Involvement of the Reductase Domain of Neuronal Nitric Oxide Synthase in Superoxide Anion Production†

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ABSTRACT: Neuronal nitric oxide synthase (nNOS) is a modular enzyme which consists of a flavin-containing reductase domain and a heme-containing oxygenase domain, linked by a stretch of amino acids which contains a calmodulin (CaM) binding site. CaM binding to nNOS facilitates the transfer of NADPH-derived electrons from the reductase domain to the oxygenase domain, resulting in the conversion of L-arginine to L-citrulline with the concomitant formation of a guanylate cyclase activating factor, putatively nitric oxide. Numerous studies have established that peroxynitrite-derived nitrogen oxides are present following nNOS turnover. Since peroxynitrite is formed by the diffusion-limited reaction between the two radical species, nitric oxide and $O_2^{\bullet-}$, we employed the adrenochrome assay to examine whether nNOS was capable of producing $O_2^{\bullet-}$ during catalytic turnover in the presence of L-arginine. To differentiate between the role played by the reductase domain and that of the oxygenase domain in $O_2^{\bullet-}$ production, we compared its production by nNOS against that of a nNOS mutant (CYS-331), which was unable to transfer NADPH-derived electrons efficiently to the heme iron under special conditions, and against that of a flavoprotein module construct of nNOS. We report that $O_2^{\bullet-}$ production by nNOS and the CYS-331 mutant is CaM-dependent and that $O_2^{\bullet-}$ production can be modulated by substrates and inhibitors of nNOS. $O_2^{\bullet-}$ was also produced by the reductase domain of nNOS; however, it did not display the same CaM dependency. We conclude that both the reductase and oxygenase domains of nNOS produce $O_2^{\bullet-}$, but that the reductase domain is both necessary and sufficient for $O_2^{\bullet-}$ production.

Neuronal nitric oxide synthase (nNOS)[1] catalyzes the NADPH-dependent conversion of L-arginine to L-citrulline with the concomitant formation of a guanylate cyclase activating factor, believed to be nitric oxide ($NO^{\bullet}$) (*1*−*4*). nNOS possesses a bidomain structure in which a flavin-containing reductase domain is fused to a heme-containing oxygenase domain (*5*). The flavin-containing reductase domain contains FAD and FMN in equimolar amounts (*6*) and accepts electrons from NADPH, structurally and functionally resembling cytochrome P-450 reductase (CPR) (*7*). The N-terminal oxygenase domain is a cytochrome P-450-type hemoprotein containing iron protoporphyrin IX (*8*−*11*) and binding sites for the substrate L-arginine and the cofactor tetrahydrobiopterin (BH_4) (*12*). The role of BH_4 in the reaction mechanism of NOS is unclear. Contradictory studies have appeared which have failed to resolve the role of BH_4 as either a stoichiometric reactant or a cofactor which protects loss of enzyme activity by allosteric and/or redox mechanisms (*13*−*15*). Formation of L-citrulline from L-arginine with the concomitant formation of $NO^{\bullet}$ is a calcium/calmodulin (Ca/CaM)-dependent process (*16*), and CaM binding has been postulated and later proved to occur in the region between the oxygenase domain and the reductase domain of nNOS (*5*, *7*, *17*). As a result of CaM binding, electron transfer between the flavins as well as flavin to heme electron transfer is facilitated (*18*).

In addition to the Ca/CaM-dependent formation of $NO^{\bullet}$, nNOS catalyzes several other reactions, such as the reduction of cytochrome *c*, 2,6-dichlorophenolindophenol (DCIP), and ferricyanide and the oxidation of NADPH by reaction mechanisms which are stimulated by Ca/CaM (*5*, *11*, *18*). The reduction of these artificial electron acceptors is mediated by the C-terminal reductase domain of nNOS (*5*, *19*, *20*). Furthermore, nNOS produces H_2O_2 in a Ca/CaM-dependent manner by a mechanism which has been proposed to be mediated by the heme reaction center (*18*, *21*, *22*). Support for heme-mediated activation of molecular oxygen has come from studies utilizing inhibitors of nNOS function (*21*) and from studies with L-arginine-depleted cells (*23*). Pou et al. (*24*) were able to detect $O_2^{\bullet-}$ production by nNOS using spin trapping with DMPO, but no effort was made to quantitate the amounts formed. Additionally, Pou et al. (*24*) suggested that the failure to detect a superoxide−DMPO adduct upon addition of L-arginine might be due to formation of peroxynitrite. Alternatively, support for the involvement of the flavin domain in the activation of molecular oxygen has come from many laboratories. By lowering the oxygen tension for the cytochrome *c* assay, Richards et al. (*22*) were able to show a decrease in the rate of cytochrome *c* reduction

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[1] Abbreviations: nNOS, rat neuronal nitric oxide synthase; $NO^{\bullet}$, nitric oxide; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; BH_4, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; Ca/CaM, calcium/calmodulin; CPR, NADPH−cytochrome P-450 reductase; C1′, flavoprotein module of rat neuronal nitric oxide synthase consisting of amino acid residues 722−1429; CYS-331, rat neuronal nitric oxide synthase in which cysteine-331 has been mutated to an alanine; DCIP, dichlorophenolindophenol; DTT, dithiothreitol; BME, 2-mercaptoethanol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; SOD, superoxide dismutase; CAT, catalase.

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by CYS-415 mutants of nNOS which did not contain heme or BH_4. Additionally, Sheta et al. (*5*) were able to show a superoxide dismutase (SOD)-inhibitable component of the reduction of cytochrome *c* by nNOS. Furthermore, Gachhui et al. (*20*) demonstrated NADPH oxidation catalyzed by a reductase construct of nNOS. However, all of these studies have failed to reconcile whether more global structural changes were occurring within the nNOS protein upon binding of substrate, inhibitor, or CaM, thereby altering the redox chemistry of the protein.

Therefore, considering (i) the homology between the nNOS reductase domain and CPR (*7*), (ii) the ability of flavoproteins to activate molecular oxygen (reviewed in ref 25), (iii) the ability of CaM to induce conformational changes in nNOS and nNOS reductase structures (*26*), and (iv) the possibility that substrate analogs induce further conformational changes in nNOS (and/or the flavoprotein module) which affect transfer of electrons to molecular oxygen, we sought to investigate whether formation of reactive oxygen species could also be mediated at the flavin centers during nNOS turnover. To differentiate between the contributions of the heme and flavin prosthetic groups in activating molecular oxygen, nNOS was compared with (i) a nNOS mutant (CYS-331) which had a limited ability to reduce heme iron in the absence of added cofactors and (ii) the flavoprotein domain of nNOS.

EXPERIMENTAL PROCEDURES

Chemicals and Enzymes. Epinephrine bitartrate, NADPH, HEPES, dithiothreitol, 2-mercaptoethanol, bovine brain calmodulin, imidazole, catalase (25 000 units/mg), and $CaCl_2 \cdot 2H_2O$ were obtained from Sigma Chemical Co. (St. Louis, MO). Potassium periodate was obtained from Aldrich Chemical Co. (Milwaukee, WI). (6*R*)-5,6,7,8-Tetrahydro-L-biopterin was from Research Biochemicals International (Natick, MA). Highly purified Cu/Zn superoxide dismutase (3400 units/mg) was a generous gift from J. Crow (University of Alabama at Birmingham). The *o*-quinone of epinephrine was prepared by adding a few grains of potassium periodate to a 10 mM solution of epinephrine in 1 mM HCl (10 mL; 4° C) with constant stirring. Following formation of the bright yellow *o*-quinone, the reaction was quenched with 1 mL of MeOH.

nNOS Constructs. Rat brain nNOS was prepared as described by Roman et al. (*27*), and the rat brain nNOS Cys331Ala mutant (CYS-331) was prepared using oligonucleotide-specific mutagenesis and confirmed by sequencing.[2] CPR was prepared as described by Narayanasami et al. (*28*), and the flavoprotein module of rat brain nNOS (C1′), including amino acid residues 722−1429, was prepared as described by Narayanasami et al. (*26*).

Adrenochrome Assay. The formation of adrenochrome from epinephrine was followed at 480 nM using an extinction coefficient of 4.02 mM^{-1} cm^{-1}, essentially as described by Prough and Masters (*29*). A typical assay consisted of 0.0625−0.125 μM enzyme, 0.125 μM calmodulin, 400 μM $CaCl_2$, 200 μM epinephrine, and 100 μM NADPH in 50 mM HEPES/KOH at pH 7.6. In studies comparing the effects of L-arginine and imidazole, final concentrations of 100 μM and 10 mM were used, respectively. To compare wild-type nNOS with CYS-331 during the same assay period, 0.0625 μM nNOS was used as compared to 0.125 μM CYS-331. All data were recorded on a Shimadzu UV 2101PC spectrophotometer.

NADPH Oxidation. For determination of NADPH consumption, cuvettes contained 0.0625 μM nNOS, 0.125 μM calmodulin, 400 μM $CaCl_2$, 200 μM epinephrine, 100 μM N^G-nitro-L-arginine, and 100 μM NADPH in 50 mM HEPES/KOH at pH 7.6. The rate of NADPH oxidation during the initial linear phase of the reaction was monitored at 340 nM using an extinction coefficient of 6.22 mM^{-1} cm^{-1}.

CO Difference Spectroscopy following Reduction with NADPH. For determination of reduced, CO difference spectra, sealed cuvettes contained 0.33 μM enzyme, 0.5 μM calmodulin, 400 μM $CaCl_2$, 200 μM NADPH, and 5 μM BH_4 (when used) in CO-saturated 50 mM HEPES/KOH at pH 7.6. Difference spectra were recorded 5 min after CaM addition, measuring the CO-reduced vs CO samples between 400 and 500 nm.

Hemoglobin Capture Assay. The rate of methemoglobin formation from oxyhemoglobin was measured as the rate of absorbance change between 401 and 411 nm per minute using an extinction coefficient of 0.038 μM^{-1} as described by Sheta et al. (*5*) with the exception that the DTT concentration was 5 μM instead of 100 μM.

Protein Determination. The protein concentration of nNOS and CYS-331 was determined on the basis of the heme content by reduced CO difference spectra using an extinction coefficient of 100 mM^{-1} cm^{-1} for a $\Delta\epsilon$ of 444−475 (*30*). CO difference spectra were obtained after reducing the enzyme with a few grains of dithionite and then bubbling with CO. Concentrations of CPR and C1′ were determined by total flavin absorbance at 455 nM using an extinction coefficient of 21.6 mM^{-1} cm^{-1}, based on the presence of two flavins per mole. These determinations are probably underestimations of protein concentration since apoprotein (minus heme or flavin) exists in all preparations to some degree.

RESULTS AND DISCUSSION

Characteristics of CYS-331. The nNOS CYS-331 mutant binds BH_4 only weakly[3] and, therefore, cannot efficiently have its heme reduced by NADPH-derived electrons under the conditions of these assays. Reductase activities, however, of CYS-331 are identical with those of wild-type nNOS.[3] Therefore, CYS-331 was used in these experiments as a construct which retained the characteristics of wild-type nNOS except for the ability to efficiently transfer electrons to the heme, further enabling us to test the hypothesis that $O_2^{\bullet -}$ can be produced by the reductase domain of nNOS.

Kinetics of the Adrenochrome Assay. Two distinct phases are evident in the oxidation of epinephrine to adrenochrome by nNOS, CYS-331, C1′, and CPR (Figure 1). Phase I is the initial lag period which represents transfer of electrons from NADPH to molecular oxygen, forming $O_2^{\bullet -}$. $O_2^{\bullet -}$ then oxidizes epinephrine to epinephrine semiquinone and epinephrine *o*-quinone. Phase II is characterized by the coupling of the *o*-quinone or adrenochrome to the enzyme, resulting in a stimulated transfer of electrons to molecular oxygen,

[2] P. Martásek, personal communication.

[3] P. Martásek and B. S. S. Masters, unpublished observations.

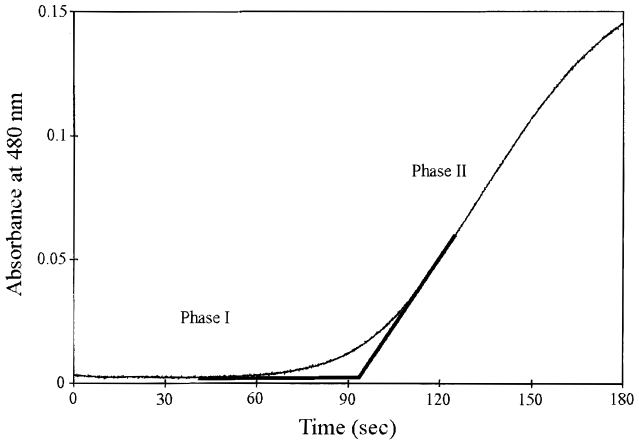


FIGURE 1: Kinetics of the adrenochrome assay. Phase I represents enzyme-mediated superoxide production leading to quinone formation. Phase II represents quinone-stimulated redox cycling and adrenochrome formation. nNOS (0.0625 μM) was assayed in the presence of 0.125 μM CaM, 400 μM $CaCl_2$, 200 μM epinephrine, 100 μM L-arginine, and 100 μM NADPH at 25 °C.

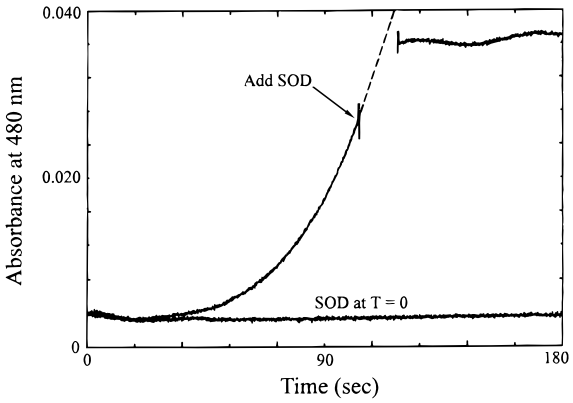
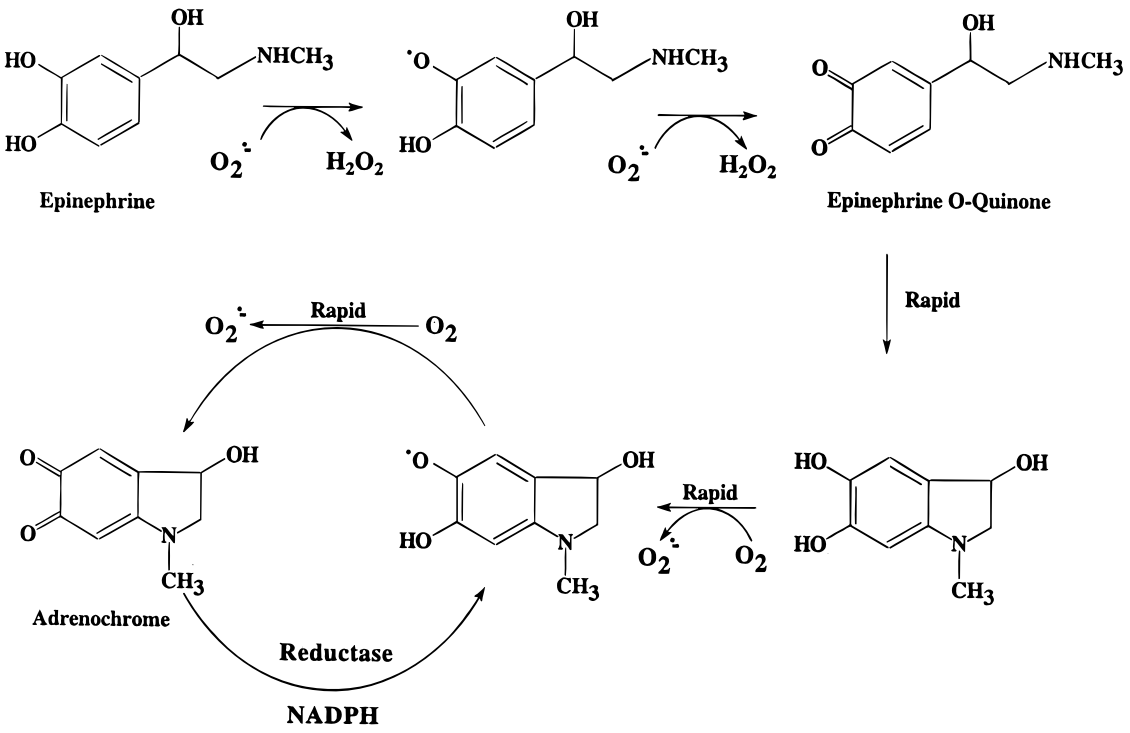


FIGURE 2: Effect of SOD on adrenochrome formation. SOD (87 units) was added either at the beginning of the assay or during phase II of adrenochrome formation. nNOS (0.0625 μM) was assayed at 25 °C in the presence of 0.125 μM CaM, 400 μM $CaCl_2$, 200 μM epinephrine, and 100 μM NADPH in a total volume of 1 mL. Identical results were obtained with CPR and C1′.

so-called "redox cycling", and ultimately leading to a stimulated rate of adrenochrome formation (Scheme 1). Both phases are completely dependent upon $O_2^{\bullet-}$ since addition of superoxide dismutase (87 units/mL) at the beginning of the assay completely prevented adrenochrome formation and addition of SOD during phase II prevented further adrenochrome formation (Figure 2). Addition of catalase (105 units/mL) at the beginning of the assay had no effect on either phase of the reaction (not shown). The duration of the initial lag period (phase I) was inversely proportional to the concentration of enzyme present in the incubation mixture and linear over the range of 0.0625 to 1 μM enzyme (data not shown). On the basis of the NADPH consumption measured over the length of the lag period, 7.3 ± 0.2 nmol (mean ± standard error; $n = 3$) of NADPH was required to achieve the threshold level of *o*-quinone required to initiate phase II. Considering that the formation of the *o*-quinone of epinephrine is a two-electron process (Scheme 1), the threshold concentration of *o*-quinone is also 7.3 μM. This corresponds to 14.6 nmol of $O_2^{\bullet-}$. Since not all of the $O_2^{\bullet-}$ formed may be able to interact with epinephrine because of spontaneous dismutation to H_2O_2, our calculated value for the threshold concentration of *o*-quinone is likely to be an overestimate. Furthermore, these observations are supported by the ability of chemically synthesized epinephrine *o*-quinone (approximately 25 nmol) to completely abolish the initial lag period when added at the beginning of the assay (Figure 3A−C) and are consistent with the interaction between CPR and menadione (*31*, *32*) and CPR with epinephrine (*29*). To exclude the possibility that trace amounts of copper were acting catalytically and affecting the amount of adrenochrome formed, we performed control experiments with C1′ in the presence of 50 μM EDTA and observed no effect on the amount or rate of adrenochrome formation.

Scheme 1



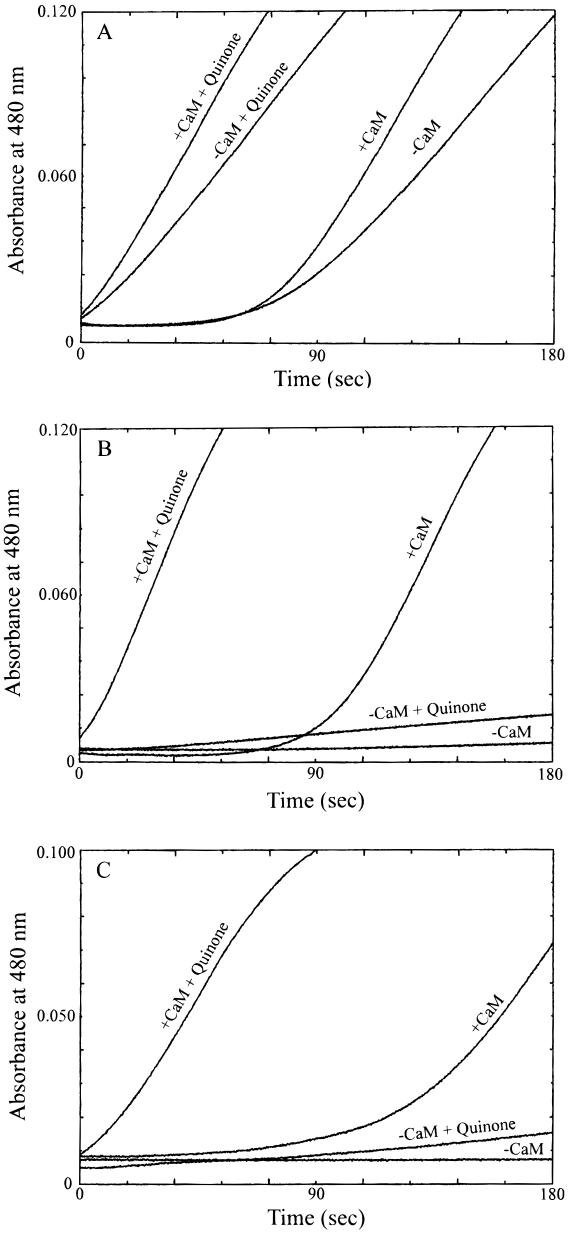


FIGURE 3: Effect of CaM on adrenochrome formation by nNOS, CYS-331, and C1′ in the presence and absence of added epinephrine *o*-quinone: (A) C1′ (0.125 μM flavin), (B) nNOS (0.0625 μM heme), and (C) CYS-331 (0.125 μM heme) as isolated. Assays were run at 25 °C at the indicated enzyme concentrations in the presence or absence of excess CaM and in the presence of 400 μM $CaCl_2$, 200 μM epinephrine, 100 μM L-arginine, and 100 μM NADPH.

The formation of adrenochrome from epinephrine has been recognized as a useful assay for detection of $O_2^{\bullet-}$ under certain experimental conditions (*33*, *34*). Although the adrenochrome assay has been utilized to measure $O_2^{\bullet-}$ generation by enzyme systems such as CPR (*29*), care must be taken in interpreting the results obtained when using these systems since cycling of quinone intermediates can occur, resulting in a false rate of NADPH oxidation due to $O_2^{\bullet-}$ production. This was first observed in studies with various electron acceptors for CPR. For example, it was noticed that the turnover number with menadione by CPR was greater than that with other electron acceptors such as DCIP or cytochrome *c* (*31*). Additionally, menadione was able to oxidize the semiquinone flavin of CPR to the fully oxidized form (*31*), whereas the semiquinone of CPR was able to reduce cytochrome *c* only extremely slowly and incompletely (*31*), suggesting differences in the mechanisms and/or redox potentials involved in the reduction of the different electron acceptors. Furthermore, menadione was found to cause a stimulation of NADPH oxidation, whereas DCIP and cytochrome *c* did not (*31*), further suggesting differences among these electron acceptors. Under anaerobic conditions, CPR catalyzes the NADPH-linked reduction of menadione to the quinol, but under these conditions, it cannot oxidize the quinol to the quinone (*35*). Aerobic studies suggest that the stimulation of NADPH oxidation by quinones is mediated by enzymatic reduction of the quinone to the semiquinone which acts as a one-electron carrier to oxygen (*32*, *35*).

Two semiquinone intermediates are formed during the oxidation of epinephrine to adrenochrome (epinephrine semiquinone and adrenochrome semiquinone); therefore, extensive redox cycling involving epinephrine quinone/epinephrine semiquinone and adrenochrome/adrenochrome semiquinone could be envisioned. However, this is probably not the case since epinephrine does not autoxidize at a considerable rate at physiological pH (*34*). The rate constants for autoxidation of epinephrine semiquinone to epinephrine *o*-quinone ($\leq 1 \times 10^5$ M^{-1} s^{-1}) (*36*), intramolecular cyclization of epinephrine *o*-quinone (1×10^{10} M^{-1} s^{-1}) (*37*) to leucoadrenochrome, leucoadrenochrome oxidation to adrenochrome semiquinone (0.14×10^8 M^{-1} s^{-1}), and adrenochrome semiquinone oxidation to adrenochrome (9×10^8 M^{-1} s^{-1}) (*38*) characterize the step(s) that require an oxidant to drive the reaction. All of the aforementioned rate constants refer to reactions with oxygen except for the intramolecular cyclization rate constant which is derived from a combination of three processes corresponding to a reversible rate-limiting deprotonation reaction and a pH-dependent rate constant (*37*). Additionally, the semiquinone radicals in this pathway can dimerize or dismutate, as well as react with oxygen at near identical rates. The rate constant for the dismutation reaction is 5×10^8 M^{-1} s^{-1}, while that for the reaction with oxygen is 9×10^8 M^{-1} s^{-1} (*38*); the initial formation of epinephrine *o*-quinone is the slowest step, while all the other steps in adrenochrome formation proceed at exceedingly fast rates. Therefore, it appears that in our experiments, formation of epinephrine *o*-quinone is the rate-limiting step since addition of epinephrine *o*-quinone at the beginning of the assay completely abolished the initial lag period. Furthermore, addition of epinephrine *o*-quinone to the assay most likely resulted in either the spontaneous coupling of epinephrine *o*-quinone to the enzyme or formation of a small amount of adrenochrome which then couples with the enzyme preparation to initiate the redox cycling mechanism (*39*). Either process is feasible since both compounds are quinones.

Iyanagi et al. (*40*) assigned values to the single-electron redox couples of CPR at −110, −270, −290, and −365 mV which, according to the work of Vermilion and Coon (*41*), represent the couples FMN/$FMNH^{\bullet}$, $FMNH^{\bullet}$/$FMNH_2$, FAD/$FADH^{\bullet}$, and $FADH^{\bullet}$/$FADH_2$, respectively. Since the lower, limiting single-electron reduction potential of quinones undergoing reduction by CPR is approximately −240 mV, $FMNH_2$ and $FADH^{\bullet}$ are most likely to mediate the reduction. However, $FADH^{\bullet}$ is an unlikely species since FMN-depleted CPR is unable to catalyze quinone reduction (*41*). Therefore, electron transfer to the quinone acceptor is most likely mediated by $FMNH_2$.

Similar redox active compounds have been utilized in studies investigating different aspects of $O_2^{\bullet-}$ radicals. For example, the CPR−paraquat system has been used as a standard system for the production of $O_2^{\bullet-}$ radicals to clarify the relationships between enzymatic reactions and spin trapping data since the only product produced from this reaction is thought to be $O_2^{\bullet-}$ (*42*). Also, in a recent report, the frequently used $O_2^{\bullet-}$ probe, lucigenin, was shown to stimulate NADPH-dependent $O_2^{\bullet-}$ production by endothelial nitric oxide synthase (*43*) and the semiquinone intermediate (lucigenin$^{\bullet+}$) was implicated in mediating the one-electron transfer to oxygen.

Effect of Ca/CaM on Adrenochrome Formation by Different nNOS Constructs. The effect of Ca/CaM on phase I and II was different among C1′, nNOS, and CYS-331. The addition of Ca/CaM had little or no effect on phase I with C1′; however, the rate of phase II was moderately stimulated (Figure 3A). Addition of Ca/CaM to C1′ also moderately stimulated the rate of phase II when the reaction was run in the presence of epinephrine *o*-quinone. In sharp contrast, the nNOS-catalyzed formation of adrenochrome from epinephrine was completely dependent on the presence of Ca/CaM. Additionally, formation of adrenochrome from epinephrine in the presence of exogenously added *o*-quinone was also Ca/CaM-dependent (Figure 3B). Similarly, assays performed with CYS-331 in the presence or absence of exogenously added *o*-quinone showed adrenochrome formation was Ca/CaM-dependent (Figure 3C), although not to the same extent as native nNOS. Although adrenochrome formation by both nNOS and CYS-331 is Ca/CaM-dependent, the rate of formation of adrenochrome is lower with CYS-331. This experiment demonstrated that $O_2^{\bullet-}$ formation as well as quinone coupling is Ca/CaM-dependent with nNOS and CYS-331 and that the reductase domain is both necessary and sufficient for adrenochrome formation.

The differences in rates of adrenochrome formation observed among the different enzyme constructs upon Ca/CaM addition might be explained by examining differences between the structures of the constructs used. The reductase construct C1′ consists of amino acid residues 722−1429, which include the nNOS reductase domain and the CaM-binding domain, whereas nNOS and CYS-331 contain both the reductase domain and oxygenase domains linked by the CaM binding domain. The lack of an effect on phase I ($O_2^{\bullet-}$ production) and a slight effect on phase II (adrenochrome formation) due to CaM binding with C1′ are in sharp contrast to absolute dependency on CaM binding of both phases I and II with nNOS and CYS-331. At first glance, it would appear that CaM binding to nNOS or CYS-331 triggers transfer of electrons to the heme where molecular oxygen can be activated, and this has been shown to be the case with wild-type nNOS (*18*, *21*); however, CYS-331, without preincubation with L-arginine, is only able to transfer NADPH-derived electrons to the heme iron very slowly. Therefore, efficient activation of oxygen by the heme is greatly diminished, yet formation of adrenochrome is still CaM-dependent. It is suggested that CaM is profoundly affecting the flavoprotein domain in the intact enzyme and enhancing the production of $O_2^{\bullet-}$. The qualitative behavior of this construct is virtually indistinguishable from that of native nNOS. Furthermore, Richards et al. (*22*), using nNOS and C415 mutants of nNOS, provided evidence that an oxygen-derived product, produced by the reductase domain

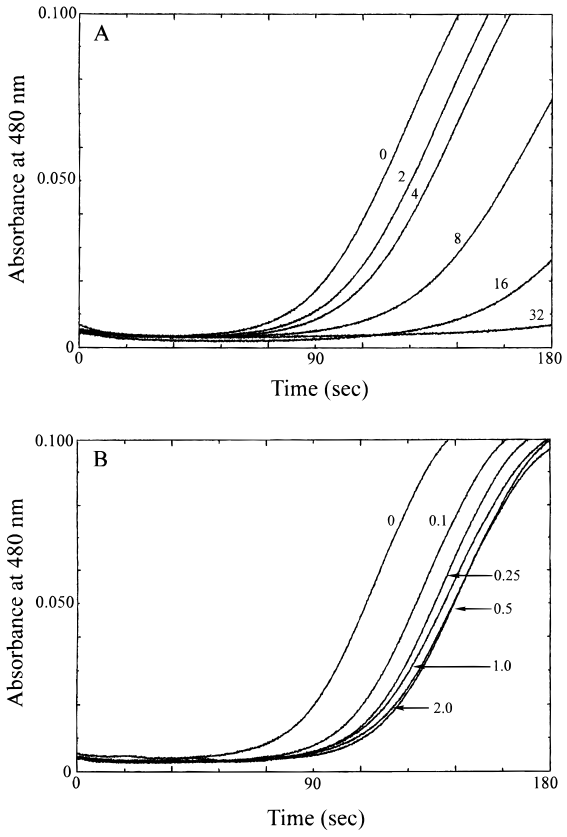


FIGURE 4: Effect of reducing agents on the initial lag period of the adrenochrome assay. nNOS (0.0625 μM) was assayed in the presence of various concentrations of DTT (A) or in the presence of various concentrations of DTT-free BH_4 (B). The numbers accompanying each trace represent the micromolar concentration of the indicated reducing agent. Assays were conducted at 25 °C in the presence of excess CaM, 400 μM $CaCl_2$, 200 μM epinephrine, 100 μM L-arginine, and 100 μM NADPH.

of nNOS, is involved in the reduction of cytochrome *c*. These authors demonstrated that the rate of cytochrome *c* reduction by nNOS was decreased when the assays were made slightly anaerobic and that upon CaM addition, no further increase in the rate of cytochrome *c* reduction was observed. Collectively, these observations suggest that some portion of the nNOS protein is interacting with the reductase domain in such a way that electron transfer to molecular oxygen is prevented in the absence of Ca/CaM binding.

Effect of Reducing Agents on Adrenochrome Formation. To investigate the effect of common reducing agents on the adrenochrome assay, we included 2-mercaptoethanol (BME) and dithiothreitol (DTT) in the assay. Addition of either BME or DTT at low micromolar concentrations to the reaction mixtures resulted in concentration-dependent increases in the length of phase I (Figure 4A). In contrast, addition of sulfhydryl reagent-free BH_4 had only a minor effect on the length of phase I, which was not concentration-dependent (Figure 4B). The lack of an effect of exogenously added BH_4 is most likely a measure of the pterin repleteness of this enzyme preparation. These results suggest that DTT and BME, but not BH_4, act to reduce the concentration of semiquinone and *o*-quinone either by scavenging $O_2^{\bullet-}$ or by directly reducing the semiquinones or *o*-quinones which are formed. Although $O_2^{\bullet-}$ does not usually oxidize thiols, this process is thermodynamically possible. However, the more likely explanation is that BME and DTT chemically reduce adrenochrome and/or its intermediate reaction products, and

preliminary experiments from this laboratory have shown that this process is possible (not shown). Therefore, when the adrenochrome assay is used, great caution must be taken when adding sulfhydryl reagents to these enzyme preparations. As shown in Figure 4B, addition of exogenous sulfhydryl reagent-free BH_4 to nNOS (BH_4 replete) had only a slight effect of increasing the duration of phase I, suggesting that BH_4 acts in some manner to couple NADPH-derived electrons more tightly to citrulline formation. However, in contrast to the findings of Heinzel et al. (*21*), $O_2^{\bullet-}$ is still produced in the presence of saturating concentrations of BH_4 and L-arginine, and the $O_2^{\bullet-}$ produced is not due to autoxidation of BH_4. If this were the case, phase I would have become shorter upon titration of BH_4 into the assay, not longer.

Enzymatic Activity of nNOS and CYS-331. To investigate the ability of nNOS and CYS-331 to transfer electrons to the heme, and therefore probe the involvement of reduced heme in the production of $O_2^{\bullet-}$, two approaches were used. First, $NO^{\bullet}$ production was measured by the hemoglobin capture assay, and second, the ability of nNOS and CYS-331 to form a reduced, CO difference spectrum following reduction with NADPH was determined. Native nNOS was able to form $NO^{\bullet}$ (not shown) and produce a reduced CO difference spectrum upon reduction with NADPH (Figure 5A), indicating that NADPH-derived electrons were being transferred to the heme iron. Results with CYS-331 differed. CYS-331, as isolated, was neither able to produce $NO^{\bullet}$ (not shown) nor able to form a reduced CO difference spectrum upon reduction with NADPH (Figure 5B), indicating that NADPH-derived electrons could not be efficiently transferred to the heme center. However, the possibility that the Cys331Ala mutation of nNOS has altered the kinetics for CO binding cannot be excluded at this time.[3] In fact, recently, Migita et al. (*44*) have elucidated interactions between bound $NO^{\bullet}$ and substrate or analogs which affect the Fe−NO geometry in nNOS, and Scheele et al. (*45*) have shown that the kinetics of CO binding are affected by substrate or analogs.

Stimulation of NADPH Oxidase Activity. Previous investigations into the NADPH oxidase activity of a similar reductase construct (*20*), an apo-NOS construct (*18*), and CYS-415 mutants of nNOS (*22*) reported no stimulation of NADPH oxidation upon CaM binding. Gachhui et al. (*20*) discounted the possibility that flavin-mediated oxygen reduction contributed to the cytochrome *c* reductase activity of the flavoprotein module of nNOS; however, in this report, we clearly show that $O_2^{\bullet-}$ is produced during catalytic turnover of nNOS and can be produced by the flavoprotein module. Additionally, we provide evidence that the activation of oxygen does not occur exclusively at the heme reaction center. These observations are supported by the findings of Mayer et al. (*46*) in which synthetic peptides blocked electron transfer to the heme iron of nNOS but were unable to completely block NADPH oxidation. Additionally, neither apo-NOS (*18*) nor CYS-415 mutants (*22*) contained bound BH_4 or heme, thus differing from the CYS-331 mutant which contains heme but does not bind BH_4. The stimulated rate of NADPH oxidation (and $O_2^{\bullet-}$ production) observed upon CaM binding to nNOS might not occur unless electrons are transferred to the heme iron, and the involvement of heme (*21*, *47*) in $O_2^{\bullet-}$ production has been suggested. However, in this report, we clearly show CaM binding is necessary for quinone coupling to nNOS and CYS-331, suggesting a conformational change is occurring upon CaM binding which then facilitates quinone coupling to some otherwise protected site on the enzyme.

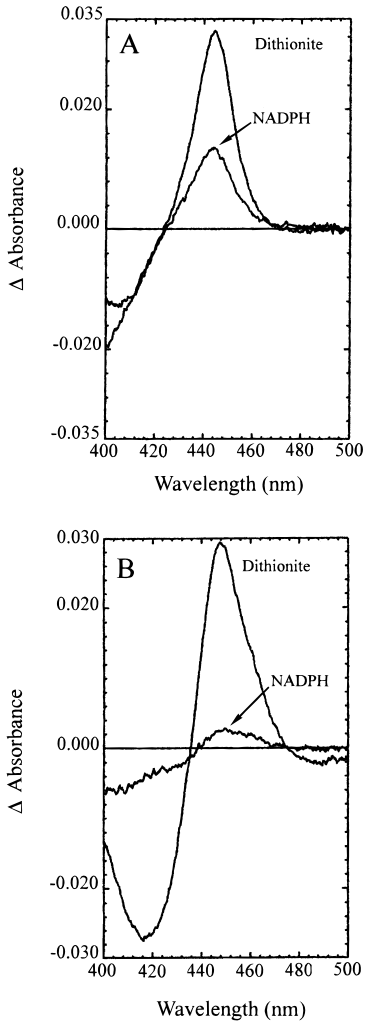


FIGURE 5: Reduced CO difference spectrum formation by nNOS and CYS-331 following addition of NADPH: (A) nNOS and (B) CYS-331 as isolated. Enzyme (0.33 μM) was incubated as described in Experimental Procedures in sealed cuvettes under an atmosphere of CO for 5 min, at which time the spectra were recorded. After the initial spectra were recorded, a few grains of dithionite were added to the cuvettes and the dithionite-reduced CO difference spectra were recorded.

Effect of Epinephrine and Epinephrine o-Quinone on the $NO^{\bullet}$ Capture Assay. Inclusion of epinephrine in the hemoglobin capture assay with wild-type nNOS did not significantly interfere with the assay at early time points. However, as the assay progressed, deviation from control values became increasingly apparent (Figure 6A), suggesting the formation of a secondary species which could interfere in some way with the assay. $O_2^{\bullet-}$ radical reacts with $NO^{\bullet}$ to form $ONOO^-$ (6×10^9 M^{-1} s^{-1}) (*48*) or can dismutate spontaneously to give hydrogen peroxide (2×10^7 M^{-1} s^{-1}) (*49*), both of which could potentially interfere with the assay. Furthermore, the rate of reaction of $NO^{\bullet}$ with oxyhemoglobin to form methemoglobin (3×10^7 M^{-1} s^{-1}) (*50*, *51*) is far slower than the reaction between $NO^{\bullet}$ and $O_2^{\bullet-}$. Examination of these rate constants suggests that when the steady-state $O_2^{\bullet-}$ concentration is approximately 0.5% of that of oxyhemoglobin, then equal amounts of peroxynitrite and methemoglobin are formed. Consistent with the reaction of $O_2^{\bullet-}$

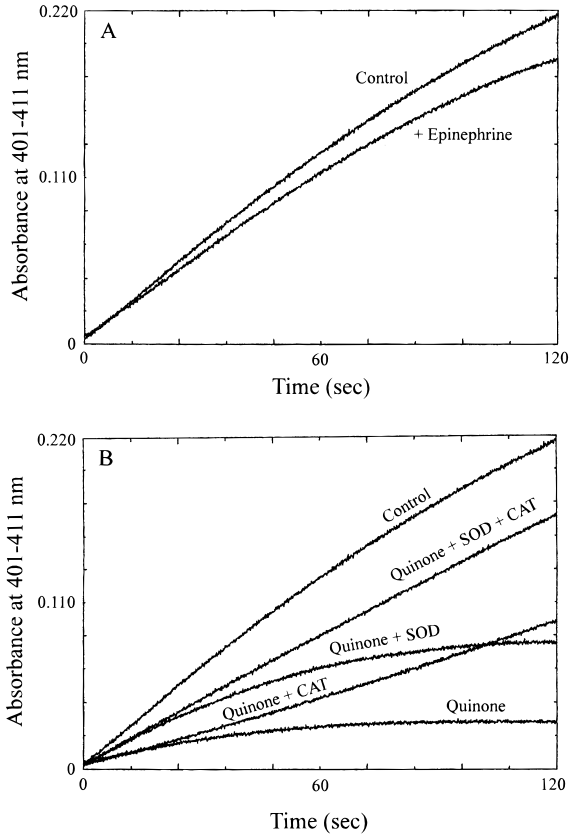


FIGURE 6: Effect of epinephrine (200 μM) (A) or epinephrine *o*-quinone (25 μM) with or without SOD (87 units) and/or catalase (46 units) (B) on the hemoglobin capture assay. nNOS (0.0625 μM) was assayed in the presence of 0.125 μM CaM, 400 μM $CaCl_2$, 100 μM L-arginine, 80 μM oxyhemoglobin, 5 μM DTT, 5 μM BH_4, and 100 μM NADPH at 25 °C. The total assay volume was 1 mL.

with $NO^{\bullet}$, addition of epinephrine *o*-quinone, which immediately results in a stimulated rate of $O_2^{\bullet-}$ production, interfered with the hemoglobin capture assay (Figure 6B), possibly by trapping $NO^{\bullet}$ to form peroxynitrite and/or due to formation of other reactive oxygen species such as H_2O_2. Superoxide dismutase provided some protection at very early time points, but as the reaction progressed, this protective effect was lost, perhaps due to the inability of superoxide dismutase to capture all of the $O_2^{\bullet-}$ being produced. The loss of protection was most likely due to accumulation of H_2O_2, one of the products of superoxide dismutase, which can react with reduced heme iron and produce the highly reactive hydroxyl radical, thereby destroying the heme absorbance.[4] Consistent with this view, catalase or a combination of catalase and superoxide dismutase afforded partial protection against the effects of the *o*-quinone. These experiments demonstrate that reactive oxygen species are produced upon the addition of epinephrine *o*-quinone and that $NO^{\bullet}$ production is not severely altered by the presence of epinephrine in the assay, at least at early time points. Radical−radical reactions between $NO^{\bullet}$ and the semiquinone radicals could also have implications for the ability to detect product formation from nNOS. These reactions are rapid and are another potential source of interference with the hemoglobin capture assay.

In conclusion, we have shown that the adrenochrome assay can be used to estimate the amount of superoxide formed during catalytic turnover of nNOS. Inclusion of thiol-based reducing agents can greatly affect the results of this assay by reducing $O_2^{\bullet-}$ and/or semiquinone intermediates. From our results, Ca/CaM appears not only to affect transfer of NADPH-derived electrons from the flavin domain to the heme domain but also to play a role in structurally altering nNOS in such a way that upon binding of NADPH, $O_2^{\bullet-}$ formation occurs and coupling of exogenously added quinone is facilitated. The role of the flavin domain in this process was confirmed by the use of a CYS-331 mutant which does not efficiently transfer NADPH-derived electrons to the heme iron and the mimicking of this process by the flavoprotein module construct alone. The addition of L-arginine to nNOS resulted in slower formation of $O_2^{\bullet-}$, whereas imidazole addition resulted in decreased coupling. In contrast to nNOS, mutation of CYS-331 resulted in a nNOS construct in which imidazole binding produced slower transfer of electrons to molecular oxygen than L-arginine binding.

[4] R. T. Miller and B. S. S. Masters., unpublished observations.

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