Cu²⁺ and Zn²⁺ Inhibit Nitric-oxide Synthase through an Interaction with the Reductase Domain*

Received for publication, December 10, 1999, and in revised form, February 15, 2000

Jason M. Perry‡, Yunde Zhao§, and Michael A. Marletta¶

From the ‡Division of Medicinal Chemistry, College of Pharmacy, §Department of Biological Chemistry, School of Medicine, and the ¶Howard Hughes Medical Institute, University of Michigan, Ann Arbor, Michigan 48109-0606

Cu²⁺ and Zn²⁺ inhibit all of the NADPH-dependent reactions catalyzed by neuronal nitric-oxide synthase (nNOS) including ferricytochrome c reduction, NADPH oxidation, and citrulline formation. Cu²⁺ and Zn²⁺ also inhibit ferricytochrome c reduction by the independent reductase domain. Zn²⁺ affects all activities of the full-length nNOS and the reductase domain to the same extent (estimated IC₅₀ values from 9 to 31 μM), suggesting Zn²⁺ occupation of a single site in the reductase domain. Citrulline formation and NADPH oxidation by the full-length nNOS and ferricytochrome c reduction by the reductase domain are affected similarly by Cu²⁺, with estimated IC₅₀ values ranging from 6 to 33 μM. However, Cu²⁺ inhibits ferricytochrome c reduction by the full-length nNOS 2 orders of magnitude more potently, with an estimated IC₅₀ value of 0.12 μM. These data suggest the possibility that Cu²⁺ may interact with nNOS at two sites, one composed exclusively of the reductase domain (which is perhaps also involved in Zn²⁺-mediated inhibition), and another that includes components of both domains. Occupation of the second (higher affinity) site could then promote the selective inhibition of ferricytochrome c reduction in full-length nNOS. Neither the inhibition by Cu²⁺ nor that by Zn²⁺ is dependent on calmodulin.

Nitric oxide (NO)¹ has been shown to be a participant in many physiological processes ranging from intercellular signal transduction to the immune response. NO-influenced events include smooth muscle relaxation, neurotransmission, circadian shifts, apoptosis, long-term potentiation, synaptogenesis, development, and pathogen cytostasis (1–5). Nitric-oxide synthase (NOS; EC 1.14.13.39) catalyzes the biosynthesis of NO through an oxidative reaction on the amino acid L-arginine that ultimately results in carbon-nitrogen bond cleavage to yield citrulline and NO. The only known intermediate in this reaction is N⁴-hydroxy-L-arginine (6, 7). NOS is a homodimer consisting of two domains (one oxygenase and one reductase) per polypeptide chain. The NOS oxygenase domain stoichiometrically binds protoporphyrin IX type heme and (6R)-5,6,7,8-tetrahydro-L-biopterin (H₂B). The heme iron is ligated to the NOS polypeptide chain through a cysteine thiolate; thus, the heme is a member of the cytochrome P450 class of enzymes and displays optical and magnetic properties typical of other known cytochrome P450s (8–12). Together with the heme and H₂B cofactors, the oxygenase domain forms the active site of the enzyme (13).

Three sets of crystal structures of the dimeric form of the oxygenase domain have been reported recently (14–16). Although no structures are currently available for either the reductase domain or the full-length NOS, it is known that each reductase domain binds one equivalent of FMN and one equivalent of FAD (17, 18). The reductase domain shuttles NADPH-derived electrons to the active site heme to support catalysis in the oxygenase domain. Presumably, electron transfer proceeds in a manner analogous to that in cytochrome P450 reductase (CPR), another two-flavin reductase. Like CPR, the nNOS reductase domain contains an air-stable neutral semiquinone radical on its high potential flavin (known in CPR, and presumed in NOS, to be FMN) (8, 12, 19–21). Another similarity between CPR and the neuronal NOS (nNOS) reductase domain is the ability of each to reduce artificial electron acceptor species such as ferricytochrome c, ferricyanide, and 2,6-dichlorophenolindophenol (20, 22–24).

The oxygenase and reductase domains of NOS are linked by a region of the polypeptide that recognizes Cu²⁺-bound calmodulin (CaM). CaM binding accelerates both intradomain electron transfer (from NADPH through the reductase domain) and interdomain electron transfer (from the reductase to the oxygenase domain) (25–27). Because NOS has been heterologously expressed in Escherichia coli, a variety of genetic manipulations (including site-directed mutagenesis and the independent expression of each domain) are possible (28). Alternatively, CaM-responsive reductase domain can be obtained as a proteolytic byproduct of the full-length nNOS E. coli expression and purification procedure (21).

In this report, we have used the CaM-responsive nNOS reductase domain (generated by the latter method) in conjunction with the full-length nNOS to demonstrate that two known NOS inhibitors (Cu²⁺ and Zn²⁺) exert their effects on the reductase domain of NOS (29, 30). These results are significant because they reconcile existing biochemical data (that certain transition metals, including Zn²⁺, inhibit NOS catalysis) with structural arguments, which suggest that Zn²⁺ bound to the oxygenase domain may actually be pivotal (i.e. essential and noninhibitory) for proper H₂B orientation and/or L-arginine binding (15, 16). In addition, complementary results from this study have enabled us to propose a mechanism of ferricytochrome c reduction by the nNOS reductase domain that resolves the differences between previously reported sets of data (22, 24, 27). The proposed mechanism invokes the in situ production of superoxide to bridge the high potential flavin of the
Cu^{2+} and Zn^{2+} Inhibition of Nitric-oxide Synthase

reductase domain and the heme of ferricytochrome c. These results are also significant because they highlight a potential structural difference between the nNOS reductase domain and CPR, namely, that the high potential flavin of the nNOS reductase domain is not as surface-accessible as that of CPR.

EXPERIMENTAL PROCEDURES

Materials

2',5'-ADP-Sepharose, calmodulin-Sepharose 4B, and the HiLoad 26/60 Superdex-200 gel filtration column were purchased from Pharmacia. H_2O was purchased from B. Schircks Laboratories (Jona, Switzerland). L-[14C]Arginine was from Amersham Pharmacia Biotech. Isopropyl-β-thiogalactopyranoside and ampicillin were from Roche Molecular Biochemicals. The original cDNA clone for rat neuronal NOS, in the vector Bluescript (SK+), was a generous gift from Dr. Solomon H. Snyder (Johns Hopkins University, Baltimore, MD). We subcloned the nNOS cDNA into a commonly used cytochrome P450 expression vector, pCWori, which was generously provided by Dr. Michael R. Waterman (Vanderbilt University, Nashville, TN). Horse heart cytochrome c, HEPES buffer, NADPH, xanthine, xanthine oxidase, manganese superoxide dismutase (Mn-SOD), dithiothreitol (DTT), glycerol, CaM, and all other reagents were purchased from Sigma.

Enzyme Overproduction and Purification

Full-length nNOS and the nNOS reductase domain were overproduced and purified as described previously (21). Briefly, four 1-liter cultures of DH5a E. coli cells containing the pCWori-nNOS expression plasmid were grown at 37 °C to an OD_600 of 0.8 and subsequently cooled to 22 °C prior to the induction of nNOS expression by the addition of 1 mM isopropyl-β-thiogalactopyranoside. Cells were harvested by centrifugation 18–21 h postinduction. The nNOS purification involves three steps: CaM affinity chromatography followed by ADP-affinity chromatography, and ultimately, gel filtration on Superdex-200 media. CaM-responsive reductase domain copurifies with the full-length nNOS up to the gel filtration step where they are resolved from one another, each to homogeneity.

Enzyme Activity Assays

Ferricytochrome c Reduction—Both the full-length nNOS and the reductase domain can reduce ferricytochrome c. The rate of this reaction was measured by monitoring the increase in absorbance at 550 nm (Δε = 21,000 M^-1 cm^-1) over time. Each assay contained 2.5 mM CaCl_2, 20 mM cytochrome c in 50 mM HEPES buffer (pH 7.4) unless otherwise specified. Various concentrations of full-length nNOS or reductase domain were used depending on the nature of the experiment. Full-length nNOS assays also contained 5 mM H_2O and 50 mM DTT, neither of which reduced ferricytochrome c at the specified concentrations. In addition, the indicated assays contained varying concentrations of transition metals, all added as their chloride salts. All ferricytochrome c reduction assays were performed at 25 °C in a total volume of 500 μL and were initiated by the addition of 500 μM NADPH.

NADPH Oxidation—The NADPH oxidase activity of full-length nNOS was determined by following the decrease in absorbance at 340 nm (Δε = 6, 200 M^-1 cm^-1) over time. Assays contained 2.5 mM CaCl_2, 20 μg CaM, 80 mM nNOS, 5 mM H_2O, 50 mM DTT, and varying concentrations of either CuCl_2 or ZnCl_2 and were performed at 25 °C in 50 mM HEPES (pH 7.4). Reactions (total volume of 500 μL) were initiated by the addition of 150 μM NADPH.

Citrulline Formation—Assays measuring citrulline formation by nNOS in the presence of various concentrations of Cu^{2+} or Zn^{2+} were also performed. These assays (total volume of 300 μL) contained 100 μM L-[14C]Arginine (1.5 μCi/mmole), 20 μg of CaM, 2.5 mM CaCl_2, 130 mM nNOS, 50 mM H_2O, 500 μM DTT, and varying concentrations of either CuCl_2 or ZnCl_2. All citrulline assays were performed at 25 °C in 50 mM HEPES (pH 7.4) and were initiated by the addition of 1 mM NADPH.

SOD Activity—SOD was assayed following a method similar to that published by Beyer and Fridovich (31). In this assay, the activity of SOD is measured as the inhibition of superoxide-dependent reduction of ferricytochrome c. The xanthine/xanthine oxidase system is used to generate superoxide, and the reaction is monitored at 550 nm. The assay contained 200 μM xanthine, 10 mM xanthine oxidase, 50 μM cytochrome c, and 100 units of Mn-SOD. SOD assays (total volume of 500 μL) were run at 25 °C in 50 mM HEPES (pH 7.4). Xanthine oxidase activity was confirmed prior to the SOD assays by following the increase in absorbance at 293 nm resulting from the conversion of xanthine to urate. Xanthine oxidase assays (total volume of 1 mL) contained 200 μM xanthine and 10 mM xanthine oxidase and were run at 25 °C in 50 mM HEPES (pH 7.4).

Electronic Absorption Spectroscopy

All spectra and kinetic traces were recorded on a Varian Cary 3E spectrophotometer at a constant temperature of 25 °C maintained by a Neslab circulating water bath.

Stopped-flow Kinetic Analysis

nNOS reductase domain (100 μL, 16 μM in 50 mM HEPES (pH 7.4), 10% (v/v) glycerol, 3 mM CaCl_2, 60 μM CaM) was mixed with 100 μL of various concentrations of either ZnCl_2 or CuCl_2 in 50 mM HEPES buffer (pH 7.4). IC_{50} values generated from these data are as follows: reductase domain, Zn^{2+} = 22 μM, Cu^{2+} = 12 μM; full length nNOS, Zn^{2+} = 15 μM, Cu^{2+} = 0.12 μM.

RESULTS

The Inhibition of Ferricytochrome c Reduction by Cu^{2+} and Zn^{2+}—Inhibition of ferricytochrome c reduction activity is shown in Fig. 1. Cu^{2+} and Zn^{2+} each inhibit this activity in both the reductase domain and the full-length enzyme in a concentration dependent manner; however, the inhibition brought about by Cu^{2+} is more pronounced in the presence of the oxygenase domain (with the full-length nNOS). That is, while Zn^{2+} inhibited ferricytochrome c reduction by the full-length nNOS and the independent reductase domain to similar extents, the inhibition observed with Cu^{2+} was two orders of magnitude more potent with the full-length nNOS than it was with the reductase domain (the IC_{50} values were 0.12 and 12 μM, respectively).

The Effect of Cu^{2+} and Zn^{2+} on CaM—Ferricytochrome c reduction activities in both full-length nNOS and the reductase
domain are greatly enhanced by association with Ca$^{2+}$-bound CaM. We therefore had to rule out the possibility that the observed inhibition was simply a consequence of the transition metals either (i) competing for the required Ca$^{2+}$ or (ii) binding to one or more of the six auxiliary cation binding sites on CaM, which could distort its structure, thereby rendering it inactive.

In the presence of 1 mM Ca$^{2+}$, it had previously been shown that each of these auxiliary sites can bind Zn$^{2+}$ with a $K_d$ of approximately 832 $\mu$M (32). This concentration of Zn$^{2+}$ greatly exceeds the levels used here; however, it is conceivable that, upon association with nNOS (or its reductase domain), CaM attains a conformation more amenable to Zn$^{2+}$ binding, which in turn results in the observed inhibition. Fig. 2 examines the possibility of CaM inhibition by using the reductase domain. The figure demonstrates that the inhibition of reductase domain mediated ferricytochrome c reduction by Cu$^{2+}$ and Zn$^{2+}$ does not, in fact, even require CaM. This result is consistent with the metals interacting with the reductase domain itself.

Inhibition of NADPH Oxidation by Cu$^{2+}$ and Zn$^{2+}$—It was also possible that the observed inhibition of ferricytochrome c reduction was related to Cu$^{2+}$ and Zn$^{2+}$ modulating the ability of ferricytochrome c to accept electrons from the reductase domain. This could happen either by (i) metal binding to and altering the conformation of ferricytochrome c, somehow making it less susceptible to reduction or (ii) by a metal-induced disruption of the presumed interaction between nNOS and cytochrome c (24). To address this possibility, we performed NADPH oxidation assays on the full-length nNOS. In this case, the electron acceptor is the nNOS heme, and ultimately, molecular oxygen. Therefore, it is an internal (interdomain) electron transfer that does not rely on any interaction with, or structural integrity of, an artificial acceptor molecule. Furthermore, inhibition of NADPH oxidation by Cu$^{2+}$ and Zn$^{2+}$ would suggest that inhibition of ferricytochrome c reduction was not caused by an effect on the metals on either cytochrome c itself, or cytochrome c-NOS docking. Fig. 3 shows that NADPH oxidation by the full-length nNOS is in fact inhibited by both Cu$^{2+}$ and Zn$^{2+}$ in a concentration-dependent manner. The estimated IC$_{50}$ values for the observed inhibition are 6 and 9 $\mu$M, respectively.

Activity Correlation—Having demonstrated that the function of the reductase domain is impaired by Cu$^{2+}$ and Zn$^{2+}$, we sought to correlate the inhibition of NADPH oxidation and ferricytochrome c reduction with the previously reported inhibition of NOS-catalyzed citrulline formation by these metals (29, 30). Such a correlation for each metal is shown in Fig. 4, and the IC$_{50}$ values for the inhibition of each activity are summarized in Table I. With Zn$^{2+}$ (Fig. 4A), the inhibition data for all three full-length nNOS activities are similar, which suggests that the ability of Zn$^{2+}$ to inhibit citrulline formation can be traced to an interaction that involves the reductase domain, exclusively. The situation with Cu$^{2+}$ (Fig. 4B) is not quite as simple, indicating that Cu$^{2+}$ may not interact with nNOS in precisely the same manner as Zn$^{2+}$. Nevertheless, the results with Cu$^{2+}$ remain consistent with the conclusion that the observed inhibition of citrulline formation is also a consequence of an interaction between the added metal and the reductase domain.

The Effect of Cu$^{2+}$ or Zn$^{2+}$ on Flavin Reduction—A stopped-flow kinetic analysis of flavin reduction shows that NADPH can efficiently reduce the low potential flavin of the reductase domain in the presence of an inhibitory concentration of either Cu$^{2+}$ (50 $\mu$M) or Zn$^{2+}$ (100 $\mu$M). Fig. 5A compares the rates of flavin reduction (as measured by the change in absorbance at 454 nm) observed under these conditions with that observed in the absence of exogenous transition metals. Data collected from reactions that contained no exogenous transition metals as well as those collected from reactions that included 100 $\mu$M Zn$^{2+}$ were best fit to single exponential equations to generate pseudo first-order rate constants ($k_{\text{obs}}$) of 60 and 46 s$^{-1}$, respectively (at 25°C). Data collected from the reactions that contained Cu$^{2+}$ were not well fit by a similar equation; however, full spectra collected by diode array 2.5 s postmixing confirmed the reduction of the low potential flavin (Fig. 5B).

The Effect of Air on Ferricytochrome c Reduction—Electronic absorption spectra of the visible region of cytochrome c in the presence of CaM-bound reductase domain (Fig. 6A) demonstrate the dramatic effect of air (presumably mediated by oxygen) on the rate of ferricytochrome c reduction. Fig. 6B shows the anaerobic chemical reduction of ferricytochrome c, demonstrating that the emerging species in the upper panel is indeed the reduced form of cytochrome c and that the formation of this species does not require oxygen. In addition, we show in Fig. 7 that the effect of air on the rate of ferricytochrome c reduction is not exclusive to the CaM-bound reductase domain. Although
The Effect of SOD on Ferricytochrome c Reduction—Whether or not ferricytochrome c reduction by nNOS can be inhibited by superoxide dismutase (SOD) has been a source of considerable debate in the literature (22, 24, 27, 33). Because we observed an air-dependent rate with the reductase domain, experiments addressing the effect of SOD were carried out. When assaying the nNOS reductase domain, the inclusion of up to 1000 units of SOD had virtually no effect on the rate of ferricytochrome c reduction (data not shown). In contrast to this result, 100 units of the same preparation of SOD completely inhibited the superoxide-mediated reduction of ferricytochrome c in the presence of 10 nM xanthine oxidase and 200 μM xanthine (0.3 nmol min⁻¹ inhibited to 0.0 nmol min⁻¹, data not shown).

The Effect of Zn²⁺ on Anaerobic Ferricytochrome c Reduction—To test whether the inhibition by Cu²⁺ and Zn²⁺ was air-dependent, assays measuring the anaerobic rate of ferricytochrome c reduction in the presence of various amounts of Zn²⁺ were carried out. The results are compared with similar assays performed under aerobic conditions in Table II. For these experiments, the use of Cu²⁺ was avoided to circumvent the potential complication that under anaerobic conditions Cu²⁺ could serve as a stable electron trap (Cu⁺), and in so doing, give an overestimation of the inhibition of ferricytochrome c reduction. Table II shows that Zn²⁺ does in fact inhibit anaerobic ferricytochrome c reduction to an extent similar to that observed in aerobic assays. In fact, the potency of Zn²⁺ seems, if anything, slightly greater in the anaerobic assays. The observed inhibition in the presence of 20 μM Zn²⁺ is approximately 50% in aerobic assays and approximately 75% in anaerobic assays. At 80 μM Zn²⁺, the observed inhibition is 95% and 99%, respectively.

**DISCUSSION**

The data presented in this report all support the conclusion that Cu²⁺ and Zn²⁺ inhibit nNOS catalysis as a result of a direct interaction with the reductase domain of the enzyme. All three commonly assayed full-length NOS activities (citrulline formation, ferricytochrome c reduction, and NADPH oxidation) were inhibited by both Cu²⁺ and Zn²⁺. Likewise, Cu²⁺ and Zn²⁺ were inhibited to 0.0 nmol min⁻¹ and 0.20 nmol min⁻¹, respectively. The observed inhibition in the presence of 20 μM Cu²⁺ and 100 μM Zn²⁺ is superimposable with the dotted line, and are therefore omitted for clarity.

**TABLE I**

| Enzyme                  | Assay               | IC₅₀ value (μM) Cu²⁺ | IC₅₀ value (μM) Zn²⁺ |
|-------------------------|---------------------|----------------------|----------------------|
| Full-length nNOS        | citrulline formation| 19                   | 31                   |
|                         | cyt-c reduction     | 0.12                 | 15                   |
|                         | NADPH oxidation     | 6                    | 9                    |
| Reductase domain (+CaM) | cyt-c reduction     | 12                   | 22                   |
| Reductase domain (−CaM) | cyt-c reduction     | 85                   | 12                   |

*Cu²⁺ and Zn²⁺ were both added as the dichloride salt.

Cyt-c reduction, ferricytochrome c reduction.

The Effect of Zn²⁺ on Anaerobic Ferricytochrome c Reduction—Whether or not ferricytochrome c reduction by nNOS can be inhibited by superoxide dismutase (SOD) has been a source of considerable debate in the literature (22, 24, 27, 33). Because we observed an air-dependent rate with the reductase domain, experiments addressing the effect of SOD were carried out. When assaying the nNOS reductase domain, the inclusion of up to 1000 units of SOD had virtually no effect on the rate of ferricytochrome c reduction (data not shown). In contrast to this result, 100 units of the same preparation of SOD completely inhibited the superoxide-mediated reduction of ferricytochrome c in the presence of 10 nM xanthine oxidase and 200 μM xanthine (0.3 nmol min⁻¹ inhibited to 0.0 nmol min⁻¹, data not shown).
Zn$^{2+}$ each inhibited ferricytochrome $c$ reduction by the independent reductase domain. The estimated IC$_{50}$ values for all of these reactions (with the exception of the inhibition of full-length nNOS ferricytochrome $c$ reduction by Cu$^{2+}$) were in the low $\mu$M (6–33 $\mu$M) range. Interestingly, Cu$^{2+}$ inhibited full-length NOS ferricytochrome $c$ reduction more potently (by two orders of magnitude) than it inhibited the same activity of the reductase domain (IC$_{50}$ values of 0.12 and 12 $\mu$M, respectively). The reason for this difference is not clear; however, because the components of the assay are otherwise equivalent, it suggests that the oxygenase domain of NOS influences Cu$^{2+}$ binding to the reductase domain. There are many ways in which the oxygenase domain could exert such an effect. For example, it could contribute to Cu$^{2+}$ binding directly, by (for example) donating a metal ligand. Alternatively, the oxygenase domain could influence the structure of reductase domain by domain-domain contacts, perhaps thereby altering its conformation to a state more amenable to Cu$^{2+}$ binding. A third possibility is that the conformation of the full-length nNOS is modified by the docking of cytochrome $c$. In this case, the apparent high affinity for Cu$^{2+}$ (which is manifested by its effect on ferricytochrome $c$ reduction) results from a composite of opposing structural effects; that is, the oxygenase domain and cytochrome $c$ synergistically affect Cu$^{2+}$ binding to the reductase domain. This explanation is consistent with the data for Cu$^{2+}$-mediated inhibition of citrulline formation and NADPH oxidation in the full-length nNOS (the estimated IC$_{50}$ values were 19 and 6 $\mu$M, respectively), because cytochrome $c$ is present in neither of these assays. Yet another possibility is that there is more than one binding site available to Cu$^{2+}$ in the full-length nNOS, one of which (that of apparent higher affinity) requires the presence of both domains. Because electrons are presumably routed in different directions depending on whether they serve to reduce the NOS heme or that of ferricytochrome $c$, it is conceivable that Cu$^{2+}$ could bind to one site (that of higher affinity) that would result in the selective inhibition of ferricytochrome $c$ reduction without eliciting an inhibitory effect on electron transfer going in the other direction; hence, NADPH oxidation and citrulline formation (both of which are dependent on NOS heme reduction) are not affected. At higher concentrations of Cu$^{2+}$, when the second (lower affinity) site would be occupied, electron transfer from the reductase domain is interrupted, manifested by the inhibition of all three activities. This not only explains the difference between the various activities presented in the Cu$^{2+}$ activity correlation curve but also the

**FIG. 6.** The effect of air on ferricytochrome $c$ reduction by the nNOS reductase domain. Visible (Q) band electronic absorption spectra of cytochrome $c$ by the nNOS reductase domain. Spectrum 1 (solid line) was recorded following the evacuation of an aqueous ferricytochrome $c$ solution containing 20 $\mu$g of CaM, 5 $\mu$M reductase domain, 2.5 mM CaCl$_2$, and 50 mM HEPES (pH 7.4). Spectrum 2 (dotted line) was recorded following the anaerobic addition of NADPH to 500 $\mu$M and a subsequent 5-min incubation period at 25 °C. Spectrum 3 (—— —) was recorded within 1 min of spectrum 2, following removal of the seal on the cuvette and gentle agitation. $B$, anaerobic chemical reduction of ferricytochrome $c$ by sodium dithionite. The solid line spectrum is ferricytochrome $c$, whereas the dashed line spectrum shows full conversion to ferrocytochrome $c$. 

**FIG. 7.** The air-induced rate enhancement of ferricytochrome $c$ reduction by the nNOS reductase domain: the effect of CaM. Q band electronic absorption spectra of cytochrome $c$ during reductase domain turnover in the absence of CaM. Spectra were recorded every 10 min for 70 min following the addition of 500 $\mu$M NADPH. Data in $A$ were collected in an unsealed cuvette, and data in $B$ were collected in the same cuvette after it had been sealed and exposed to repeated cycles of evacuation followed by argon purging. Both reactions contained 5 $\mu$M reductase domain, 50 $\mu$M cytochrome $c$, and 50 mM HEPES (pH 7.4).

**TABLE II**

| Zn$^{2+}$ | Aerobic | Anaerobic$^b$ |
|-----------|--------|--------------|
| $\mu$M    |        |              |
| 0         | 9.756 ± 0.483 ($n = 22$) | 0.451 ± 0.015 ($n = 3$) |
| 20        | 4.890 ± 0.170 ($n = 6$)   | 0.106 ± 0.014 ($n = 3$) |
| 80        | 0.507 ± 0.020 ($n = 6$)   | 0.008 ± 0.001 ($n = 3$) |

All assays were run in the presence of 2.5 mM Ca$^{2+}$ and 20 $\mu$g of CaM, and the activities are expressed as $\mu$mol min$^{-1}$ mg$^{-1}$. $^a$ The first column refers to the final concentration of Zn$^{2+}$ (added as ZnCl$_2$) in the assay reaction cuvette. $^b$ Anaerobic samples were prepared by using repeated cycles of alternate evacuation and argon purging of a sealed reaction cuvette using a gas train constructed in our laboratory.
difference between the magnitude of Cu$^{2+}$-mediated inhibition of ferricytochrome $c$ reduction observed with the full-length NOS as compared with that observed with the reductase domain. The independent domain would not have the first (higher affinity) site intact, and the observed inhibition would necessarily be a consequence of Cu$^{2+}$ interacting with the second (lower affinity) site. In support of this, the estimated IC$_{50}$ of Cu$^{2+}$ with the reductase domain was 12 $\mu$M, which is similar to the IC$_{50}$ values observed for both citrulline formation and NADPH oxidation by the full-length NOS (19 and 6 $\mu$M, respectively). In addition, the presence of a high affinity Cu$^{2+}$ binding site in the full-length nNOS is consistent with our previous findings that nNOS overproduced in E. coli can be purified with one equivalent of round copper per subunit (30). Because the final step of the nNOS purification is gel filtration, one would not expect the second (lower affinity) site to be occupied until the enzyme is assayed in the presence of micromolar levels of Cu$^{2+}$.

The situation with Zn$^{2+}$ is less complicated; all three full-length NOS activities and the reductase domain activity are modulated similarly. The estimated IC$_{50}$ values of Zn$^{2+}$ required to affect these processes fall between 9 and 31 $\mu$M, in the same range as the IC$_{50}$ values for the Cu$^{2+}$ effects caused by its interaction at the hypothesized second (lower affinity) site. Because Zn$^{2+}$ inhibition of the reductase domain correlates well with its inhibition of full-length NOS activities (citrulline formation, NADPH oxidation, ferricytochrome $c$ reduction), it is reasonable to conclude that an interaction between Zn$^{2+}$ and the reductase domain can account for all of its observed inhibitory effects on the full-length NOS. This is of considerable significance because recent crystallographic studies of the NOS oxygenase domain have demonstrated that a Zn$^{2+}$ atom can bind at the dimeric interface of the enzyme (15, 16), and this site had previously been hypothesized to be the site of Zn$^{2+}$ inhibition (29). Structural studies have led to the conclusion that Zn$^{2+}$ binding to the oxygenase domain is essential to the stability of the dimer and, in addition, serves to maintain the proper orientation of $\delta$-HB and L-arginine. The current results are not inconsistent with such an argument because they point to a different site that can account for the observed NOS inhibition by Zn$^{2+}$, i.e., in the reductase domain.

The precise mechanism of inhibition of nNOS and its reductase domain by Cu$^{2+}$ and Zn$^{2+}$ is not completely clear; however, the results presented here combined with previous results from our laboratory and others enable us to make some suggestions. From this report it is clear that an electron transfer reaction of the reductase domain is inhibited. This inhibition is not exclusive to the CaM-activated domain, nor does it require oxygen. In addition, we have ruled out the possibility that the observed inhibition is a consequence of the inhibition of NADPH-mediated reduction of the low-potential flavin. These results are consistent with previous studies using electronic absorption spectroscopy to show that in the presence of 100 $\mu$M Cu$^{2+}$ (enough to completely inhibit citrulline formation by NOS), NADPH could not reduce the heme iron, although flavin reduction was observed (30). From the current data there are two possibilities: (i) inhibition of interflavin electron transfer or (ii) inhibition of electron transfer from the high-potential flavin to the acceptor molecule. The redox potentials of the two semiquinones are comparable and metal binding somehow inhibits interflavin electron transfer, this situation could be possible. Additionally, it would be impossible to make a definitive distinction between FMNH/FADH$_2$ and FMNH$_2$/FADH$^+$ from absorption spectra of full-length NOS. In short, the possibility of inhibition of interflavin electron transfer seems unlikely, but it cannot be eliminated. The more likely explanation (see (ii) above) is that Cu$^{2+}$ and Zn$^{2+}$ inhibit electron transfer from the high-potential flavin to the acceptor molecule (either the heme of nNOS or that of ferricytochrome $c$). There are many ways in which this could happen. Three possibilities are: 1) a metal-induced rearrangement of the nNOS polypeptide that (in 3-dimensional space) is between the high potential flavin and the acceptor molecule, perhaps thereby repositioning participating amino acid residues or setting up a local environment that is not conducive to electron transfer; 2) a structural perturbation that results in the movement of the high potential flavin further away from the acceptor molecule; or 3) the binding of the metal directly to the N5 and/or O4 of the isoalloxazine ring of the high potential flavin, sequestering the redox-active portion of the molecule, and thereby interfering with electron transfer. It is known that Cu$^{2+}$ can indeed bind to a flavin in this manner, and a structure of this species crystallized from aqueous solution has been solved (34). Zn$^{2+}$ can also bind to flavin in this manner, but its binding is more favored in hydrophobic environments; however, this environment may very well be provided by the reductase domain (34). An interesting possibility with particular relevance to Cu$^{2+}$ is that metal binding occurs with a semiquinone radical during the electron transfer process. In this instance, the complex may be more favorable because the Cu$^{2+}$/semiquinone is resonance-stabilized with a Cu$^{2+}$/oxidized flavin species (35, 36). Such an hypothesis could be further substantiated by electron paramagnetic resonance experiments (performed anaerobically) in which the reductase domain was shown to reduce Cu$^{2+}$ following the anaerobic addition of NADPH.

It has previously been hypothesized that the reason for the observed CaM-dependent enhancement of ferricytochrome $c$ reduction by full-length NOS was that electrons could pass to the heme and subsequently be consumed by oxygen. This process would generate superoxide which could then diffuse in solution to the terminal acceptor, ferricytochrome $c$ (22). It was later shown that a heme-ligand mutant (nNOS C415A) retained the property of CaM-dependent acceleration of ferricytochrome $c$ reduction; however, the potential role of dioxygen, if any, was unclear (33). Our results showing the air enhancement of ferricytochrome $c$ reduction by the reductase domain suggest that dioxygen can play a role in this process, and, that the high potential flavin of NOS may be positioned further away from the acceptor molecule (and hence the surface of the enzyme) than that of CPR. This hypothesis is consistent with the results presented in our previous work which show that the rapid relaxation properties of the high potential flavin radical are imparted by elements of the reductase domain, implying that the flavin is somewhat buried (21). If the high potential flavin was at the surface of the enzyme, the radical spin would be expected to resemble that of an isolated free radical, and relax much more slowly. CPR, however, is believed to directly reduce its substrates by one electron from FADH$_2$/FMNH$_2$, transiently yielding FADH/FMNH; which rearranges to FAD/FMN$_2$, donates another electron, and returns to FAD/FMNH; the species that is reduced by NADPH (19, 20, 23). For dioxygen to mediate the one electron reduction of ferricytochrome $c$, it is presumably reduced to superoxide by the hydroquinone of the high potential flavin of nNOS. However, the
reaction was not inhibited by SOD, which suggests that superoxide does not escape the flavin “active site”. Rather, superoxide must be generated in situ as dioxygen can serve to bridge the two redox centers.

In conclusion, we have shown that Cu$^{2+}$ and Zn$^{2+}$ interact with the reductase domain of nNOS to elicit their inhibitory effects. The underlying cause of all of the observed effects of these metals (inhibition of ferricytochrome $c$ reduction, NADPH oxidation, and citrulline formation) is the inhibition of electron transfer, presumably that from the high potential flavin of the reductase domain to the acceptor heme iron of either ferricytochrome $c$ or nNOS. In addition, we have given evidence that dioxygen facilitates electron transfer to ferricytochrome $c$.

Acknowledgments—We thank Professor David P. Ballou (University of Michigan) for the use of his stopped-flow apparatus as well as for his advice on experimental conditions. We also wish to thank members of the Marletta laboratory for their critical comments on this manuscript.

REFERENCES

1. Wang, T., Xie, Z., and Lu, B. (1995) Nature 374, 262–266
2. Kantor, D. B., Lanzrein, M., Stary, S. J., Sandoval, G. M., Smith, W. B., Sullivan, B. M., Davidson, N., and Schuman, E. M. (1996) Science 274, 1744–1748
3. DeGroote, M. A., Testerman, T., Xu, Y., Stauffer, G., and Fang, F. C. (1996) Science 272, 414–417
4. Ding, J. M., Chen, D., Getzoff, E. D., Rea, M. A., and Gillette, M. U. (1994) Science 266, 1713–1717
5. Jaffrey, S. R., and Snyder, S. H. (1996) Science 274, 774–776
6. Pufahl, R. A., Najarppan, P. G., Woodard, R. W., and Marletta, M. A. (1992) Biochemistry 31, 6522–6528
7. Stuehr, D. J., Kwon, N. S., Nathan, C. F., Griffith, O. W., Feldman, P. L., and Wiseman, J. (1991) J. Biol. Chem. 266, 6259–6263
8. Richards, M. K., and Marletta, M. A. (1994) Biochemistry 33, 14723–14732
9. McMillan, K., and Masters, B. S. S. (1995) Biochemistry 34, 3686–3693
10. Crane, B. R., Arvai, A. S., Gachhui, R., Wu, C., Ghosh, D. K., Getzoff, E. D., Stuehr, D. J., and Tainer, J. A. (1997) J. Biol. Chem. 272, 425–431
11. White, K. A., and Marletta, M. A. (1992) Biochemistry 31, 6627–6631
12. Stuehr, D. J., and Ikeda-Saito, M. (1992) J. Biol. Chem. 267, 20547–20550
13. Marletta, M. A. (1994) Cell 78, 927–930
14. Crane, B. R., Arvai, A. S., Ghosh, D. K., Wu, C., Getzoff, E. D., Stuehr, D. J., and Tainer, J. A. (1998) Science 279, 2121–2126
15. Raman, C. S., Li, H., Martasek, P., Král, V., Masters, B. S. S., and Poulos, T. L. (1999) Cell 95, 939–950
16. Fischmann, T. O., Hruza, A., Niu, X. D., Fassetta, J. D., Lunn, C. A., Dolphin, E., Prongay, A. J., Reichert, P., Lundell, D. J., Narula, S. K., and Weber, P. C. (1999) Nat. Struct. Biol. 6, 233–242
17. Hevel, J. M., White, K. A., and Marletta, M. A. (1992) in The Biology of Nitric Oxide. 2. Enzymology, Biochemistry and Immunology (Moncada, S., Marletta, M. A., Hibbs Jr, J. B., and Higgs, E. A., eds) Portland Press, London
18. Stuehr, D. J., Cho, H. J., Kwon, N. S., Weise, M. F., and Nathan, C. F. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 7773–7777
19. Vermilion, J. L., and Coon, M. J. (1978) J. Biol. Chem. 253, 8812–8819
20. Vermilion, J. L., Ballou, D. P., Massey, V., and Coon, M. J. (1981) J. Biol. Chem. 256, 266–277
21. Perry, J. M., Moon, N., Zhao, Y., Dunham, W. R., and Marletta, M. A. (1998) Chem. & Biol. 5, 355–364
22. Sheta, E. A., McMillan, K., and Masters, B. S. S. (1994) J. Biol. Chem. 269, 15147–15153
23. Kurzban, G. P., and Strobel, H. W. (1986) J. Biol. Chem. 261, 7824–7830
24. Klatt, P., Heinzl, B., John, M., Kastner, M., Bohme, E., and Mayer, B. (1992) J. Biol. Chem. 267, 11374–11378
25. Abu-Soud, H. M., Yoho, L. L., and Stuehr, D. J. (1994) J. Biol. Chem. 269, 32047–32050
26. Abu-Soud, H. M., Feldman, P. L., Clark, P., and Stuehr, D. J. (1994) J. Biol. Chem. 269, 25318–25326
27. Gachhui, R., Presta, A., Bentley, D. F., Abu-Soud, H. M., McArthur, R., Brudvig, G., Ghosh, D. K., and Stuehr, D. J. (1996) J. Biol. Chem. 271, 20594–20602
28. Gerber, N. C., and Ortiz de Montellano, P. R. (1995) J. Biol. Chem. 270, 17791–17796
29. Persechini, A., McMillan, K., and Masters, B. S. S. (1995) Biochemistry 34, 15091–15095
30. Perry, J. M., and Marletta, M. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11101–11106
31. Beyer, W. F., and Fridovich, I. (1987) Anal. Biochem. 161, 559–566
32. Milos, M., Comte, M., Schaer, J. J., and Cox, J. A. (1989) Eur. J. Biochem. 185–196
33. Richards, M. K., Clague, M. J., and Marletta, M. A. (1996) Biochemistry 35, 7772–7780
34. Fritchie, C. J., Jr. (1973) J. Biol. Chem. 248, 7516–7521
35. Muller, F., Hemmerich, P., Ehrenberg, A., Palmer, G., and Massey, V. (1970) Eur. J. Biochem. 14, 185–196
36. Muller, F., Hemmerich, P., and Ehrenberg, A. (1968) Eur. J. Biochem. 5, 158–164
Cu\(^{2+}\) and Zn\(^{2+}\) Inhibit Nitric-oxide Synthase through an Interaction with the Reductase Domain

Jason M. Perry, Yunde Zhao and Michael A. Marletta

*J. Biol. Chem. 2000, 275:14070-14076.*

doi: 10.1074/jbc.275.19.14070

Access the most updated version of this article at http://www.jbc.org/content/275/19/14070

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 35 references, 20 of which can be accessed free at http://www.jbc.org/content/275/19/14070.full.html#ref-list-1