukumori, Y.; Tanaka, N. *Nat. Struct. Biol.* **1997**, *4*, 276−284.
(8) Arciero, D. M.; Hooper, A. B. *J. Biol. Chem.* **1993**, *268*, 14645−14654.
(9) Hooper, A. B.; Terry, K. R. *Biochemistry* **1977**, *16* (3), 455−459.
(10) Hendrich, M. P.; Logan, M.; Andersson, K. K.; Arciero, D. M.; Lipscomb, J. D.; Hooper, A. B. *J. Am. Chem. Soc.* **1994**, *116*, 11961−11968.
(11) Logan, M. S. P.; Balny, C.; Hooper, A. B. *Biochemistry* **1995**, *34*, 9028−9037.
(12) Logan, M. S. P.; Hooper, A. B. *Biochemistry* **1995**, *34*, 9257−9264.
(13) Arciero, D. M.; Balny, C.; Hooper, A. B. *Biochemistry* **1991**, *30*, 11466−11472.
(14) Wehrfritz, J.-M.; Reilly, A.; Spiro, S., Richardson. *FEBS Lett.* **1993**, *335*, 246−250.
(15) Moir, J. W. B.; Crossman, L. C.; Spiro, S.; Richardson, D. J. *FEBS Lett.* **1996**, *387*, 71−74.

10.1021/ic025779n CCC: $25.00
Published on Web 12/24/2002

for the P460 active site. Hydrolysis of the Fe−NO moiety in {Fe(NO)}^{6} hemes, as shown in Scheme 2, has extensive precedent in heme chemistry.[18,19] Herein we present results that demonstrate that NO• can indeed act as a 1-e$^-$ reductant of HAO, as suggested in Scheme 2. This result assumes a heightened importance in light of a recent article that reported NO• to be incapable of reducing HAO.[20]

Using a methodology first reported by Namiki et al.,[21] NO• for reaction with HAO was generated in situ by the photolysis of *N*,*N*′-bis(carboxymethyl)-*N*,*N*′-dinitroso-1,4-phenylenediamine (**1**), as depicted in Scheme 3. A 10 ns pulse from a XeCl excimer laser was used to generate initial concentrations [**2**]$_0$ and [NO•]$_0$ from **1**, that could be readily controlled either by varying the intensity of the laser pulse, or by maintaining the laser pulse constant and varying the concentration of **1**.[22] The [NO•] at any subsequent time could then be calculated using the recently determined values for k_r and k_d.[22] After at most 1−2 ms, [**2**] decreased to ∼0, and [NO•] decreased to a constant concentration [NO•]$_f$. Species **2** and **3** in Scheme 3 are both oxidizing agents,[23] so NO• is the only potential reductant generated in the photolysis.

Scheme 3

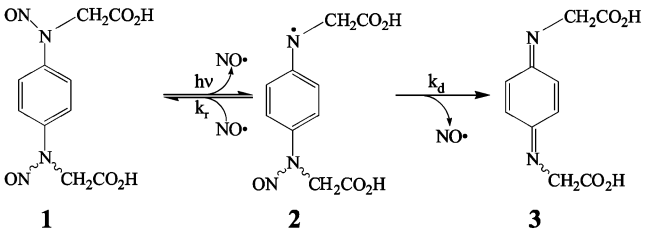

Figure 1 shows typical changes observed in the visible difference spectrum when a thoroughly anaerobic solution containing **1** (47 μM) and HAO (87 nM),[24] in phosphate buffer pH = 7.4, was irradiated as described above.[25] The prominent signal seen at 420 nm, together with weaker features at 523 and 552 nm, grows exponentially, while over the same time frame a signal at 404 nm decreases exponentially. Such spectral changes are characteristically seen during the reduction of all the *c*-hemes of HAO, except one that has a maximum ΔA at 560 nm instead of at 552 nm.[26−28] Figure 1 shows no evidence of spectral changes involving the P460 center, which has visible spectroscopic characteristics that are very different from those of the *c*-hemes.[11,26,28,29]

The molar absorptivity differences at 420 nm ($\Delta\epsilon_{420}$) associated with reduction of the *c*-hemes of HAO are known to fall between 48 and 114 mM^{-1} cm^{-1}, depending on the

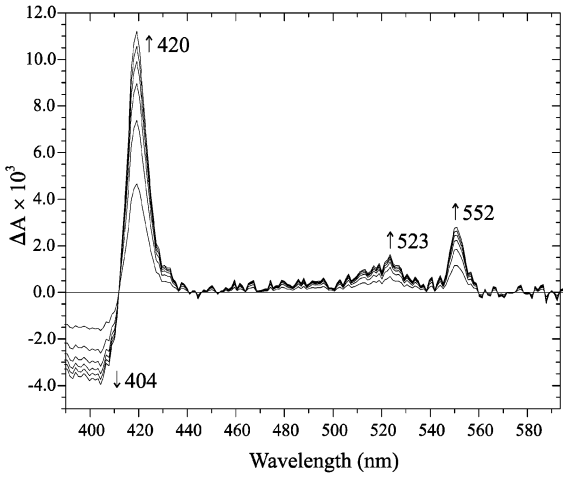

Figure 1. Changes observed in the visible spectrum after photoexcitation of a solution initially containing 47 μM **1** and 87 nM HAO, in 50 mM phosphate buffer pH = 7.4 (path length = 0.5 cm[30]). Under these conditions, [NO•]$_f$, the concentration of NO• present after 1 ms, would be approximately 2.4 μM.[22] In this and all other experiments [NO•]$_f$ was taken to be roughly constant during the subsequent reaction, because it was 5−10× greater than [HAO]. The first five spectra shown are at 300 ms intervals; the final spectrum is one obtained after 6 s.

heme.[28] Application of Beer's law to the final value of ΔA_{420} seen in Figure 1 (corresponding to $t = 6$ s), using the smaller of the $\Delta\epsilon_{420}$ values and a path length of 0.5 cm,[30] shows that at most two *c*-hemes out of the seven available per subunit were reduced following the laser irradiation. However, the three most readily reducible hemes all have $\Delta\epsilon_{420}$ clustered around 105 mM^{-1} cm^{-1},[28] and, if this value is used to analyze ΔA_{420} as above, then the conclusion is that only one *c*-heme per subunit is reduced following the laser pulse.

An apparent rate constant $k_{obs} = 1.7$ s^{-1} was obtained by fitting the data in Figure 1 to an exponential function.

(16) Enemark, J. H.; Feltham, R. D. *Coord. Chem. Rev.* **1974**, *13*, 339−406.
(17) In the designation {M(NO)}^{n}, *n* is the sum of the d e$^-$ that would be counted if the ligand were NO•, plus the unpaired NO e$^-$.
(18) Hoshino, M.; Maeda, M.; Konishi, R.; Seki, H.; Ford, P. C. *J. Am. Chem. Soc.* **1996**, *118*, 5702−5707.
(19) Ford, P. C.; Lorkovic, I. M. *Chem. Rev.* **2002**, *102*, 993−1017.
(20) Hendrich, M. P.; Upadhyay, A. K.; Riga, J.; Arciero, D. M.; Hooper, A. B. *Biochemistry* **2002**, *41*, 4603−4611.
(21) Namiki, S.; Arai, T.; Fujimori, K. *J. Am. Chem. Soc.* **1997**, *119*, 3840−3841.
(22) Cabail, M. Z.; Lace, P. J.; Uselding, J.; Pacheco, A. A. *J. Photochem. Photobiol., A* **2002**, *152*, 109−121.
(23) Ellis, L. M.; Lace, P. J.; Patel, N. K.; Ver Haag, M. P.; Pacheco, A. A. Manuscript in preparation.
(24) HAO was purified as follows. After a 40% $(NH_4)_2SO_4$ fractionation step, the 10000*g* supernatant fraction was chromatographed on an octyl-Sepharose (Amersham Pharmacia Biotech AB) column equilibrated with 50 mM Tris-SO_4^{2-} buffer, pH 7.5, containing 1.7 M $(NH_4)_2SO_4$, and developed with a 1.7−0.0 M $(NH_4)_2SO_4$ linear gradient. Fractions of the eluate with high hydroxylamine cytochrome *c* oxidoreductase activity[1] were pooled and concentrated by ultrafiltration (Amicon YM-10 membrane) and then subjected to chromatography on a Sephacryl S-200 column (Amersham Pharmacia Biotech AB) equilibrated with 50 mM Tris-SO_4^{2-}, pH 7.5, buffer containing 60 mM $(NH_4)_2SO_4$. Fractions of the eluate with high HAO specific hydroxylamine cytochrome *c* oxidoreductase activity were pooled and concentrated as before. The eluate thus obtained showed a single band in SDS/PAGE electrophoresis, due to a ∼200 kDa protein (the molecular mass of HAO is 204 kDa[34]). The concentration of the purified protein was determined from the absorbance of the Soret band at 408 nm, using a molar absorptivity value of 2100 mM^{-1} cm^{-1}, which is 3× the value reported in ref 10 for the molar absorptivity of the individual monomers.
(25) Spectra were collected every millisecond for 30 s, using an Olis RSM-1000 rapid-scanning spectrophotometer. The data were analyzed using the Specfit 32 Global Analysis System (Version 3.0), Spectrum Software Associates, Marlborough, MA.
(26) Hooper, A. B.; Maxwell, P. C.; Terry, K. R. *Biochemistry* **1978**, *17*, 2984−2989.
(27) Yamanaka, T.; Shinra, M.; Takahashi, K.; Shibasaka, M. *J. Biochem.* **1979**, *86*, 1101−1108.
(28) Collins, M. J.; Arciero, D. M.; Hooper, A. B. *J. Biol. Chem.* **1993**, *268*, 14655−14662.
(29) Arciero, D. M.; Golombeck, A.; Hendrich, M. P.; Hooper, A. B. *Biochemistry* **1998**, *37*, 523−529.
(30) The width of photoexcited solution that the spectrophotometer beam must traverse is 0.5 cm, as explained in ref 22. Therefore 0.5 cm is taken to be the solution path length in the application of Beer's law.

Halving $[NO^{\bullet}]_f$ decreased k_{obs} by roughly 50%, suggesting that, in the limited range examined so far, reduction of HAO is first order with respect to $[NO^{\bullet}]_f$. In the absence of **1**, irradiation of HAO with 308 nm laser light did not result in any absorbance changes. The final intensities of the spectral features vary linearly with [HAO]. Adding an excess of up to 1 mM NO_2^- to the reaction mixture had no effect on either k_{obs} or the final intensities of the spectral features.

HAO has two solvent-accessible hemes per subunit that could be directly reduced by NO•. One is P460, and the other is a *c*-heme that has a highly exposed heme edge.[7] The remaining *c*-hemes of HAO are buried within the protein[7] and could only be reduced indirectly by intramolecular electron transfer (IET) either from P460 or from the exposed *c*-heme, once either of these had been reduced by the NO•. Direct reduction of the exposed *c*-heme, on the time scale observed in the present experiments, would necessarily involve outer-sphere e^- transfer from NO• to the heme edge, because the Fe^{III} center is 6-coordinate and not directly available to the reducing ligand. NO• has been shown to displace one of the axial ligands of ferricytochrome *c*, a process that can ultimately lead to cytochrome reduction, but the ligand substitution is slow ($k_{on} = 720\ M^{-1}\ s^{-1}$).[18,31] For its part outer-sphere e^- transfer from NO• to the heme edge is highly unlikely, because such transfer would generate the intermediate NO^+, which has the high 1-e^- reduction potential of 1.2 V at physiological pH.[19,32] In support of this reasoning, separate experiments verified that horse heart ferricytochrome *c* at least does not react in any detectable way with NO• on sub-second time scales.

Given that direct outer-sphere e^- transfer from NO• to the solvent-exposed *c*-heme edge is highly unlikely, the most plausible alternative is that the spectral changes seen in Figure 1 are due to reduction of a *c*-heme by IET from reduced P460, which has itself been previously reduced by NO• as suggested in Scheme 2. As mentioned earlier, such a scheme has extensive precedent in heme chemistry.[18,19,31] An alternative possibility that cannot yet be ruled out is that an impurity enzyme, present in concentrations too small to be detected, could be catalytically oxidizing the NO, and then in turn reducing the HAO.

Two things about the results presented here appear to be at odds with a very recent article that also reported on the interaction of NO• with HAO.[20] The earlier article concluded that NO• is incapable of reducing HAO, and moreover, it presented excellent IR, Mössbauer, and EPR evidence for a stable $\{Fe(NO)\}^6$ moiety in HAO P460 that does *not* hydrolyze to give reduced HAO.[20] The lack of evidence for a $\{Fe(NO)\}^6$ intermediate in the work presented here is not surprising, given the low [NO•] used in the experiments ($\leq 2.4\ \mu M$, as compared to ~ 1 mM in ref 20). The stability constant that governs binding of NO• to ferrihemes is much smaller than that for ferrohemes,[31] and the first equilibrium in Scheme 2 is likely to lie far to the left in solutions with micromole/liter concentrations of NO•. Moreover, the lack of effect of NO_2^- on HAO reduction by NO• (see above) indicates that the back-reaction in the second equilibrium of Scheme 2 is not a significant source of $\{Fe(NO)\}^6$, probably because the subsequent IET reaction is fast and essentially irreversible (the reduction potential for P460 is -260 mV at pH 7, while one of the *c*-hemes has a potential as high as 288 mV[28]). It is not so clear why Hendrich et al. saw no evidence for HAO reduction by NO•.[20] In a preliminary experiment at higher [NO•], we obtained a visible spectrum for HAO under 0.68 atm of NO• gas[33] that clearly showed a signal at 552 nm, attributable to reduced *c*-heme(s) (data not shown). Further studies are currently under way to investigate the reaction of HAO with NO• under a broader range of conditions, including higher [NO•], and different pH values. Hopefully these investigations will clear up the apparent discrepancy.

Acknowledgment. We thank John Enemark and Lara Ellis for proofreading the manuscript. Financial support received from the University of Wisconsin—Milwaukee Graduate School is gratefully acknowledged.

IC025779N

(31) Hoshino, M.; Ozawa, K.; Seki, H.; Ford, P. C. *J. Am. Chem. Soc.* **1993**, *115*, 9568−9575.
(32) Beckman, J. S. In *Nitric Oxide, Principles and Actions*; Lancaster, J., Ed.; Academic Press: New York, 1996; pp 1−82.
(33) NO• gas was Matheson CP grade 99.5%. It was manipulated using an all-glass and metal gas handling apparatus fully described in ref 22 and scrubbed using a column of NaOH pellets prior to use.
(34) Hendrich M. P.; Petasis, D.; Arciero, D. M.; Hooper, A. B. *J. Am. Chem. Soc.* **2001**, *123*, 2997−3005.