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Reactivity of the Flavin Semiquinone of Nitric Oxide Synthase in the Oxygenation of Arginine to N^G-Hydroxyarginine, the First Step of Nitric Oxide Synthesis

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Nitric oxide synthase (NOS) is a heme protein that catalyzes the oxygenation of L-arginine in the presence of NADPH to form nitric oxide, L-citrulline and $NADP^+$, and proceeds via two partial reactions:

L-Arginine → N^G-hydroxy-L-arginine (1)

N^G-Hydroxy-L-arginine → L-citrulline + nitric oxide (2)

Calmodulin, FAD, FMN and tetrahydrobiopterin are required for both reactions. Reactions 1 and 2 require the input of 2 and 1 electron equivalents, respectively. Under normal multiple turnover conditions, these electrons are ultimately derived from NADPH. We previously reported that NOS contains an endogenous reductant that, in the absence of NADPH, can support the single-turnover oxygenation of L-arginine to N^G-hydroxy-L-arginine and a relatively small amount of L-citrulline [Campos, K. L., Giovanelli, J., and Kaufman, S. (1995) *J. Biol. Chem.* 270, 1721–1728]. This reductant has now been identified as the stable flavin semiquinone free radical (FSQ). Its oxidation appears to be coupled to the formation of N^G-hydroxy-L-arginine and L-citrulline. The rate of FSQ oxidation is two orders of magnitude slower than the flux of electrons from NADPH through NOS during normal turnover of the enzyme, indicating that FSQ is not the proximal electron donor for heme under these conditions. © 1998 Academic Press

Nitric oxide synthase (NOS) catalyzes the oxygenation of L-arginine in the presence of NADPH to form nitric oxide, L-citrulline and $NADP^+$. Based largely on the assumption that the path of electron flow during the NOS-catalyzed oxygenation reaction is the same as in the cytochrome P450 system, it has been postulated that during the NOS-catalyzed reaction, electrons are transferred from NADPH via the flavins FAD and FMN to heme. Reduction of heme iron leads to the binding and subsequent reduction of oxygen to generate the ultimate oxidant (1,2). NOS differs from the P450 enzymes in that both Ca^{2+}/calmodulin (CaM) and 6-(L-erythro-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropterin (BH_4) are essential for nitric oxide synthesis (3). The overall reaction catalyzed by NOS proceeds by two partial reactions:

L-Arginine → N^G-hydroxy-L-arginine (NHA) (1)

NHA → L-citrulline + NO (2)

Stoichiometry measurements show that approximately 1.5 mol (3 electron equivalents) of NADPH are consumed during the oxidation of each mol of L-arginine to L-citrulline and nitric oxide, and 0.5 mol of NADPH (1 electron equivalent) during the oxidation of each mol of NHA to L-citrulline and nitric oxide (4,5), i.e. reactions 1 and 2 require the input of 2 and 1 electron equivalents, respectively.

Campos *et al.* (6) have reported that *in the absence of NADPH,* neuronal NOS catalyzes a single-turnover oxygenation of L-arginine to NHA (Reaction 1). These studies indicated the presence of an NOS-bound reductant that can substitute for NADPH in the single-turnover oxygenation of L-arginine to NHA. The nature of this reductant was not determined. Neither NADPH,

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reduced heme nor photoreduction appeared to be involved; reduced flavin and BH_4 remained as possible candidates.

This system was considered worthy of further study, since it allows direct examination of the sources of reducing potential in NOS under single-turnover conditions without the complication of regeneration of electron carriers by NADPH. Here we show that the Ca^{2+}/CaM-dependent oxidation of NOS-bound flavin semiquinone free radical, FMNH· (FSQ) can account for all the electrons required for the observed oxygenation of L-arginine to NHA and of NHA to L-citrulline, suggesting a coupling between FSQ oxidation and the oxygenation reactions under these single turnover conditions. The kinetics of FSQ oxidation also provide insights into the nature of the flavin species involved in the normal turnover of NOS.

EXPERIMENTAL PROCEDURES

Materials. The following materials were obtained from the sources shown in parentheses: Beef liver catalase (Boehringer Mannheim, Indianapolis, IN); N^G–nitro–L–arginine, Amberlite CG-50, ophthaldialdehyde (OPA), and Chelex 100 resin (Sigma Chemical Corp., St. Louis, MO); high purity bovine brain CaM (Calbiochem, San Diego, CA). BH_4 (B. Schircks Laboratories, Jona, Switzerland); [2,3,4,5–^{3}H]-L-arginine (Amersham Corp.); AG 50W-X8 200-400 mesh, an analytical grade of Dowex 50 (Bio Rad Laboratories, Richmond, CA); 4-(2-aminoethyl)-benzenesulfonylfluoride (AEBSF), leupeptin and pepstatin (ICN Biochemicals, Aurora, OH); NHA (Alexis Corp., San Diego, CA) was purified as described below.

Preparation of ^{3}H-L-citrulline. Radioactive ^{3}H–L-citrulline of high specific activity and radiopurity was used to monitor the recovery of L-citrulline formed by oxygenation of NHA. ^{3}H–L-citrulline was prepared by incubation of ^{3}H–L-arginine (specific activity of 63 Ci mmol^{-1}) with NOS under the conditions of the assay of Bredt and Snyder (7). ^{3}H–L-citrulline so formed was purified by negative adsorption on Dowex 50 equilibrated with Na acetate, pH 5.5, followed by adsorption on Dowex 50–H^+ and elution with 2N NH_4OH. The eluate from Dowex 50 was then subjected to thin layer chromatography with n-butanol/acetone/diethylamine/water (70/70/14/35, v/v) on an Eastman Kodak Chromagram cellulose plate that had been previously washed by development first with the same solvent, then with water. Radioactivity corresponding with ^{3}H–L-citrulline was eluted with water.

Purification of NHA. NHA was applied to a column of the Amberlite CG-50 resin equilibrated with Na acetate, pH 5.5 in a 0.1 N solution of the same buffer. The resin was washed with water, then eluted with 0.1 N HCl to recover NHA. All procedures were carried out at 40C. High performance liquid chromatography (HPLC) of the OPA derivative of the purified material yielded a single peak corresponding in retention time to NHA, with no fluorescing material corresponding to L-citrulline.

Enzymes. Rat brain NOS was purified from transfected human kidney 293 cells (8) by the procedure of McMillan *et al.* (9), with the following modifications. The homogenization buffer contained 50 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 1 mM EDTA, 0.5 mM DTT, 0.5 mM L-arginine, 5 μM BH_4, 0.1 mM AEBSF, 10 μg/ml antipain, and 0.5 μM leupeptin and pepstatin. Buffer B used for equilibration and the first wash (10 bed volumes) of the 2′,5′-ADP-Sepharose 4B column was as described by McMillan et al. (9) except for addition in our work of 0.5 mM L-arginine, 5 μM BH_4, and substitution of AEBSF (0.1 mM), leupeptin (0.5 μM) and pepstatin (0.5 μM) for the protease inhibitors described by McMillan et al. (9). NaCl (0.4M) was added to buffer B for the next wash (4 bed volumes) of the affinity column, which was then eluted with buffer B containing 0.4 M NaCl and 25 mM 2′-AMP. Buffer C used for gel filtration consisted of 50 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 0.1 mM EDTA, 100 mM NaCl, together with the protease inhibitors present in buffer B. The enzyme was more than 95% pure as judged by SDS-PAGE and typically had a specific activity of 250-400 nmol min^{-1} mg^{-1} as determined by L-citrulline formation during a 15 min incubation at 250C (7). The BH_4 content of the purified NOS was approximately 0.5 mol per mol subunit as determined on HPLC after oxidation of the sample (10). Molar concentrations of NOS are reported for the subunit. The flavin domain of rat brain NOS (amino acids 724-1429, which includes the CaM binding site) was prepared according to Gachhui *et al.* (11). Protein was determined by the bicinchoninic acid method (Pierce manual 23225X) with the use of a bovine serum albumin standard.

Assay conditions. Except where noted, incubations were at 200C and compositions of reaction mixtures include any contribution of components (buffers, etc.) present in NOS.

Single turnover oxygenation of L-arginine to NHA. The formation of NHA in the absence of added NADPH was measured as described in Campos *et al.* (6).

Comparison of the single turnover of L-arginine to NHA and of NHA to L-citrulline. For comparison of the conversion of NHA to L-citrulline relative to that of L-arginine to NHA, each reaction mixture contained (in a final volume of 30 μl) NOS (8 μM subunit), 50 μM NHA or ^{3}H-L-arginine, 18 μM CaM, 0.5 mM Ca^{2+}, 35 μM BH_4, 42 mM Tris–HCl (pH 7.5), 83 mM NaCl, 83 μM EDTA, 8.3% (v/v) glycerol, 83 μM AEBSF, and 420 nM leupeptin and pepstatin. Incubation was for 10 min. Products of the oxygenation of L-arginine were assayed as described above. Reaction mixtures for assay of NHA oxygenation were quenched with perchloric acid to a final concentration of 45 mM followed by addition of ^{3}H-L-citrulline (18600 dpm, 0.13 pmol). The mixture was centrifuged and the supernatant solution titrated with $KHCO_3$ to a pH of approximately 5.5 using methyl red as an internal indicator. The precipitate of potassium perchlorate was removed by centrifugation, and sodium acetate (pH 5.5) added to a final concentration of approximately 24 mM. Compounds other than citrulline that can react with OPA (NHA, Tris, BH_4) were removed by passing the solution through a column containing 50 μl of Dowex 50 equilibrated with Na acetate, pH 5.5. The resin was washed with 50 μl of water and the ^{3}H content of an aliquot of the combined material not retained on the column was measured for determination of ^{3}H-L-citrulline recovery. Another aliquot of the combined material was derivatized with a freshly prepared solution of OPA (12) and the products analyzed for the fluorescent derivative of L-citrulline by reverse phase HPLC on a Beckman Ultrasphere 5 μm column (4.6 mm x 150 mm), eluted isocratically with 34 mM NaH_2PO_4: 32% methanol (v/v) (12). These values were corrected for recovery of ^{3}H-L-citrulline and for a blank reaction in which perchloric acid was added before addition of NOS. A blank reaction in which Ca^{2+}/CaM was omitted gave comparable results. L-citrulline was quantified by means of a calibration curve established on the same day as sample analysis.

Optical measurements of FSQ. Spectra were taken every 5 seconds with a Hewlett Packard 8450A or 8453 diode array spectrophotometer. Oxidation of FSQ was determined from the decrease in absorbance at 590 nm (11,13). Any instrumental drift was corrected by an absorbance value at 590 nm that was calculated from the relatively small changes in absorbance at the isosbestic point (500 nm) and at 700 nm, assuming a linear change in absorbance between 500 nm and 700 nm. First order rate constants were calculated by fitting the data to the equation $Y = Ae^{-kt}+B$, where k is the first order rate constant, Y is the absorbance at time t, A is an amplitude factor, and B is a constant. The absolute concentration of FSQ was estimated by using an extinction coefficient (2.95 mM^{-1}cm^{-1}) based on the average of 3.5 and 2.4 mM^{-1}cm^{-1} determined respectively by titration with ferricyanide of the reductase domain of neuronal NOS

(11), and a mutant neuronal NOS that lacked heme and BH_4 (14). Attempts at determining an extinction coefficient for the native enzyme by titration with ferricyanide were unsuccessful because of the simultaneous oxidation by this compound of enzyme-bound BH_4.

Electron paramagnetic resonance (EPR) measurements of FSQ. For EPR measurements, NOS was first dialyzed against Buffer C containing a suspension of Chelex 100 resin to remove metal ions, then concentrated by microfiltration with a Centricon 100 Microconcentrator (Amicon, Beverly, MA). This procedure appeared to result in a loss of FSQ, since these preparations showed no EPR signal or decrease in the absorbance at 590 nm upon addition of Ca^{2+}/CaM. FSQ was regenerated by preincubation of NOS with NADPH (13) in air at 15°C for 2 min. Addition of NADPH (1 mol NADPH/mol NOS subunit) resulted in an initial rapid formation of reduced flavins, that were slowly oxidized to yield FSQ as the dominant reduced flavin species at the end of the preincubation.[3]

Reactions were carried out as described in the legend to Fig. 2. EPR spectra were recorded on a Bruker ESP 300 spectrometer with TE_{102} resonator operated at 9.78 GHz with 100 kHz magnetic field modulation. The quartz capillary (0.6 mm internal diameter) was used as a sample tube (sample volume = 20 μl). The conditions for the acquisition of spectral data were as follows: temperature, 25°C; microwave power, 10 mW; modulation amplitude, 3.0 G; conversion time, 41 ms; scan time, 42 s; sweep width, 1,000 G with 1024 resolution. Data from 20 spectral scans were accumulated.

RESULTS

Oxidation of endogenous FSQ of NOS. Figure 1 illustrates the oxidation of the endogenous FSQ of NOS and its dependence on CaM binding. Addition of Ca^{2+} at zero time to a reaction mixture containing NOS, CaM, L-arginine and the remaining components listed in the legend to Fig. 1 leads to a sharp decrease in absorbance at 590 nm indicative of a decrease in FSQ content. This decrease was arrested by addition of excess EDTA; upon subsequent addition of excess Ca^{2+}, the oxidation of FSQ resumed. As illustrated in the *Insert*, the time course of oxidation of FSQ shown only with the data points taken in the presence of sufficient Ca^{2+} for CaM binding is first order with a rate constant of 0.78 min^{-1}.

These decreases in FSQ based on optical determinations were confirmed by EPR measurements (Fig. 2). The spectrum of NOS (Fig. 2, spectrum A) exhibits one line absorption at g=2.002 that has a peak-to-peak width of 19 G. The g value and the line width are similar to those obtained from flavin semiquinone free radicals (13,15-17). Previous EPR studies carried out at liquid helium temperature have shown that the FSQ of NOS is spin-spin coupled to both the ferric ion of the oxygenase domain and dissolved oxygen (13,15). It is, therefore, surprising that EPR spectra of FSQ were observed at 250C. To confirm the identity of FSQ, a similar experiment was carried out with a sample containing NOS flavin domain protein in place of native NOS. The spectrum obtained from this sample (Fig. 2, spectrum B) exhibits a g value, line width, and line shape identical to spectrum A. The g value and line width (20 G) reported by Iyanagi and Mason (16) for the FSQ of NADPH cytochrome P450 reductase measured at room temperature also closely resemble those observed with NOS. These results together indicate that at room temperature, the interaction of the flavin and heme domains in the NOS preparation used in these studies is minimal in the absence of Ca^{2+}/CaM.

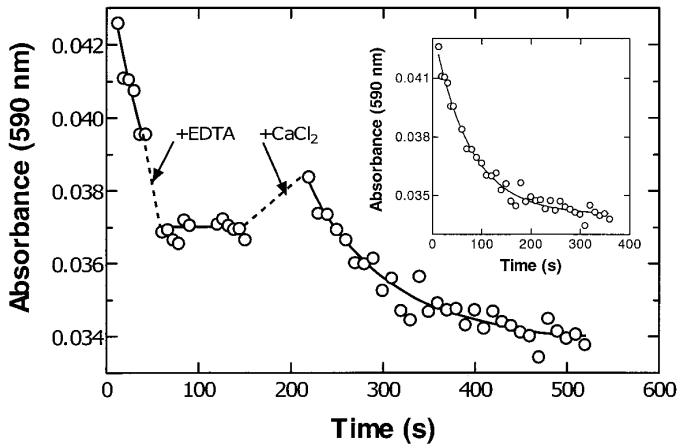

FIG. 1. Optical studies of oxidation of FSQ of NOS and requirement for CaM binding. The reaction was started by adding 0.5 μl of 40 mM $CaCl_2$ (200 μM final concentration) to a reaction mixture containing 5 μM NOS, 29 μM CaM, 50 μM L-arginine, 25 μM BH_4, 45 mM Tris-HCl (pH 7.5), 9% (v/v) glycerol, 90 mM NaCl, 90 μM EDTA, 90 μM AEBSF, and 450 nM leupeptin and pepstatin; final volume, 100 μl. At 50 s, 0.5 μl of 100 mM EDTA (additional 200 μM) was added; at 210 s, 0.5 μl of 200 mM $CaCl_2$ (additional 1 mM) was added. Data were not recorded during the addition of EDTA and excess $CaCl_2$. Time courses were extrapolated during these periods, and are shown as broken lines. The insert shows a time course reconstructed by excluding data points taken under conditions that do not allow CaM binding. The solid line in the insert is a first order line of best fit, with a first order rate constant of 0.78 min^{-1}.

The signal observed in Fig. 2, spectrum A, was discharged upon addition of Ca^{2+}/CaM (Fig. 2, spectrum C). We considered the possibility that the loss of signal in Fig. 2C might be caused by Ca^{2+}/CaM resulting in broadening of the signal by spin-spin interaction due to a closer proximity of the heme and the flavins. This possibility was excluded by determining the spectrum of enzyme that had been incubated for 5 minutes at 250C with Ca^{2+}/CaM and to which EDTA was subsequently added to block binding of CaM to NOS (Fig. 2, spectrum D). No signal was observed, confirming that FSQ oxidation had indeed occurred as a result of the conformational change induced by Ca^{2+}/CaM. To demonstrate that a signal can be observed in this mixture, we added an additional 40 μM NADPH to reaction mixture D (Fig. 2, spectrum E), which resulted in restoration of the FSQ signal. These combined EPR data confirm the optical determination of the oxidation of endogenous FSQ upon binding of CaM (Fig. 1), and further show that the oxidation is complete under these conditions.

[3] C.F.B. Witteveen, J. Giovanelli, and S. Kaufman, manuscript in preparation.

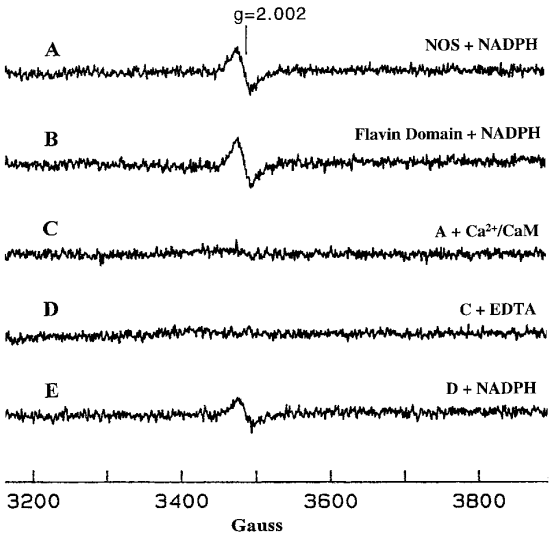

FIG. 2. EPR studies of discharge of FSQ upon addition of Ca^{2+}/CaM. A is a spectrum of a reaction mixture containing 50 μM NOS, 23 μM NADPH, 59 μM CaM, 100 μM L-arginine, 40 mM Tris.HCl (pH 7.5), 8% glycerol (v/v), 80 mM NaCl, 80 μM EDTA, 80 μM AEBSF, and 400 nM leupeptin and pepstatin. **B** is a spectrum of a reaction mixture containing 59 μM flavin domain, 43 μM NADPH, 30 mM MOPS (pH 7.5), 11 mM Tris. HCl (pH 7.5), 133 mM NaCl, 9.6% (v/v) glycerol, 22 μM EDTA, 22 μM AEBSF and 109 nM leupeptin and pepstatin. **C** is a spectrum of reaction mixture *A* to which $CaCl_2$ was added to a final concentration of 1 mM, followed by incubation for 5 min at 200C. **D** is a spectrum of reaction mixture **C** to which EDTA was added to a final concentration of 2.5 mM. **E** is a spectrum of reaction mixture **D** to which NADPH was added to a final concentration of 40 μM. FSQ exhibits a broad one-line signal at room temperature (16).

The effects of L-arginine, NHA, N^G-nitro-L-arginine and BH_4 on the rate of oxidation of FSQ are shown in Table 1. As can be seen, L-arginine and NHA each increase the rate constant for oxidation, whereas N^G-nitro-L-arginine is a potent inhibitor. Addition of BH_4 had no effect, but it should be noted that the enzyme as isolated contains approximately 0.5 mol bound BH_4 per mol NOS subunit. In all cases, oxidation of FSQ followed first order kinetics. The discrepancy between the rate constant in the presence of L-arginine and BH_4 calculated from Fig. 1 (0.78 min^{-1}) and the corresponding value in Table I (1.26 min^{-1}) results from the variation in rates of FSQ oxidation observed with different NOS preparations.

Single-turnover synthesis of NHA and oxidation of FSQ. In order to prevent misinterpretation (18), it should be emphasized at the outset that the reducing potential in these single-turnover studies is derived from an NOS-bound reductant, and that substrate amounts of NOS are therefore required for oxygenation (6). NOS-bound reductant is not adequate to support a catalytic function of NOS; this would require substrate amounts of the ultimate electron donor, NADPH. Fig. 3A shows the rates of oxidation of FSQ and synthesis of NHA and L-citrulline. In Fig. 3B these variables are converted to electron equivalents, as described in the legend. The time courses for FSQ oxidation and synthesis of NHA are each first order with similar rate constants of 0.74 min^{-1} and 0.82 min^{-1}, respectively; the relatively small amount of L-citrulline synthesized follows a linear time course. Fig. 3B shows that the utilization of electrons during the formation of the products of Reaction 1 accounts for approximately 70% of the loss of electrons from FSQ. When the relatively small utilization of electrons that occurs during the formation of the products of Reaction 2 is taken in account, this value increases to 80%. In two other experiments, electrons utilized to form the products of Reactions 1 and 2 accounted for 70 and 77% of those lost from FSQ during its oxidation. These results indicate that FSQ can provide all the electrons required for Reactions 1 and 2. Gachhui *et al.* (11) reported the presence of FSQ in only 50% of the isolated NOS flavin domains. A value of 0.6 (2.4/4) FSQ per NOS subunit was calculated from the data of Fig. 3, and other NOS preparations used in our work were found to contain between 0.4 to 0.6 FSQ per NOS subunit. As noted in the legend to Fig. 3, all the values listed above, except for the rate constants, are subject to a range of +/− 20% resulting from the range in the two extinction coefficients available for the FSQ of NOS (see Experimental Procedures).

Comparison of single turnover oxygenation of L-arginine to NHA and of NHA to L-citrulline. In a previous study (6), conversion of NHA to L-citrulline catalyzed by NOS was detected only in the presence of added NADPH. Since very low concentrations of NHA relative to those of L-arginine were employed in these earlier experiments, we decided to re-examine the relative amounts of oxygenation of each of these two substrates at the same concentration (50 μM) and under identical conditions of NOS concentration, etc. The limited sensitivity of the L-citrulline assay required that the amount of

TABLE 1

First Order Rate Constants of FSQ Oxidation

Additions	Rate constant for FSQ oxidation (min^{-1})	
	$-BH_4$	$+BH_4$
None	0.76 (0.72; 0.80)	0.67 (0.66; 0.67)
L-Arginine	1.28 (1.25; 1.30)	1.26 (1.22; 1.30)
NHA	1.40 (1.37; 1.43)	1.53 (1.48; 1.58)
N^G-nitro-L-arginine	<0.1	<0.1

Note. Each reaction mixture contained 6 μM NOS, 21 μM CaM, 547 μM EDTA, 48 mM Tris-HCl (pH 7.4), 4.5% (v/v) glycerol, 95 mM NaCl, 95 μM AEBSF, and 0.48 μM leupeptin and pepstatin. Reactions were started by adding $CaCl_2$ to a concentration of 1 mM. L-Arginine or NHA at 45 μM concentration, 100 μM N^G-nitro-L-arginine, or 23 μM BH_4 was added as indicated. Values are the means of duplicate determinations shown in parentheses. The same preparation of NOS was used for all determinations.

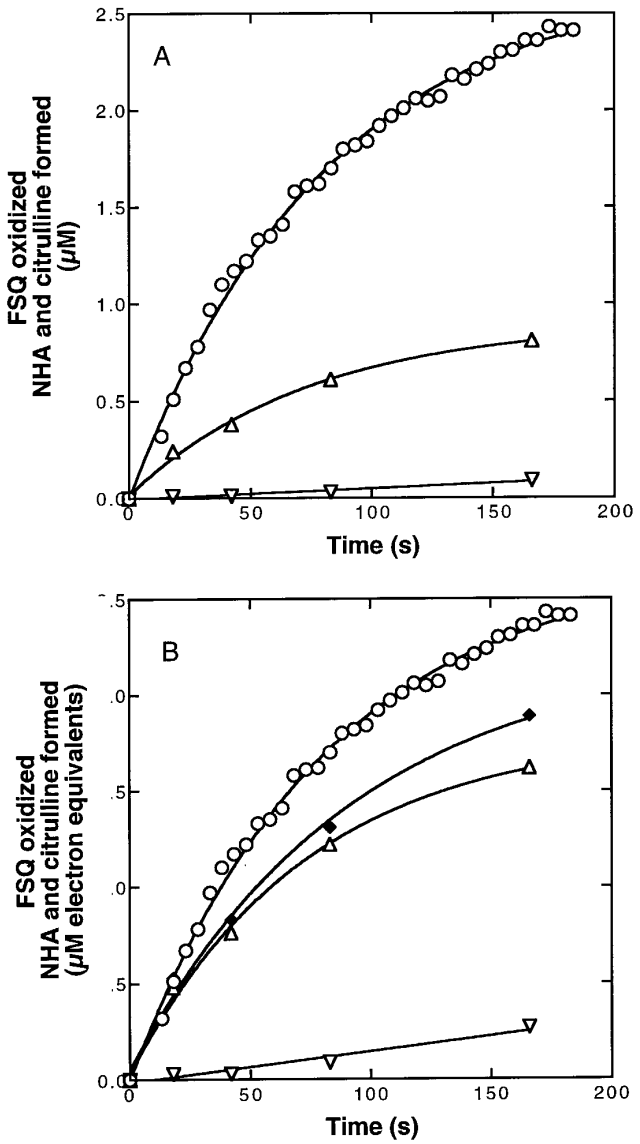

FIG. 3. NHA synthesis and oxidation of FSQ of NOS. The reaction mixture contained 4.0 μM NOS, 109 μM $CaCl_2$, 4.7 μM CaM, 50 μM ^{3}H-L-arginine, 35 μM BH_4, 9.6 mM Tris-HCl (pH 7.5), 1.9% (v/v) glycerol, 19 mM NaCl, 19 μM EDTA, 19 μM AEBSF, and 96 nM leupeptin and pepstatin. Except for L-citrulline, all curves are first order lines of best fit. A shows (in μM) FSQ oxidation (0), NHA synthesis (q), and L-citrulline synthesis (u). B compares the loss (in μM electron equivalents) of FSQ during its oxidation (0) with utilization (in μM electron equivalents) to form the products of Reaction 1 (q), Reaction 2 (u), and these products combined (u). Calculations are based on one electron equivalent per FSQ oxidized, and 2 and 3 electron equivalents per formation of NHA and L-citrulline, respectively. The values of FSQ oxidized are subject to a range of +/− 20% resulting from the variation in the two extinction coefficients available for the FSQ of NOS (see Experimental Procedures). Note that the calculation of the rate constant of FSQ oxidation is not subject to this error.

oxygenation of each substrate be determined after a 10 min incubation, which allowed the amount of products formed to reach their maximal values. Triplicate determinations of the values for the oxygenation of NHA to L-citrulline were 0.26, 0.31 and 0.32 (mean 0.30) mol L-citrulline per mol NOS subunit. The corresponding values for oxygenation of L-arginine were 0.15 and 0.15 (mean 0.15) mol NHA per mol NOS subunit. The significance of the ratio of 2 for the amount of product formed in these two partial reactions is discussed below.

DISCUSSION

The endogenous reductant that is capable of supporting the single-turnover oxygenation of L-arginine to NHA (6) has been characterized as FSQ by the following criteria:

(i) Spectrophotometric studies (Fig. 1) demonstrate that oxidation of FSQ requires CaM binding, as does the oxygenation of L-arginine to NHA (6). The EPR studies (Fig. 2) confirm that the spectrophotometric measurements reflect the presence of FSQ on the protein.

(ii) Both the oxidation of FSQ and the formation of NHA follow first order kinetics with similar rate constants (Fig. 3).

(iii) No NHA synthesis was observed with an NOS preparation that had been preincubated with Ca^{2+}/CaM (in the absence of L-arginine) in order to discharge the FSQ (data not shown). Normal NHA synthesis was observed in a parallel preincubation in which Ca^{2+}/CaM was omitted.

The observed inhibition of FSQ oxidation by N-nitro-arginine (Table I), which blocks electron transfer between reduced flavin and heme (19), indicates FSQ oxidation proceeds through heme. The stimulatory effect of L-arginine on FSQ oxidation is consistent with a similar effect observed on electron transfer from NADPH to heme (20). Whether or not NHA also acts like L-arginine in facilitating electron transfer into heme remains to be determined. A proposed scheme for the single-turnover oxidation of FSQ and its associated oxygenation reactions is given in Fig. 4.

A number of important conclusions may be drawn from this work. A minimum of two FSQs oxidized for each L-arginine oxygenated shows that all the electrons required in the first half reaction can be supplied by FSQ, and that no other source of electrons is required. Subject to the uncertainty of the extinction coefficient of FSQ (see Experimental Procedures), it was estimated that utilization of electrons during the formation of NHA accounts for 57 to 83% of the loss of electrons from FSQ. Electrons not utilized for NHA formation are most likely used for superoxide formation (21). Although the close similarity in rate constants for FSQ oxidation and NHA synthesis may be coincidental, it indicates that the quantitative electron relationship between these two reactions remains constant with time, consistent with the two reactions being coupled. Our finding of a ratio of 2 for product formation in the second half reaction to product formation in the first half reaction, a value equal to that expected

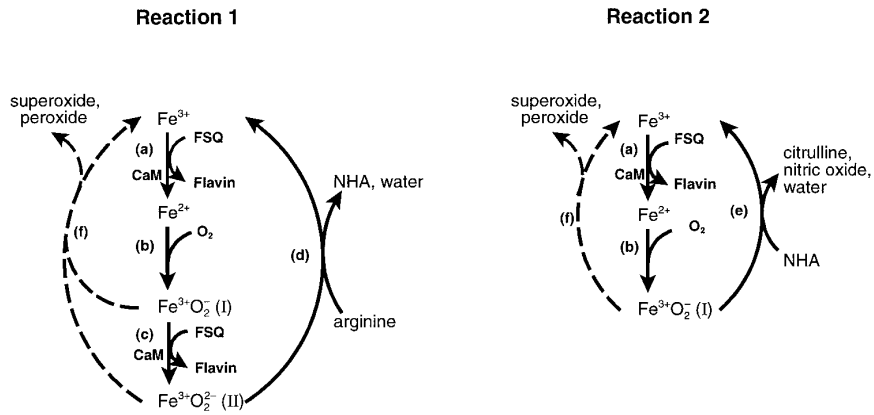

FIG. 4. Proposed sequence of reactions catalyzed by NOS in the single-turnover oxygenation of NHA to L-citrulline and of L-arginine to NHA. Reaction 1 illustrates a proposed reaction sequence for the single-turnover oxygenation of L-arginine to NHA, requiring two electron equivalents. Reaction 2 illustrates a proposed reaction sequence for the single-turnover oxygenation of NHA to L-citrulline, requiring one electron equivalent. Reactions a and c show FSQ donating an electron, accompanied by its conversion to flavin. The steps involved in the activation of oxygen to form the ferric superoxide species (**I**) and ferric peroxo species (**II**) are based largely on studies of cytochrome P-450 (30). These forms can also exist in their respective canonical forms, $Fe^{2+}O_2$ and $Fe^{2+}O_2^-$ (30). In Reaction 2, species **II** that is believed to be formed from the one-electron reduction of species **I** by NHA (1,5,31-33) has been omitted for the sake of clarity. In Reaction 1, species **II** may be protonated and lose water to form a high valence iron-oxo complex that is the actual oxygenating species (1,30). It should also be noted that L-arginine and NHA, which are shown reacting in steps d and e, respectively, are initially bound to the ferric form of NOS (20,34,35). Coupled reactions resulting in the oxygenation of L-arginine and NHA are shown by solid lines. Uncoupled reactions resulting in the formation of superoxide and peroxide are shown by broken lines. As discussed in the text, FSQ is unlikely to be the source of electrons during normal catalytic turnover of NOS.

(4,5), is a strong indication that electrons are supplied by a single source, i.e. all the electrons required for both reactions (1) and (2) can be provided by FSQ. Our studies, however, do not exclude the possibility of other electron donors such as BH_4 acting as an electron source.

Two sequential reductions take place in the first half reaction . As illustrated in Fig. 4, during the first reduction, Fe^{3+} is converted to Fe^{2+} which then reacts with oxygen to form the unstable ferric superoxide species (**I**) (21). **I** can either be reduced during the second reduction to the oxygenating species **II,** or be degraded to superoxide and peroxide. A coupling of 60-90% between FSQ oxidation and formation of L-arginine oxygenation products suggests that **I** is rapidly reduced to **II** rather than being degraded to superoxide. The mechanism of this second reduction is not entirely clear. Since our preparations of NOS contain approximately 1 FSQ per NOS dimer, electrons must be transferred between dimers. Such a transfer could be subject to a number of constraints. For example, it appears that each of the two hemes of a dimer can accept electrons from only one of the flavin domains, and that these hemes do not readily exchange electrons (22). Furthermore, transfer of electrons between flavin species can be slow in the absence of the mediator, methyl viologen (11,23).

Indirect evidence strongly implicates flavins in the electron transfer sequence of NOS (3,24). Here we provide evidence for the coupling of flavin oxidation with product formation catalyzed by this enzyme. In addition, these studies provide insights into the possible flavin species involved in NOS turnover. To date, the mechanism of electron flow in the flavins of NOS has been based mainly on studies with the microsomal cytochrome P-450 system (3,24). In this system, it has been clearly demonstrated that the proximal reductant for heme is $FMNH_2$, and not FSQ (25,26). Recent studies (27,28) of the fatty acid hydroxylase P-450 system from *Bacillus megaterium* have shown that FSQ is the reductant of the heme iron in this system, and $FMNH_2$ is incapable of acting as a reductant. This fatty acid hydroxylase P-450 system resembles NOS in that it contains both its reductase and oxygenase domains on a single polypeptide chain (29). Whereas the work described here indicates that FSQ can indeed provide electrons for the oxygenation reactions of NOS under certain experimental conditions, the rate of FSQ oxidation is two orders of magnitude slower than the flux of electrons from NADPH through NOS during normal turnover of the enzyme.[4] These preliminary observations therefore indicate that NOS resembles the microsomal cytochrome P-450 system in using $FMNH_2$ as the proximal electron donor for heme, rather than FSQ used in the *B. megatherium* system.

[4] An oxidation rate of 1.26 μM electron equivalents min^{-1} (μM NOS subunit)$^{-1}$ was calculated from a rate constant for FSQ oxidation of 1.26 min^{-1} (at 200C) (Table I) and one FSQ per NOS subunit. This rate was compared to a catalytic turnover of NOS in the presence of excess NADPH of 250 nmol L-citrulline formed min^{-1} (mg protein)$^{-1}$, or 37 μM L-citrulline min^{-1} (μM NOS subunit)$^{-1}$. Based on the requirement of 3 electrons from NADPH for each L-citrulline formed, the latter value is equivalent to 112 μM electron equivalents min^{-1} (μM NOS subunit)$^{-1}$, or 89-fold the corresponding rate of FSQ oxidation.

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