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Analysis of Neuronal NO Synthase under Single-Turnover Conditions: Conversion of N^{ω}-Hydroxyarginine to Nitric Oxide and Citrulline†

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ABSTRACT: Nitric oxide synthases (NOSs) are proposed to generate NO and citrulline from L-arginine in two steps: initial N-hydroxylation to generate N^{ω}-hydroxyarginine (NOHA) followed by a three-electron oxidation of the hydroxylated nitrogen to form products. Both steps consume NADPH and may involve heme iron-based activation of O_2. Studies done under multiple-turnover conditions suggest that 0.5 mol of NADPH is consumed to convert 1 mol of NOHA to products, implying that one electron from NADPH may be sufficient. To test this, we studied NOHA oxidation under single-turnover conditions using neuronal NOS (nNOS), whose heme iron reduction requires bound calmodulin. The heme iron in calmodulin-bound nNOS was reduced with excess NADPH under anaerobic conditions, calmodulin was then dissociated from nNOS to prevent subsequent heme iron reduction, NOHA was added, and the reaction initiated by exposure to air. Spectra obtained at each step were consistent with buildup of NOHA-bound ferrous nNOS prior to air exposure. Reactions containing graded amounts of nNOS produced L-citrulline in linear relation (1.2 ± 0.1 mol of citrulline per mole of nNOS). Nitrite and nitrate also accumulated as NO-derived products. Control reactions that contained L-arginine instead of NOHA, no enzyme, or ferric nNOS did not generate products. Thus, supplying a single electron from NADPH to the heme iron permits nNOS to catalyze one full round of citrulline and NO synthesis from NOHA upon exposure to O_2. These data provide a molecular explanation for the NADPH requirement in the second step of the biosynthetic reaction, implicate ferrous−dioxy nNOS as a critical reactant in that step, and eliminate a number of possible alternative catalytic mechanisms or products.

Nitric oxide (NO)[1] is a widespread mediator in physiology and pathology [reviewed in Schmidt and Walter (1994) and Vincent (1994)]. Three NO synthase (NOS) isoforms have been cloned from a variety of sources. Although the NOS isoforms differ regarding their primary sequence, mode of expression, and post-translational control (Brenman et al., 1996; Nathan & Xie, 1994; Bredt & Snyder, 1994; Albakri & Stuehr, 1996), they are structurally and catalytically similar. For example, all NOS are homodimers with each subunit being comprised of a N-terminal oxygenase domain that binds tetrahydrobiopterin (H4B), iron protoporphyrin IX (heme), and L-arginine, and a C-terminal reductase domain that binds calmodulin (CaM), FMN, FAD, and NADPH (Chen et al., 1996; Masters et al., 1996; Ghosh et al., 1996; Xie et al; 1994; McMillan & Masters, 1995). During NO synthesis electrons from NADPH are transferred through the flavins to the heme iron, whose reduction is associated with increased O_2 consumption and initiation of NO synthesis (Abu-Soud et al., 1994, 1995; Heinzel et al., 1992; Pou et al., 1992; Griffith & Stuehr, 1995). Indeed, a direct role for the heme in oxygen binding and activation is supported by recent work showing a transient ferrous−O_2 complex forms upon mixing the ferrous nNOS oxygenase domain with O_2 (Abu-Soud et al., 1997).

All NOS generate NO by oxidizing a terminal guanidino nitrogen of L-arginine. The reaction occurs in two steps and generates N^{ω}-hydroxyarginine (NOHA) as an intermediate (Stuehr et al., 1991; Klatt et al., 1993; Pufahl et al., 1992). Both steps consume NADPH and O_2. Although the O_2 stoichiometry has not been reported, measurement of the NADPH stoichiometry for all three isoforms under multiple-turnover conditions (Stuehr et al., 1991; Klatt et al., 1993; Presta et al., 1997) suggests that 1.5 mol of NADPH is consumed to generate 1 mol of NO from L-arginine. Of this, 1 mol of NADPH appears to be utilized to convert L-arginine to NOHA, while the remainder (0.5 mole) of NADPH is utilized to convert NOHA to NO and citrulline (Figure 1).

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[1] Abbreviations: CaM, calmodulin; DTT, dithiothreitol; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; H_4B, (6*R*,6*S*)-2-amino-4-hydroxy-6-(L-*erythro*-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropteridine; MOPS, 2-(*N*-morpholino)propanesulfonic acid; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOHA, N^{ω}-hydroxy-L-arginine; OPA, *o*-phthaldialdehyde; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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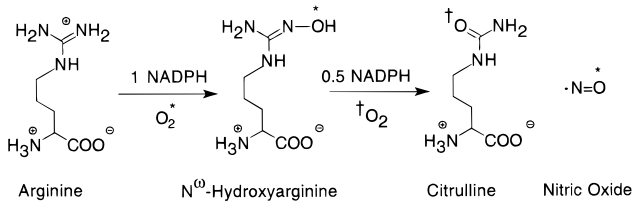

FIGURE 1: NADPH utilization and oxygen incorporation during NO synthesis from L-arginine. Source of oxygen in each product is marked by a symbol.

Related studies using $^{18}O_2$ (Leone et al., 1991; Kwon et al., 1990) showed that the oxygen atoms incorporated into NO and citrulline derive from different molecules of O_2 (Figure 1), suggesting that each step may consume 1 mol of O_2. This led to proposals [reviewed in Kerwin et al., (1995)] that NOHA may form via mixed-function hydroxylation of L-arginine as is typically catalyzed by the cytochrome P-450 class of heme proteins [reviewed in Ortiz de Montellano (1995)]. Such reactions require that two electrons be provided to the heme iron and consume 1 mol of O_2. Mixed-function hydroxylation of L-arginine is supported by studies showing cytochrome P-450's can carry out similar N-hydroxylation reactions with amidines and guanidines (Clement & Jung, 1994; Clement et al., 1993). In contrast, the second step of NO synthesis is less straightforward because it involves a three-electron oxidation of NOHA's hydroxylated nitrogen that is apparently coupled to consumption of only 0.5 mol of NADPH. Although this stoichiometry predicts that a single NADPH-derived electron when provided to the heme should enable NOS to convert NOHA to NO plus citrulline, direct evidence for this transformation has not been presented. In addition, the basis for this prediction has been questioned by reports of different NADPH stoichiometries (Schmidt et al., 1996; Wang et al; 1995; Chen & Rosazza, 1994; Hevel & Marletta, 1993) and by data suggesting that nitroxyl (NO^-) may form as a primary product of NOS catalysis (Schmidt et al., 1996).

To address these issues, we studied the second step of NO synthesis under single-turnover conditions. We used nNOS as a model, because in this particular isoform electron transfer to the heme iron is completely dependent on CaM binding (Abu-Soud & Stuehr, 1993), which in turn is Ca^{2+}-dependent and can be rapidly reversed by adding a Ca^{2+} chelator (Schmidt et al., 1991; Persechini et al., 1995). CaM control of nNOS heme iron reduction provided a means to load a single NADPH-derived electron onto the heme iron and a means to prevent subsequent electrons from transferring onto it. In this report we describe the catalytic properties of ferrous nNOS under single-turnover conditions and discuss our findings in relation to the mechanism of NO synthesis.

MATERIALS AND METHODS

Materials and Enzyme Preparation. NOHA was obtained from Alexis. All other materials were obtained from sources as reported previously (Presta et al., 1997). The nNOS was purified from baculovirus-infected insect cells in the presence of of H4B as described previously (Harteneck et al., 1994). The protein was >90% pure as judged by SDS−PAGE and by its specific activity of 1200 nmol of NO/min per mg in the oxyhemoglobin NO capture assay. For some experiments 0.5 mL of purified enzyme was dialyzed to remove exogenous H4B against 500 mL MOPS, pH 7.4, containing 2 mM DTT, 10% glycerol, and 100 mM NaCl. The H4B content of the dialysis fluid and enzyme was then determined as previously described (Giovanelli et al., 1991; Baek et al., 1993).

Preparation of Reaction Solutions. Anaerobic reaction solutions were prepared in a two-neck, septum-sealed, pear-shaped flask that could be attached through a ground-glass joint to a vacuum gas train. Reactions (120 μL) initially contained 0−20 μM nNOS, a 5-fold molar excess of CaM and H4B relative to nNOS, 0.6 mM EDTA, and 300 μM DTT dissolved in 40 mM Hepes buffer, pH 7.4, and were made anaerobic by repeated cycles of evacuation and equilibration with catalyst-deoxygenated N_2, with vessels finally maintained under positive pressure. Concentrated (15×) stock solutions of EDTA, NADPH, Ca^{2+}, NOHA, or L-arginine were dissolved in buffer, evacuated, and gassed with N_2 in separate vessels. Liquid transfers were done using gas-tight syringes. Final concentrations of Ca^{2+}, EDTA, NADPH, NOHA, and L-arginine in the reaction solutions were 1 mM, 1.8 mM, 200, 150, and 150 μM, respectively. Reactions were initiated by exposing the solutions to air. In some cases, experiments were run in 1 mL septum-sealed quartz cuvettes that could be attached to the vacuum gas train in order to collect spectra at each stage in the experiment.

Optical Spectroscopy. Spectra were recorded with a Hitachi 3110 spectrophotometer at 15 °C.

Enzyme and Amino Acid Analysis. Half of the contents of each reaction were used to determine the nNOS concentration using an estimated extinction coefficient at 398 nm of 72 mM^{-1} cm^{-1} (Abu-Soud & Stuehr, 1993). The remaining reaction solution was immediately filtered through Millipore Ultrafree-MC filter units (10 000 MW cutoff) by centrifuging at 6500 rpm for 30 min. A small amount of 1 M HCl was added to the filtrate to prevent slow nonenzymatic hydrolysis of NOHA. The amino acids in 20 μL of the filtrate were precolumn derivatized by adding 80 μL of *o*-phthaldialdehyde (OPA) reagent solution (35 mM OPA, 10 v/v MeOH, 1 v/v mercaptoethanol in 0.1 M sodium borate). After 2 min of derivatization, a 50 μL sample was injected onto a Hewlett-Packard ODS-Hypersil, 5 μm particle size, 2.1 × 100 mm reverse-phase HPLC column. The column was eluted at a flow rate of 0.75 mL/min with linear gradients of buffers A and B (buffer A = 5% CH_3CN in 15 mM sodium borate with 0.1 v/v trifluoroacetic acid, pH 9.5; buffer B = 50% CH_3CN in 8 mM sodium borate with 0.1 v/v trifluoroacetic acid, pH 9.5) as noted. The solvent gradient was 0−12% B at t = 0−10 min, maintaining this composition at t = 10−15 min, then increasing from 12% to 85% B at t = 15−20 min. This composition was maintained until t = 25 min, before being reduced to the initial 0% B composition. Under these conditions, OPA-derivatized NOHA, citrulline, and arginine were completely resolved and eluted at 16, 19, and 23 min, respectively, and were detected using a Hitachi F-2000 fluorescence spectrophotometer set at 360 nm excitation and 455 nm emission. Citrulline was quantified by means of a calibration curve prepared on the same day as sample analysis. Each filtrate was analyzed in duplicate or triplicate.

Nitrite and Nitrate Measurement. Total nitrite and nitrate in reaction filtrates was measured simultaneously using a Sievers nitrogen oxide analyzer (Boulder, CO) equipped with an in-line vanadium reflux vessel that converted nitrite and

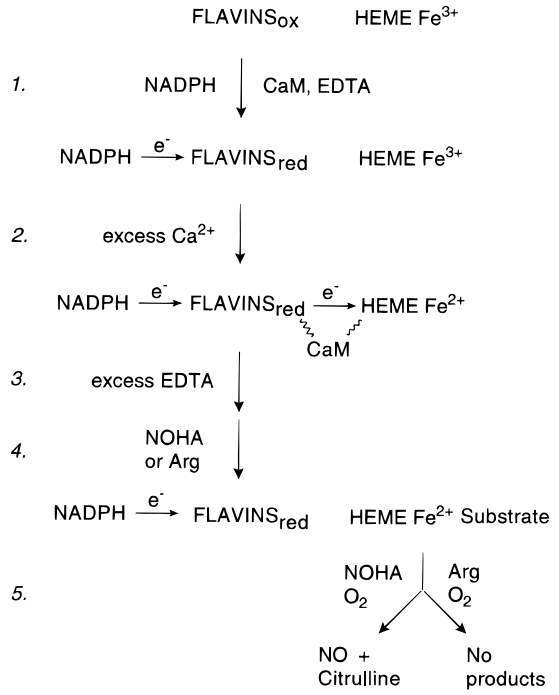

FIGURE 2: Strategy to study NOHA oxidation in a single-turnover reaction. Excess NADPH was added to anaerobic, H4B-saturated ferric nNOS to reduce enzyme bound FAD and FMN (*1*). Excess Ca^{2+} was then added to promote CaM binding, which triggers electron transfer between the nNOS flavins and heme iron (*2*). After full heme iron reduction was achieved (determined spectrophotometrically), excess EDTA was added to remove bound CaM from nNOS (*3*). Excess substrate (NOHA or L-arginine) was added (*4*), and the reaction was initiated by exposure to air (*5*). The predicted outcome using each substrate is shown.

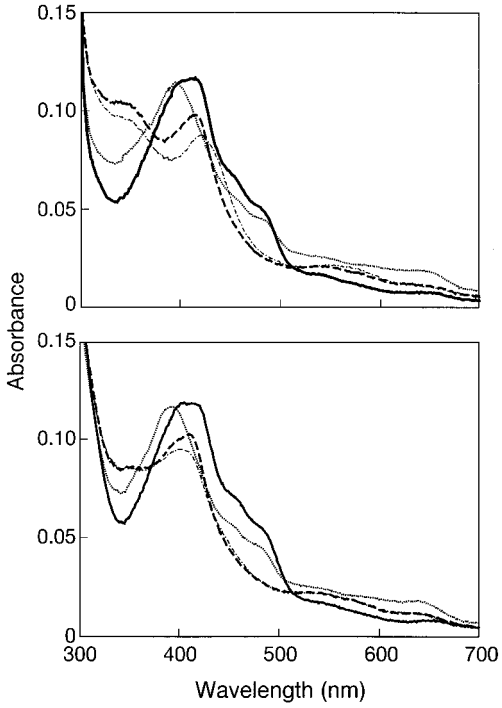

FIGURE 3: Spectroscopic confirmation of steps involved in the single turnover reaction. Cuvettes contained ~1 μM nNOS and a 5-fold molar excess of H4B under a N_2 atmosphere. Either NOHA (upper panel) or L-arginine (lower panel) was added as substrate. Traces were obtained sequentially under the following conditions: ferric nNOS under anaerobic conditions (—); ferrous nNOS after adding 20 μM NADPH and adding Ca^{2+} to promote CaM binding (- - -); ferrous nNOS 5 min after dissociating CaM with EDTA and adding substrate (— . —), and ferric nNOS after exposing the anaerobic reaction solution to air (···). The traces shown are representative of four experiments.

nitrate to NO prior to entering the instrument (Hampl et al., 1996). Values obtained were quantitated on the basis of nitrite and nitrate standards.

RESULTS

Figure 2 illustrates the method used to study nNOS catalysis under single-turnover conditions. Our strategy was to fully reduce the heme iron in a flavin-dependent manner by adding excess NADPH to CaM-bound NOS (steps 1 and 2), then dissociate CaM from nNOS with excess EDTA to prevent subsequent heme iron reduction (step 3), add NOHA or L-arginine (step 4), and initiate catalysis by exposing the sample to air (step 5). Under these conditions, the moles of NOHA converted to citrulline should equal the moles of ferrous hemeprotein present in the reaction, if this step truly requires that only one NADPH-derived electron be provided to the heme iron. In contrast, a reaction in which L-arginine substitutes for NOHA should yield no amino acid products, because the NADPH stoichiometry suggests that L-arginine oxidation requires that either two or three NADPH-derived electrons be provided to the heme to generate NOHA or citrulline as products, respectively.

We employed light absorbance spectroscopy to monitor each step in experimental sequence described in Figure 2. The spectrum of ferric nNOS exhibited a broad Soret peak centered at 400 nm with shoulders at 456 and 485 nm (Figure 3, upper and lower panels), consistent with its containing oxidized flavins and a mixture of high- and low-spin heme iron in the H4B-saturated, substrate-free state (McMillan & Masters, 1995; Abu-Soud & Stuehr, 1993). Addition of excess NADPH followed by CaM binding caused reduction of nNOS flavins and heme iron as indicated by a disappearance of flavin absorbance between 450−480 nm, a decrease in Soret extinction, and a shift in its maxima to 417 nm (Abu-Soud et al., 1994). Dissociating bound CaM from ferrous nNOS with 1.8 mM EDTA did not result in a spectral change for up to 60 min, confirming that our anaerobic conditions were sufficient to maintain the heme iron in its reduced state. Addition of NOHA or L-arginine to the reduced nNOS caused a shift in Soret absorbance in both cases, confirming that they bound to the ferrous enzyme. Exposing the solution to air resulted in a shift in Soret absorbance to 390 nm (L-arginine) or 395 nm (NOHA) and a return of the flavin visible absorbance peaks in both cases. Thus, air exposure converted nNOS to its substrate-bound ferric form, which is the expected result following single catalytic turnover in the presence of excess substrate.

The experiment described above was repeated using 200 μM NADPH and different nNOS concentrations ranging from 0 to 20 μM, and the final NOHA and citrulline concentrations in each sample were determined by HPLC analysis. As shown in Figure 4, the citrulline concentration increased in a linear manner with increasing nNOS concentration. Least-squares analysis gave a line with a slope of 1.2 ± 0.1 and a correlation coefficient of 0.93. We observed a corresponding decrease in NOHA concentration, and no other amino acid products were detected (data not shown). The concentration of nitrite plus nitrate generated in some of the reactions was also measured by chemiluminescence

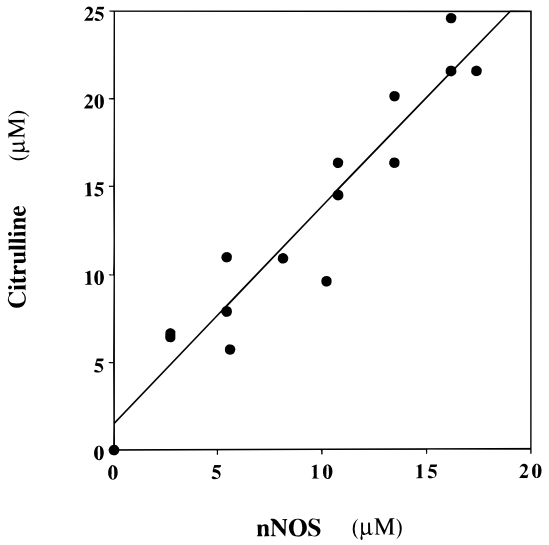

FIGURE 4: Citrulline formed from NOHA as a function of nNOS concentration in the single turnover reaction. The reactions were run as detailed in Materials and Methods and contained various concentrations of nNOS. The points represent the citrulline concentrations achieved in single reactions from three separate experiments. The slope and standard deviation were determined using least-squares analysis.

to determine if NO was produced along with citrulline. Reactions containing 0.8 and 1.6 nmol of nNOS formed 0.6 and 1.4 nmol nitrite plus nitrate, respectively. To determine the dependence on bound H4B, single turnover reactions were run in the absence of added H4B using nNOS that had been dialyzed in H4B-free buffer. Reactions containing 20 μM nNOS generated 13.2 $\pm$ 3 μM citrulline (n = 5), consistent with the dialyzed nNOS containing only 0.5 $\pm$ 0.1 mol of bound H4B per mole of heme (n = 3). Together, the results indicate that nNOS can quantitatively convert NOHA to citrulline plus NO when its heme is provided with a single NADPH-derived electron, and catalysis is dependent on bound H4B.

Control reactions containing L-arginine in place of NOHA showed only trace or no conversion to NOHA or L-citrulline (data not shown). Ferric nNOS was unable to oxidize NOHA in the absence of an electron donor, consistent with a previous report (Pufahl & Marletta, 1993). No amino acid products were detected in the absence of substrate, and NOHA was not converted to products in the absence of nNOS (data not shown). When Ca^{2+} was allowed to remain in excess of EDTA, all of the NOHA in the reaction was converted to citrulline. This is consistent with NADPH and O_2 being provided in excess relative to NOHA and with nNOS undergoing multiple rounds of heme reduction and catalysis when CaM remains bound to the enzyme.

DISCUSSION

We studied NO synthesis under single-turnover conditions to better understand the mechanism of the second step. The NADPH requirement in this step was suggested to be 0.5 NADPH oxidized per mole of NO formed from NOHA, based on experiments done under multiple-turnover settings (Klatt et al., 1993; Stuehr et al., 1991; Presta et al., 1997). Our current results indicate that utilization of 0.5 NADPH actually reflects a need to transfer a single NADPH-derived electron to the nNOS heme iron. Once this occurs, subsequent exposure to O_2 enables ferrous nNOS to catalyze one round of NO and citrulline synthesis from NOHA and converts nNOS back to its ferric form in the process. Thus, nNOS becomes committed to catalyzing the second step in NO synthesis once its heme iron is reduced. Although ferrous nNOS could clearly oxidize bound NOHA upon exposure to air, it was completely incapable of hydroxylating L-arginine or converting it to citrulline plus NO. This is consistent with the original NADPH stoichiometry studies that suggested N-hydroxylation requires two electrons be provided by NADPH, and indicates that electron transfer between two nNOS molecules did not occur within the time course of the reaction in our system.

Given that ferrous nNOS converts NOHA to products upon exposure to O_2, what does this tell us about the mechanism of the second step in NO synthesis? To address this question it is useful to review the proposed mechanism for oxygen activation in NOS, which is thought to proceed according to the general mechanism outlined for the cytochrome P-450's [reviewed in Ortiz de Montellano (1995)]:

$$\text{heme } Fe^{III} \xrightarrow[O_2]{e^-} \underset{\mathbf{I}}{\text{heme } Fe^{II}O_2} \xrightarrow{e^-} \underset{\mathbf{II}}{\text{heme } Fe^{II}O_2^-} \xrightarrow[-H_2O]{2H^+} \underset{\mathbf{III}}{\text{heme } Fe^{IV}O}$$

Initial reduction of the nNOS heme iron enables O_2 binding and formation of the ferrous−dioxy complex (**I**). Transfer of a second electron is thought to form an iron−peroxy species (**II**), which may protonate, lose water, and form a high valence iron−oxo complex (**III**).

On the basis of the NADPH requirement (Klatt et al., 1993; Stuehr et al., 1991), model studies (Clement et al., 1993), and analogy to the cytochromes P-450 (Mansuy & Renaud, 1995; Masters et al., 1996), it is generally assumed that the first step of NO synthesis (i.e., N-hydroxylation of L-arginine) involves a reaction between the iron−oxo complex (**III**) and L-arginine. However, formation of **III** requires that two electrons be provided to the heme iron, but neither NOHA nor H4B can reduce ferric nNOS (Pufahl & Marletta, 1993). Thus, **III** cannot form under the single-turnover setting and therefore cannot be the species that initially reacts with NOHA. Rather, our results implicate the ferrous−dioxy complex (**I**) of nNOS as a possible initial reactant because it is the only species that could form when a single electron is made available to the heme. Indeed, our related work has directly demonstrated that the ferrous−dioxy complex forms as a transient species upon mixing reduced (ferrous) nNOS oxygenase domain with O_2 (Abu-Soud et al., 1997). Thus, we can now state that (1) provision of a single electron to the heme iron enables formation of ferrous−dioxy nNOS, and (2) a reaction between this species and bound NOHA generates NO and citrulline.

Although the ferrous−dioxy species appears to react directly with NOHA in the second step of NO synthesis, how it participates is not precisely known. Current proposals suggest that it may remove a hydrogen atom or electron from NOHA (Marletta, 1993; Griffith & Stuehr, 1995; Korth et al., 1994; Kerwin et al., 1995). Hydrogen atom abstraction from NOHA was originally proposed as one possible means to transfer an electron back to NOS in order to satisfy the observed reaction stoichiometry of 0.5 NADPH oxidized per

NO formed in the second step (Stuehr & Griffith, 1992). Hydrogen atom transfer to ferrous−dioxy nNOS would generate a protonated iron−peroxy species (**II**) that is widely imagined to be capable of attacking the NOHA radical at its guanidino carbon, forming a tetrahedral intermediate that rearranges to generate citrulline, NO, water, and ferric nNOS [reviewed in Kerwin et al., (1995)]. Although direct evidence for these reactions is not yet available, reaction of (**II**) with NOHA has been successfully modeled using an organic hydroxyguanidine and a peracid as an oxygen-based nucleophile (Fukuto et al., 1993). Our single turnover results are consistent with NOHA reducing ferrous−dioxy nNOS to form (**II**) as the initial event in the second step of NO synthesis and for the first time eliminate other possible sources of the electron like the nNOS flavins (Stuehr & Griffith, 1992).

Although the chemical transformations involved in NO synthesis can be fully accounted for by heme-based oxygen activation and catalysis, NOS enzyme activity under multiple-turnover settings requires H4B (Hevel & Marletta, 1992; Heinzel et al., 1992). Our current results show that bound H4B is clearly required for NO synthesis under single-turnover conditions. On the basis of the role of H4B in aromatic amino acid hydroxylation [reviewed in Dix & Benkovic, (1988)], it is conceivable that H4B could function as an electron donor or directly participate in oxygen activation during NO synthesis (Marletta, 1993; Griffith & Stuehr, 1995). However, studies that specifically looked for H4B oxidation or redox cycling during NO synthesis have thus far proven negative (Giovanelli et al., 1991; Whiteveen et al., 1996). It may be unlikely that H4B plays a redox role in NOHA oxidation for several reasons. First, as discussed in detail previously (Kerwin et al., 1995), H4B-derived electrons are not required to account for oxygen activation in the proposed reaction and would actually create a circumstance in which excess reducing equivalents must be removed from the enzyme (presumably in the form of superoxide or H_2O_2). Second, heme iron reduction is closely associated with activation of NO synthesis (Abu-Soud et al., 1995) and leads to formation of a NOS ferrous−dioxy complex (Abu-Soud et al., 1997), which eliminates the need to invoke a role for H4B in reductive oxygen activation. On the other hand, H4B affects NOS protein structure in ways that may directly impact on catalysis (Klatt et al., 1995; Ghosh et al., 1996; Baek et al., 1993) and when bound to nNOS alters the stability of the ferrous−dioxy complex (Abu-Soud et al., 1997). Thus, H4B may perform essential functions without undergoing formal oxidation or reduction.

Our single-turnover study further limits the number of catalytic mechanisms that remain consistent with the data. In particular, it is now difficult to envision how nNOS oxidation of NOHA could generate molecules other than citrulline and NO as primary products. For example, formation of NO^- instead of NO (Schmidt et al., 1996) in the single-turnover system would require that an additional electron be provided to the heme from an unknown source [discussed in Fukuto et al., (1993) and Stuehr and Griffith (1992)]. NO^- production in the absence of superoxide dismutase would also be expected to generate N_2O as a major accumulating end product (Fukuto et al., 1993) rather than nitrite and nitrate, which are instead main products of NO oxidation in solution (Wink et al., 1996) and actually accumulated in our single turnover reactions. Finally, the fact that ferrous nNOS catalyzed one full round of citrulline and NO synthesis from NOHA in a single-turnover setting indicates that superoxide and NO were not generated concurrently and thus implies that heme iron reduction and NO synthesis are tightly coupled when nNOS is provided with saturating concentrations of substrate and H4B.

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REFERENCES

Abu-Soud, H. M., & Stuehr, D. J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 10769−10772.
Abu-Soud, H. M., Yoho, L. L., & Stuehr, D. J. (1994) *J. Biol. Chem. 269*, 32047−32050.
Abu-Soud, H. M., Wang, J., Rousseau, D. L., Fukuto, J., Ignarro, L. J., & Stuehr, D. J. (1995) *J. Biol. Chem. 270*, 22997−23006.
Abu-Soud, H. M., Gachhui, R., Raushel, F. M., & Stuehr, D. J. (1997) *J. Biol. Chem. 272*, 17349−17353.
Albakri, Q. A., & Stuehr, D. J. (1996) *J. Biol. Chem. 271*, 5414−5421.
Baek, K. J., Thiel, B. A., Lucas, S., & Stuehr, D. J. (1993) *J. Biol. Chem. 268*, 21120−21129.
Bredt, D. S., & Snyder, S. H. (1994) *Annu. Rev. Biochem. 63*, 175−195.
Brenman, J. E., Chao, D. S., Gee, S. H., McGee, A. W., Craven, S. E., Santillano, D. R., Wu, Z., Huang, F., Xia, H., Peters, M. F., Froehner, S. C., & Bredt, D. S. (1996) *Cell 84*, 757−767.
Chen, P.-F., Tsai, A.-L., Berka, V., & Wu, K. K. (1996) *J. Biol. Chem. 271*, 14631−14635.
Chen, Y., & Rosazza, J. P. N. (1994) *Biochem. Biophys. Res. Commun. 203*, 1251−1258.
Clement, B., & Jung, F. (1994) *Drug Metab. Dispos. 22*, 486−497.
Clement, B., Schultze-Mosgau, M. H., & Wohlers, H. (1993) *Biochem. Pharmacol. 46*, 2249−2267.
Dix, T. A., & Benkovic, S. J. (1988) *Acc. Chem. Res. 21*, 101−107.
Feron, O., Belhassen, L., Kobzik, L., Smith, T. W., Kelley, R. A., & Michel, T. (1996) *J. Biol. Chem. 271*, 22810−22814.
Fukuto, J. M., Stuehr, D. J., Feldman, P. L., Bova, M. P., & Wong, P. (1993) *J. Med. Chem. 36*, 2666−2670.
Ghosh, D. K., Abu-Soud, H. M., & Stuehr, D. J. (1996) *Biochemistry 35*, 1444−1449.
Giovanelli, J., Campos, K. L., & Kaufman, S. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7091−7095.
Griffith, O. W., & Stuehr, D. J. (1995) *Annu. Rev. Physiol. 57*, 707−736.
Hampl, V., Walters, C. L., & Archer, S. L. (1996) in *Methods in Nitric Oxide Research* (Feelisch, M., & Stamler, J. S., Eds.) pp 309−318, John Wiley & Sons, NY.
Harteneck, C., Klatt, P., Schmidt, K., & Mayer, B. (1994) *Biochem. J. 304*, 683−686.
Heinzel, B., John, M., Klatt, P., Bohme, E., & Mayer, B. (1992) *Biochem. J. 281*, 627−630.
Hevel, J. M., & Marletta, M. A. (1992) *Biochemistry 31*, 7160−7165.
Hevel, J. M., & Marletta, M. A. (1993) in *Chemistry and Biology of Pteridines and Folates* (Ayling, J. E., Ed.) pp 285−288, Plenum Press, New York.
Kerwin, J. F., Jr., Lancaster, J. R., Jr., & Feldman, P. L. (1995) *J. Med. Chem. 38*, 4343−4362.
Klatt, P., Schmidt, K., Uray, G., & Mayer, B. (1993) *J. Biol. Chem. 268*, 14781−14787.
Klatt, P., Schmidt, K., Lehner, D., Glatter, O., Bachinger, H. P., & Mayer, B. (1995) *EMBO J. 14*, 3687−3695.

Korth, H.-G., Sustmann, R., Thater, C., Butler, A. R., & Ingold, K. (1994) *J. Biol. Chem. 269*, 17776−17779.

Kwon, N. S., Nathan, C. F., Gilker, C., Griffith, O. W., Matthews, D. E., & Stuehr, D. J. (1990) *J. Biol. Chem. 265*, 13442−13445.

Leone, A. M., Palmer, R. M. J., Knowles, R. G., Francis, P. L., Ashton, D. S., & Moncada, S. (1991) *J. Biol. Chem. 266*, 23790−23795.

Marletta, M. A. (1993) *J. Biol. Chem. 268*, 12231−12234.

Mansuy, D., & Renaud, J.-P. (1995) in *Cytochrome P450: Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P. R., Ed.) 2nd ed., pp 537−574, Plenum Press, New York.

Masters, B. S. S., McMillan, K., Sheta, E. A., Nishimura, J. S., Roman, L. J., & Martasek, P. (1996) *FASEB J. 10*, 552−558.

McMillan, K., & Masters, B. S. S. (1995) *Biochemistry 34*, 3686−3693.

Nathan, C. F., & Xie, Q.-w. (1994) *J. Biol. Chem. 269*, 13725−13728.

Ortiz de Montellano, P. R. (1995) in *Cytochrome P450: Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P. R., Ed.) 2nd ed., pp 245−304, Plenum Press, New York.

Persechini, A., White, H. D., & Gansz, K. J. (1995) *J. Biol. Chem. 271*, 62−67.

Pou, S., Pou, W. S., Bredt, D. S., Snyder, S. H., & Rosen, G. M. (1992) *J. Biol. Chem. 267*, 24173−24176.

Presta, A., Liu, J., Sessa, W. C., & Stuehr, D. J. (1997) *Nitric Oxide: Biol. Chem. 1*, 74−87.

Pufahl, R. A., & Marletta, M. A. (1993) *Biochem. Biophys. Res. Commun. 193*, 963−970.

Pufahl, R. A., Nanjappan, P. G., Woodard, R. W., & Marletta, M. A. (1992) *Biochemistry 31*, 6822−6828.

Schmidt, H. H. H. W., & Walter, U. (1994) *Cell 78*, 919−925.

Schmidt, H. H. H. W., Pollock, J. S. P., Nakane, M., Gorsky, L. D., Forstermann, U., & Murad, F. (1991) *Proc. Natl. Acad. Sci. U. S. A. 88*, 365−369.

Schmidt, H. H. H. W., Hofmann, H., Schindler, U., Shutenko, Z. S., Cunningham, D. D., & Feelisch, M. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 14492−14497.

Stuehr, D. J., Kwon, N. S., Nathan, C. F., Griffith, O. W., Feldman, P. L., & Wiseman, J. (1991) *J. Biol. Chem. 266*, 6259−6263.

Stuehr, D. J., & Griffith, O. W. (1992) *Adv. Enzymol. Rel. Areas Mol. Biol. 65*, 287−346.

Vincent, S. R. (1994) *Prog. Neurobiol. 42*, 129−160.

Wang, W., Inoue, N., Nakayama, T., Ishii, M., & Kato, T. (1995) *Anal. Biochem. 227*, 274−280.

Whiteveen, C. F. B., Giovanelli, J., & Kaufman, S. (1996) *J. Biol. Chem. 271*, 4143−4147.

Wink, D. A., Beckman, J. S., & Ford, P. C. (1996) in *Methods in Nitric Oxide Research* (Feelisch M., & Stamler J. S., Eds.) pp 29−37, John Wiley & Sons, New York.

Xie, Q.-w., Cho, H. J., Kashiwabara, Y., Baum, M., Weidner, J. R., Elliston, K., Mumford, R., & Nathan, C. (1994) *J. Biol. Chem. 269*, 28500−28505.

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