Inhibition of Nitric Oxide Synthase Activity by Zn^{2+} Ion†

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ABSTRACT: We have found neural nitric oxide synthase (nNOS) activity to be completely and reversibly inhibited by Zn^{2+} ion with an apparent K_i of 30 μM. Zn^{2+} blocks NADPH-dependent reduction of heme iron in nNOS and also blocks the calmodulin-dependent superoxide-mediated cytochrome *c* reductase activity exhibited by nNOS. However, Zn^{2+} ion has no apparent effect on the calmodulin-independent direct reduction of cytochrome *c* by nNOS. Zn^{2+} ion induces perturbation difference spectra in nNOS characterized by the appearance of a peak at ~430 nm and a trough at ~395 nm, with an apparent spectral binding constant of 50 μM. These spectral changes are consistent with a Zn^{2+}-dependent change in the spin-state equilibrium of the heme iron in nNOS. The spectral binding constant for L-arginine binding to nNOS (~1.5 μM) is not significantly affected by the presence of 50 μM Zn^{2+}, indicating that Zn^{2+}-dependent inhibition of nNOS activity is not due to interference with substrate binding. The estimated maximal change in nNOS absorbance at ~418 nm caused by the L-arginine-dependent conversion of the ferric heme iron from hexacoordinate low-spin to pentacoordinate high-spin is increased by 50% in the presence of 50 μM Zn^{2+}, which reflects the increased initial amount of low-spin ferric heme iron present. These data indicate that Zn^{2+}-dependent inhibition of nNOS activity is due to binding of Zn^{2+} to the hemoprotein domain in the enzyme and that inhibition is associated with perturbations in the environment of the heme iron that appear to block its ability to mediate oxygen reduction.

It is now well established that NO gas produced by the nitric oxide synthases has a variety of functions throughout the body including those of diffusible second messenger, neurotransmitter, and cytotoxic agent (Moncada, 1991; Nathan, 1992). The nitric oxide synthases catalyze production of NO from L-arginine by a mechanism that, while incompletely understood, appears to involve a monooxygenase reaction similar those catalyzed by members of the P-450 enzyme family (Marletta, 1994). An interesting feature of nitric oxide synthase is that in it a heme-binding oxygenation domain and a flavin-binding reductase domain are joined in a single polypeptide chain (Bredt et al., 1991). Among the P-450 enzymes, only P-450 BM-3 has this characteristic, with the rest depending for activity on the presence of an additional P-450 reductase enzyme (Narhi & Fulco, 1986). The primary structure of the reductase domain in nitric oxide synthase contains the consensus flavin- and NADPH-binding sequences seen in the reductase domain of P-450 BM-3 and in P-450 reductase (Bredt et al., 1991; Li et al., 1991). The nitric oxide synthases are known to bind 1 mole of FAD and FMN per mole of enzyme (Mayer et al., 1991; Stuehr et al., 1991; McMillan et al., 1992). Not surprisingly, neural nitric oxide synthase (nNOS)[1] is able to reduce cytochrome *c* in analogy with cytochrome P-450 reductase (Klatt et al., 1992a; Sheta et al., 1994). While the primary structure of the heme-binding domain in P-450 BM-3 clearly places it in the P-450 family, the heme-binding region in nitric oxide synthase contains no recognizable sequence similarity with this protein family (Bredt et al., 1991; Li et al., 1991). Resonance Raman spectroscopy of nitric oxide synthase indicates that its heme-binding pocket is structurally similar to those found in the P-450 enzymes, including the presence of a proximal thiolate axial ligand, which occurs in P-450 enzymes, including P-450 BM-3 (Ravichandran et al., 1993; Wang et al., 1993). Like the P-450 enzymes, the nitric oxide synthases bind 1 mole of heme per mole of protein (Klatt et al., 1992b; McMillan et al., 1992; Stuehr & Ikeda-Saito, 1992; White & Marletta, 1992). An important distinction between nitric oxide synthase and the P-450 enzymes is that the former requires stoichiometric amounts of tetrahydrobiopterin (BH_4) (Mayer et al., 1991; Hevel & Marletta, 1992) which is bound within the hemoprotein domain (McMillan & Masters, 1995). We have demonstrated that nNOS is reversibly inhibited by Zn^{2+} bound with an apparent K_i of 30 μM. Inhibition is accompanied by changes in the nNOS heme absorbance spectra, suggesting that Zn^{2+} perturbs the environment of the heme iron in a manner that blocks heme-mediated oxygenation catalyzed by nNOS. This result is significant in that it demonstrates Zn^{2+} to be a new and useful probe of nNOS function, which has revealed a potentially novel site of enzyme inhibition. In addition, it suggests that the Zn^{2+}

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[1] Abbreviations: nNOS, rat neural nitric oxide synthase; BH_4, tetrahydrobiopterin; CaM, calmodulin.

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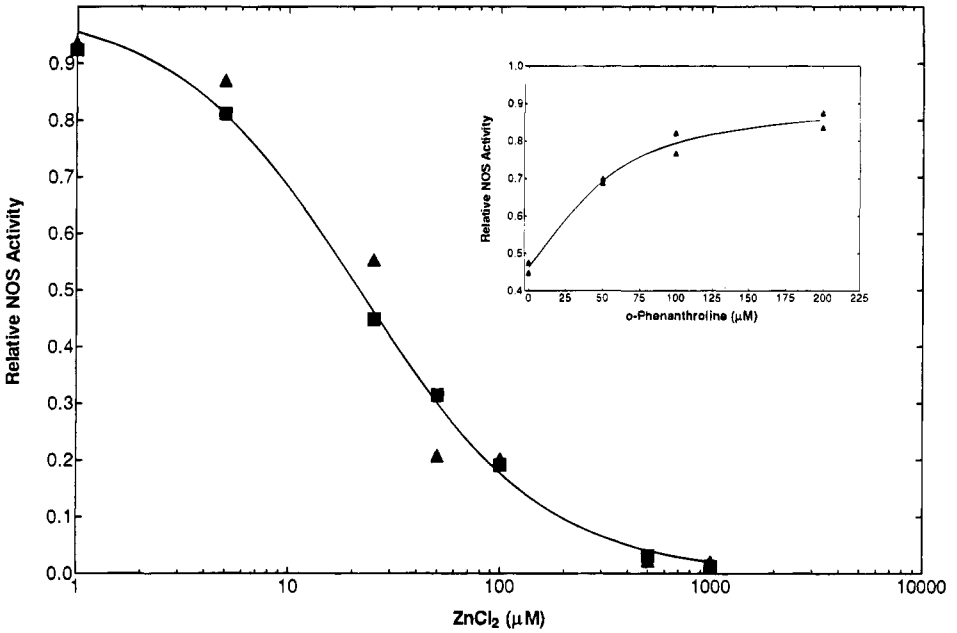

FIGURE 1: Inhibition of nNOS catalytic activity by Zn^{2+}. $ZnCl_2$ was added to give the indicated final concentrations. nNOS and CaM were present at concentrations of 1 and 200 nM, respectively, in all reactions with a $CaCl_2$ concentration of either (■) 200 μM or (▲) 7.5 mM. The curve shown was generated by a nonlinear least squares fit of the data to an equation of the form: $y = 1\text{-}[x/(K_i + x)]$, where y is the observed fractional nNOS activity, x is the concentration of Zn^{2+} ion, and K_i is the apparent dissociation constant for inhibitory Zn^{2+}. Inset: Reversal of Zn^{2+}-dependent inhibition by *o*-phenanthroline. Enzyme reactions containing 30 μM $ZnCl_2$ were incubated for 5 min after initiation of the reaction by addition of tritiated L-arginine. *o*-Phenanthroline was then added from a 10 mM stock in 50% ethanol to give the indicated final concentrations, and the enzyme reaction time course was determined 10 and 20 min after initiation of the reaction in order to establish the rate of catalysis. Catalytic activities are expressed relative to the maximum activity measured in the absence of either $ZnCl_2$ or *o*-phenanthroline. The line drawn through the data points is included for clarity and has no mechanistic significance.

coreleased with glutamate in some glutaminergic pathways in the brain may modulate nNOS activity *in vivo* (Frederickson, 1989; Frederickson & Danscher, 1990).

MATERIALS AND METHODS

Neuronal nitric oxide synthase (nNOS) was purified from human kidney 293 cells stably transfected with a gene encoding the rat brain isoform as described previously (McMillan et al., 1992). nNOS optical absorbance absolute and difference spectra, and nNOS enzyme and cytochrome *c* reductase activities were measured essentially as described previously (McMillan & Masters, 1993; Persechini et al., 1994; Sheta et al., 1994). For use in optical absorbance measurements nNOS was buffer-exchanged (Sephadex G-50) to 50 mM Tris-HCl, 10% glycerol, 0.1 M NaCl, and 1 mM β-mercaptoethanol, at pH 7.5.

Measurements of time-dependent NADPH consumption and heme iron reduction were performed as follows. Reactions were initiated by addition of $CaCl_2$ to a final concentration of 100 μM, to a reaction mixture containing 2 μM nNOS, 2 μM calmodulin (CaM), 100 μM NADPH, 100 μM L-arginine, 50 mM Tris-HCl, and 10% glycerol, at pH 7.5. All reaction mixtures were saturated with respect to CO gas and sealed under a CO atmosphere. Absorbance spectra were collected after the incubation times indicated in Figure 4.

Other experimental details are given in the figure legends.

RESULTS

Data for Zn^{2+} inhibition of nNOS activity are presented in Figure 1. These data are fit reasonably well by a single-site inhibition model with an apparent K_i for Zn^{2+} of 30 μM. Inhibition of nNOS activity was determined in the presence

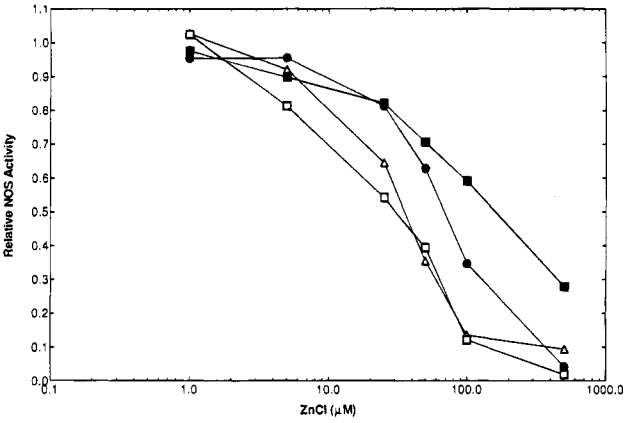

FIGURE 2: Zn^{2+}-dependent inhibition of nNOS activity measured at different NADPH concentrations. Reactions containing 1 nM nNOS and 200 nM CaM were incubated with either (△) 0.2 mM, (□) 1.0 mM, (●) 5 mM, or (■) 25 mM NADPH at the indicated Zn^{2+} concentrations. Rates are expressed relative to the maximum enzyme activity measured in the absence of added Zn^{2+}.

of either 0.2 or 7.5 mM $CaCl_2$; the lack of any significant difference between these two sets of data serves to demonstrate that inhibition does not involve competitive displacement by Zn^{2+} of Ca^{2+} bound to calmodulin. We have not determined the abilities of other divalent cations to inhibit nNOS activity. Since it has been reported that Zn^{2+} can interact with NADPH (Ludwig et al., 1980), we investigated the ability of Zn^{2+} to inhibit nNOS activity measured at a series of different NADPH concentrations (Figure 2). The data show that NADPH at a concentration of up to 1 mM does not significantly affect the concentration of Zn^{2+} needed for half-maximal inhibition. At NADPH concentrations 5 mM or higher the concentration of Zn^{2+} needed for half-

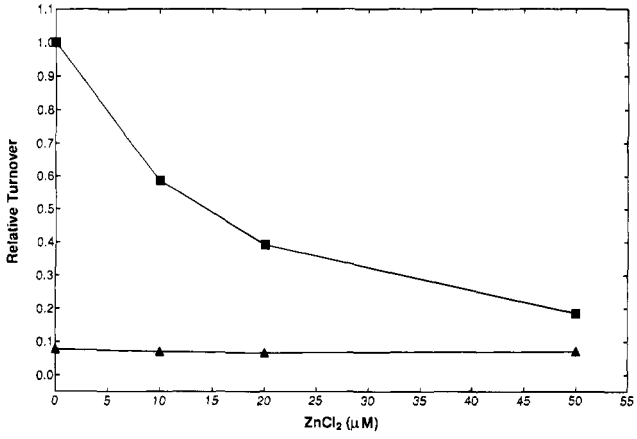

FIGURE 3: Inhibition of CaM-dependent cytochrome *c* reductase activity by Zn^{2+}. Reaction mixtures contained 10 μM $CaCl_2$, 4 nM nNOS, 50 μM cytochrome *c*, and either (▲) no added CaM or (■) 60 nM added CaM. Cytochrome *c* reductase activity is expressed relative to the maximum CaM-dependent activity observed in the absence of added $ZnCl_2$.

maximal inhibition increases and the concentration dependence of the inhibition is no longer fit by a single site model. It is our interpretation that at these higher, nonphysiological, NADPH concentrations some of the added Zn^{2+} is complexed with NADPH. Reversibility of Zn^{2+}-dependent inhibition of nNOS activity was tested by adding *o*-phenanthroline in order to avoid chelation of Ca^{2+} ion, which is required for CaM-dependent activation of this enzyme activity. We found that in the presence of 30 μM $ZnCl_2$ addition of *o*-phenanthroline to a concentration of 200 μM restores nNOS activity to nearly 90% of its maximum level (Figure 1; inset). The lack of complete restoration of activity by *o*-phenanthroline is due to its partial inhibition of nNOS activity (Stuehr & Griffith, 1992; A. Persechini, unpublished data). Data illustrating the effect of Zn^{2+} ion on nNOS-dependent cytochrome *c* reductase activity are presented in Figure 3. Zn^{2+} inhibits significantly only the Ca^{2+}/CaM-dependent fraction of this reductase activity, which is superoxide-mediated (Klatt et al., 1992a; Sheta et al., 1994). Reduction of cytochrome *c* by cytochrome P-450 reductase is unaffected by Zn^{2+} ion at a concentration of 50 μM (data not shown). Although the above data strongly suggest that inhibitory Zn^{2+} concentrations block CaM-dependent oxygen reduction occurring at the heme iron, it was necessary to show this more directly. We therefore monitored NADPH consumption and heme iron reduction in the presence and absence of 100 μM Zn^{2+} (Figure 4). In the absence of Zn^{2+}, initiation of CaM-dependent NO synthase activity is associated with rapid depletion of NADPH, as reflected in the reduction in absorbance at 340 nm. Since these reactions were performed under a CO atmosphere, the occurrence of NADPH-dependent reduction of the heme iron from its ferric to its ferrous state is evidenced by the formation of a ferrous−CO absorbance peak at ~445 nm (Figure 4). In contrast, when 100 μM Zn^{2+} is present, NADPH consumption is greatly reduced and there is no evidence of ferrous−CO formation (Figure 4).

Taken together these data suggest that Zn^{2+}-dependent inhibition of nNOS activity causes blockage of heme-iron-mediated oxygen reduction and catalysis of NO production either by blocking electron flow responsible for heme-iron reduction or by destabilizing the ferrous state of the heme iron.

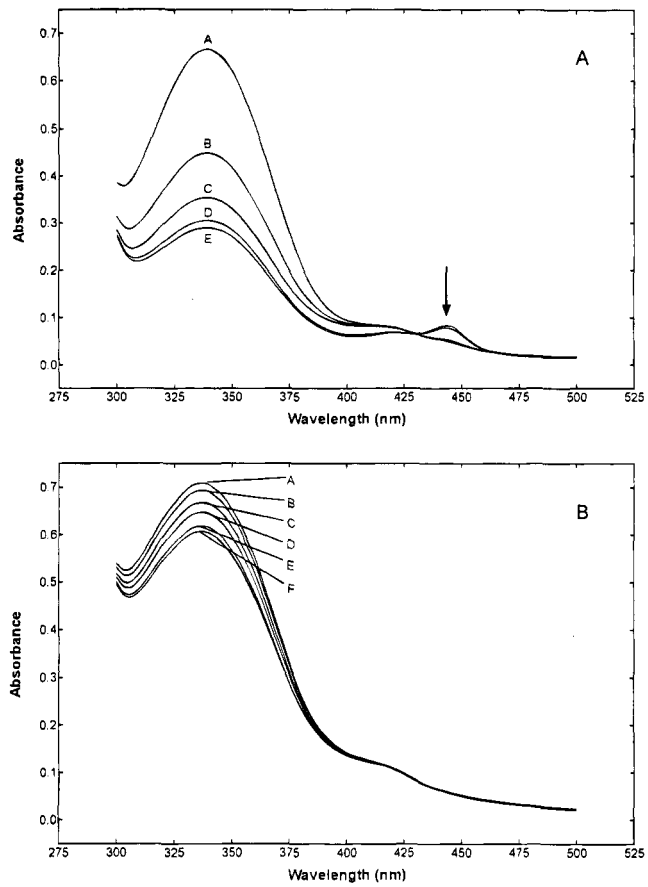

FIGURE 4: The effect of Zn^{2+} on nNOS-dependent NADPH consumption and reduction of nNOS heme iron. (A) Control absorbance spectra measured in the absence of Zn^{2+} at times equal to A, 0; B, 5; C, 10; D, 18; E, 24; and F, 31 min after addition of activating Ca^{2+} ion. (B) Spectra measured in the presence of 100 μM Zn^{2+} at times equal to A, 0; B, 5; C, 10; D, 15; E, 25; and F, 30 min after addition of activating Ca^{2+} ion. All reactions contained 2 μM nNOS, 2 μM CaM, 100 μM NADPH, and 100 μM L-arginine. Rates of NADPH consumption estimated from the change in absorbance at 340 nm were 2 nmol/min in the absence of Zn^{2+} and 0.5 nmol/min in the presence of Zn^{2+}.

In order to assess more fully the interactions between Zn^{2+} and nNOS, we have determined the enzyme plus Zn^{2+} *vs* enzyme difference spectra for nNOS at several Zn^{2+} concentrations and have observed type II difference spectra indicating a Zn^{2+}-dependent decrease in the amount of high-spin heme iron (Figure 5). The apparent spectral binding constant for Zn^{2+} based on these data is 50 μM (Figure 5; inset). The extrapolated maximal spectral shift caused by Zn^{2+} represents ~10% of the total chromophore present (Figure 5). Spectral titration of nNOS with L-arginine in the presence and absence of 50 μM Zn^{2+} gives similar spectral binding constants for L-arginine of 1.5 and 1.2 μM, respectively (Figure 6). This suggests that there is no direct competition between Zn^{2+} and L-arginine binding. In agreement with this conclusion, we find that the levels of nNOS inhibition by 30 μM Zn^{2+} measured at 1.4 and 50 μM L-arginine are not significantly different (data not shown). The extrapolated maximal spectral shifts caused by L-arginine in the presence and absence of 50 μM Zn^{2+} are, respectively, ~15% and ~10% of the total chromophore (Figure 6). Hence, the low-spin heme iron produced by Zn^{2+} addition is shifted back to the high-spin state by L-arginine.

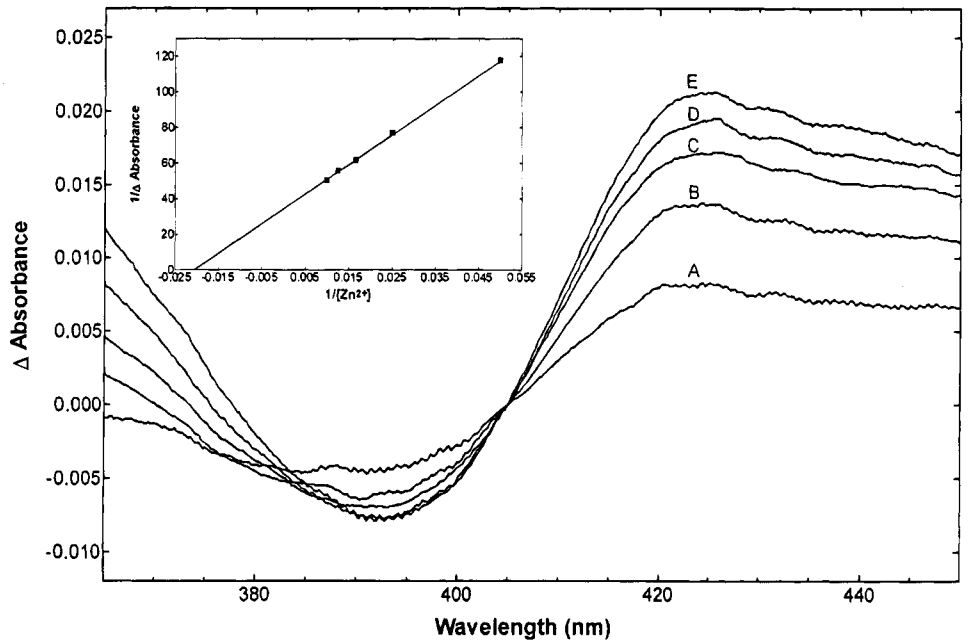

FIGURE 5: Zn^{2+} perturbation difference spectra of nNOS. Difference spectra were determined with 4 μM nNOS in the absence of added CaM at Zn^{2+} concentrations of A, 20 μM; B, 40 μM; C, 60 μM; D, 80 μM; and E, 100 μM. Inset: Double reciprocal plot of ΔO.D.$_{420}$ *vs.* $ZnCl_2$ concentration. The line shown was determined by a nonweighted least-squares fitting. The extrapolated maximal change at 420 nm (0.029 absorbance units) represents spin conversion of the heme iron in ~10% of the enzyme present. The apparent spectral binding constant for Zn^{2+} determined from these data is 50 μM.

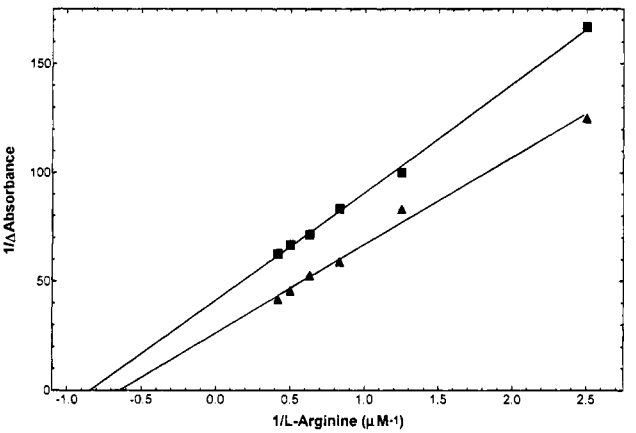

FIGURE 6: Spectral titration of nNOS with L-arginine in the presence (▲) or absence (■) of 50 μM $ZnCl_2$. Data are presented as double reciprocal plots of ΔO.D.$_{418}$ *vs.* L-arginine concentration. The lines shown were determined by nonweighted least squares fitting. The concentration of nNOS used in these experiments was 3.5 μM. CaM was not added, as it has been previously shown not to affect L-arginine binding (McMIllan & Masters, 1993). The extrapolated maximal absorbance changes in the presence and absence of Zn^{2+} are 0.037 (~15% of nNOS) and 0.024 (~10% of nNOS) absorbance units, respectively. The apparent spectral binding constants for L-arginine in the presence and absence of Zn^{2+} are, respectively, 1.2 and 1.5 μM.

DISCUSSION

We have found that Zn^{2+} ion inhibits nNOS enzyme activity more than 90% with an apparent K_i of 30 μM. Data for inhibition are fit by a single-site model suggesting that binding of one Zn^{2+} ion per molecule of nNOS is sufficient for inhibition (Figure 1). Zn^{2+} appears to act solely through interactions with the hemoprotein domain in nNOS as it has *no* effect on the direct cytochrome *c* reductase activity exhibited by nNOS, nor does it affect the reductase activity of cytochrome P-450 reductase. Zn^{2+} inhibits by greater than 90% CaM-dependent superoxide-mediated reduction of cytochrome *c* by nNOS. A direct effect of Zn^{2+} on the heme-binding domain in nNOS is suggested by the Zn^{2+}-dependent type II difference spectra presented in Figure 5. It is evident from our results that Zn^{2+} inhibits nNOS activity by acting on the hemoprotein domain in the enzyme so as to block formation of ferrous heme iron, which is required for catalysis and incidental production of superoxide occurring in the absence of the substrate, L-arginine. This could result either from blockage of electron flow to the heme iron or from destabilization of the ferrous state. Clearly, additional spectral and physical studies are necessary to resolve these issues. Nevertheless, we have at this stage demonstrated Zn^{2+} to be a useful probe of nNOS activity which has revealed a novel inhibitory site in nNOS. Zn^{2+}-dependent high-spin to low-spin conversion occurs in the absence of bound L-arginine, and we have shown that Zn^{2+} actually increases the amount of nNOS undergoing substrate-dependent spin conversion due to an increased initial amount of low-spin heme iron (Figure 6). Hence, Zn^{2+} and L-arginine would appear to be acting independently.

Zn^{2+} is known to bind to sulfhydryl groups in proteins, and our current hypothesis is that it inhibits nNOS activity through an interaction with one or more sulfhydryl groups in the vicinity of the heme ligand. Recent investigations of endothelial nitric oxide synthase indicate that Cys-99 and Cys-184 are both required for enzyme activity, although it is Cys-184 that appears to function in the proximal coordination of the heme iron (Chen et al., 1994). Using site-directed mutagenesis, Cys-415, which is homologous to Cys-184 in endothelial nitric oxide synthase, has been replaced by histidine in the expressed nNOS hemoprotein domain (McMillan & Masters, 1995). The resultant mutant protein exhibits the optical absorbance properties expected of a hemoprotein with an imidazole proximal heme iron ligand, thereby confirming that Cys-415 in nNOS contributes the proximal thiolate axial heme iron ligand. A recent report

indicates that Cys-99 in the endothelial enzyme is involved in the binding and action of BH_4 (Chen et al., 1995). Perhaps Cys-331 in nNOS, which is homologous to Cys-99 in the endothelial enzyme, plays a similar role. In support of this idea, electron paramagnetic resonance (EPR) spectroscopy of the isolated hemoprotein domain in the absence of bound tetrahydrobiopterin indicates the presence of a bis-thiolate complex with a cysteine located in the distal heme pocket (McMillan et al., 1995). This bis-thiolate is abolished by addition of tetrahydrobiopterin or L-arginine (McMillan et al., 1995). Cys-331 may correspond with the cysteine identified in these EPR experiments and may also be the target of inhibitory Zn^{2+} ion. Site-directed mutagenesis experiments to investigate this possibility are ongoing. It is clearly of high priority to conduct investigations aimed at determining directly the amino acid side chains in nNOS interacting with inhibitory Zn^{2+} ion.

An interesting aspect of these results is the finding that Zn^{2+} alters the absorbance spectrum of only about 10% of the nNOS present. Previous studies of nitric oxide synthase indicate that the heme iron in ~90% of purified nNOS is already in the ferric high spin state, leaving only about 10% to undergo a L-arginine-dependent spectral shift (McMillan & Masters, 1993; Wang et al., 1993). EPR spectra determinations indicate that Zn^{2+} ion perturbs the environment of the heme iron in a substantially greater fraction of nNOS than is indicated by optical absorbance measurements (K. McMillan, unpublished observations). The physical basis for the relatively small amounts of nNOS that undergo Zn^{2+}- and/or L-arginine-dependent optical absorbance spectral shifts is currently under investigation in this and other laboratories.

Stuehr and Griffith (1992) have reported that inducible nitric oxide synthase in macrophages is better than 90% inhibited by 100 μM Zn^{2+}; we have recently confirmed this using inducible nitric oxide synthase expressed in A293 human kidney cells (A. Persechini, unpublished results). Jeffery (1983) has previously reported that Zn^{2+} at micromolar concentrations substantially inhibits drug metabolism by rat liver microsomal preparations. This investigator noted that the absorbance spectrum of oxidized cytochrome P-450 was not affected by an inhibitory Zn^{2+} concentration, while the reduced enzyme *vs.* reduced enzyme plus CO spectrum was perturbed by the addition of Zn^{2+}. It is therefore possible that Zn^{2+} inhibits the nitric oxide synthases and some P-450 enzymes by similar mechanisms.

Nitric oxide synthase is present in large amounts in the brain, where it catalyzes the production of NO, which appears to act as a retrograde neurotransmitter, a cytotoxic agent, and a modulator of glutaminergic neurotransmission (Peters et al., 1987; Frederickson, 1989; Wu et al., 1993). It has been demonstrated that Zn^{2+} ion is colocalized with glutamate in presynaptic vesicles and is coreleased with this neurotransmitter into the synaptic cleft (Frederickson, 1989; Frederickson & Danscher, 1990). Zn^{2+} at micromolar concentrations has been demonstrated to have both pre- and postsynaptic inhibitory effects on neurotransmission, which appear to involve Zn^{2+}-dependent inhibition of several ion channel activities, including Ca^{2+} channel activity (Peters et al., 1987; Wu et al., 1993). It has also been demonstrated that glutamate-induced neurotoxicity is attenuated by Zn^{2+} at micromolar concentrations (Koh & Choi, 1988) and activation of nNOS present in glutaminergic pathways has recently been implicated in glutamate-induced neurotoxicity (Dawson & Synder, 1994). The results we present here raise the interesting possibility that, in addition to its effects on pre- and postsynaptic ion channel activities, Zn^{2+} may also directly modulate nNOS activity *in vivo*.

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