Role of Bound Zinc in Dimer Stabilization but Not Enzyme Activity of Neuronal Nitric-oxide Synthase*

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Nitrergic-oxide synthases (NOS) are homodimeric proteins and can form an intersubunit Zn(4S) cluster. We have measured zinc bound to NOS purified from pig brain (0.6 mol/mol of NOS) and baculovirus-expressed rat neuronal NOS (nNOS) (0.49 ± 0.13 mol/mol of NOS), by on-line gel-filtration/inductively coupled plasma mass spectrometry. Cobalt, manganese, molybdenum, nickel, and vanadium were all undetectable. Baculovirus-expressed nNOS also bound up to 2.00 ± 0.58 mol of copper/mol of NOS. Diethyleneetriaminepentaacetic acid (DTPA) reduced the bound zinc to 0.28 ± 0.07 and the copper to 0.97 ± 0.24 mol/mol of NOS. Desalting of samples into thiol-free buffer did not affect the zinc content but completely eliminated the bound copper (≤0.02 mol/mol of NOS). Most (≥75%) of the bound zinc was released from baculovirus-expressed rat nNOS by p-chloromercuribenzenesulfonic acid (PMPS). PMPS-treated nNOS was strongly (90 ± 5%) inactivated. To isolate functional effects of zinc release from other effects of PMPS, PMPS-substituted thiols were unblocked by excess reduced thiol in the presence of DTPA, which hindered reincorporation of zinc. The resulting enzyme contained 0.12 ± 0.05 mol of zinc but had a specific activity of 462 ± 46 nmol of citrulline.min⁻¹.mg⁻¹, corresponding to 93 ± 10% of non-PMPS-treated controls. PMPS also caused dissociation of nNOS dimers under native conditions, an effect that was blocked by the pteridine cofactor tetrahydrobiopterin (H₄biopterin). H₄biopterin did not affect zinc release. Even in the presence of H₄biopterin, PMPS prevented conversion of NOS dimers to an SDS-resistant form. We conclude that zinc binding is a prerequisite for formation of SDS-resistant NOS dimers but is not essential for catalysis.

Nitrergic-oxide synthase (NOS) catalyzes the synthesis of the physiologically effector molecule nitric oxide (NO) from l-argi-

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‡ The abbreviations used are: NOS, nitrergic-oxide synthase; nNOS, neuronal NOS; eNOS, endothelial NOS; iNOS, inducible NOS; ICP-MS, inductively coupled plasma-mass spectrometry; PMPS, p-chloromercurobenzenesulfonate; NTA, nitricotinamide; PAR, 4-(2-pyridylazo)-resorcinol; H₄biopterin, (5,6,7,8-tetrahydro-L-biopterin) (6-1,2-dihydroxypropyl)pteridine; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate; DTPA, diethyleneetriaminepentaacetic acid; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; cps, counts per second.

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per dimer and could be reactivated by preincubation with L-Arg (16). Thus, an intact zinc site may protect NOS against inactivation without being essential for activity.

If the zinc is not necessary for NOS activity, it may not be incorporated into NOS under all physiological circumstances. Because of this, and because of a claim of non-heme iron involvement in NOS catalysis (17), several groups have re-examined the metal content of NOS. For zinc, values around 0.35–0.5 mol per subunit have been found in human iNOS expressed in Escherichia coli, in nNOS and eNOS expressed in E. coli (16, 18), and in human eNOS expressed in yeast (19).

In the present study, we aimed first to examine nNOS from both a baculovirus expression system and natural tissue, for bound zinc. Second, we wanted to study its contribution to structure and function of wild-type rather than mutant NOS; if it is inessential, we reasoned, it might be possible to remove it without destroying the enzyme activity. Finally, because such an intersubunit connection would be expected to contribute to dimer stability, we wanted to check whether, and how, removal of the zinc would affect the dimerization of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials—**L-[2,3,4,5,5′H]Arginine hydrochloride (45 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc., St. Louis, MO, and further purified by HPLC as described earlier (20). H2O2 was obtained from B. Schircks Laboratories, Jona, Switzerland. Myoglobin (product as described previously (22). Because the normal enzyme storage buffer was made in new single-use plastic vessels with water ADP-Sepharose and calmodulin-Sepharose were from Amersham Pharmacia Biotech (Uppsala, Sweden). 4-(2-pyridylazo)resorcinol (PAR) was from Fluka, Vienna, Austria. 2′,5′-ADP-Sepharose and calmodulin-Sepharose were from Amersham Pharmacia Biotech, Vienna, Austria. Solutions for experiments involving zinc release were made in new single-use plastic vessels, and with water prepared by glass distillation followed by passage through a milliQ apparatus (Millipore, Billerica, Massachusetts).

**NOS Expression and Purification—**nNOS from pig cerebellum was isolated according to Mayer et al. (21). Rat nNOS was expressed in baculovirus-infected Sf9 cells and purified by sequential affinity chromatography on 2′,5′-ADP-Sepharose and then calmodulin-Sepharose, as described previously (22). Because the normal enzyme storage buffer contained 12 mM 2-mercaptoethanol and 2 mM EGTA, NOS samples for experiments involving zinc release were stripped of these compounds by gel filtration. NOS samples (3–5 mg) with phenylmethylsulfonyl fluoride (1 mM) and hen egg white trypsin inhibitor (1 mg/ml) were concentrated to about 15 mg/ml in a dialysis tube (Spectrapor, 8-mm flat width tube, 30,000 Da) placed in solid sucrose, before injection into the GFC1300 column (30 cm × 7.5 mm; Supelco) run at 0.5 ml/min in 0.05 M Tris-HCl, pH 7.4, containing 0.2 M NaCl. We sometimes (as specified below) injected samples of DTPA (90 μM, 2 mM) to purge metal ions from the chromatography system. Photometric Assay for Zinc Release—Release of zinc from thiol-free NOS was monitored using the absorbance change of the metal-binding dye PAR at 500 nm (24, 25). PAR was added to 200-μM samples to give a final concentration of 100 μM. The response was calibrated from 0 to 10 μM using solutions of zinc and/or copper acetate in the same buffer previously used for nNOS (0.05 M Tris-HCl, pH 7.4, containing 0.15 mM NaCl). The slope of the absorbance increase was 76 nm/M for zinc and 43 μM/M for copper. The chelator NTB, when added to a final concentration of 2 mM, was found to completely abolish the response to zinc, whereas the signal for copper was only reduced to 33 μM/M.

The PAR reaction was used to measure release of zinc from NOS on treatment with PMPS. PAR was added to samples of NOS (3–5 μM), and the absorbance spectrum was recorded. After adding 4 μM of DTPA (final concentration 0.16 mM), the absorbance at 500 nm was measured at intervals of 10 s for 200 s.

**ICP-MS Analysis and NOS Assay of Zinc-depleted NOS—**Samples of NOS (5 μM; 400 μl), kept on ice, were treated with the following combinations of DTPA (0.15 mM), DTPA (2 mM), and l-cysteine (10 mM). To a 500-μl sample, a total of 10 μM DTPA and 20 μM l-cysteine were added, followed 1 min later by DTPA (2 mM) and 2 min of further incubation. To a third sample (PMPS/cysteine), PMPS was added, followed 1 min later by DTPA and after a further 2 min by cysteine. Immediately after these incubations, three 110-μl portions of each sample were frozen in liquid N2 for ICP-MS analysis. A further 10-μl portion was taken from each sample for NOS assays. Of each 110-μl sample for ICP-MS, 90 μl was injected onto the SigmaChrom GFC 1300 column. Samples were injected in the following order: first, two samples of 2 mM DTPA, then standards in the range 0–10 μM, then 2 × 2 mM DTPA, then two injections of 2 mM DTPA plus 0.3 mM PMPS, and then the three PMPS-only replicate samples. Next, the system was re-equilibrated for 1 h in buffer containing 10 mM l-cysteine followed by 2 × 2 mM DTPA, and the control samples were applied. A single injection of DTPA was made between these and the PMPS/cysteine samples.

For NOS assays, the 10-μl samples were diluted 10-fold in 50 mM triethanolamine-HCl, pH 7.4, containing 1 mM CHAPS and 0.8 mM DTPA, supplemented for the control and PMPS/cysteine samples, but not for the PMPS-only samples. 10 μl of the diluted enzyme stock was added to 80 μl of assay mixture, containing (final concentrations) 100 μM [3H]-L-Arg (−50,000 cpm), 10 μM l-cysteine, 400 μM H2O2, 5 mM FMN, 200 μM NADPH, 1.5 mM CaCl2, and 50 mM triethanolamine-HCl, pH 7.0. Reactions were done at 37°C for 5 min, then stopped by adding 1 ml of chilled 20 mM sodium acetate, pH 5.0, containing 2 mM EDTA and 1 mM l-cysteine. [3H]-L-Citrulline was separated from [3H]-L-Arg on Dowex-50 columns and measured by scintillation counting as described before (26).

**Gel Filtration—**NOS dimerization was analyzed by gel filtration with a Superose 6 HR 10/30 column on an AKTA chromatography apparatus, at 10°C. The flow rate was set to 0.3 ml/min and the buffer used was 0.05 M Tris-HCl, pH 7.4, containing 0.2 mM NADPH, 1.5 mM NADH, and [3H]-L-Arg (500,000 cpm), 10 μM [3H]-l-Arg (−50,000 cpm), 10 μM l-cysteine, 400 μM H2O2, 5 mM FMN, 200 μM NADPH, 1.5 mM CaCl2, and 50 mM triethanolamine-HCl, pH 7.0. Reactions were done at 37°C for 5 min, then stopped by adding 1 ml of chilled 20 mM sodium acetate, pH 5.0, containing 2 mM EDTA and 1 mM l-cysteine. [3H]-L-Citrulline was separated from [3H]-L-Arg on Dowex-50 columns and measured by scintillation counting as described before (26).

**Gel Electrophoresis—**Formation of SDS-resistant NOS dimers was investigated using the low temperature PAGE method of Klatz et al. (8).

Enzyme samples were preincubated in a total volume of 70 μl at an NOS concentration of 1.6 μM in 50 mM Tris-HCl, pH 7.4. Some of the samples were preincubated with H2O2 (0.2 mM) for 20 min on ice; of these, some were further treated with PMPS (0.15 mM) on ice for 3 min before addition of substrate. For each sample from 70 μl, two portions of 32 μl each were taken and mixed with 8 μl of 5-fold Laemmli buffer, with (“reducing gel”) or without (“non-reducing gel”) 2-mercaptoethanol. The other components of the 5-fold sample buffer were 0.32 M Tris-HCl (pH 6.8), 0.5% glycine, 10% SDS, 50% glycerol, and 0.03% bromphenol blue (27). Samples containing 8 μg of NOS were subjected to SDS-PAGE on 5.5% SDS gels, using the Mini Protean II system from
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FIG. 1. Online gel filtration/ICP-MS of nNOS. Proteins were chromatographed on a Superose 6 HR column with on-line UV and ICP-MS detection, as described under “Experimental Procedures.” A, baculovirus-expressed rat nNOS; B, pig brain NOS. The ICP-MS signals have been offset for clarity. The ratio of the left and right y axis scales is the same in both panels. For baculovirus-expressed rat nNOS, the content of these metals was calculated based on the amount of protein injected, determined by the Bradford assay. The pig brain NOS sample contained a significant protein contaminant smaller than NOS, so that the total protein concentration of these samples could not be used as a basis for calculating its metal content; instead, this was estimated relative to the peak area of the 280-nm trace, assuming the same extinction coefficient as for baculovirus-expressed enzyme.

Bio-Rad (Vienna, Austria). Gels and buffers were equilibrated at 4 °C, and the buffer tank was cooled during electrophoresis in an ice bath. Proteins were visualized by Coomassie Brilliant Blue R staining.

RESULTS

Zinc Content of NOS—Metal content of NOS samples was measured initially by on-line gel-filtration/ICP-MS with a Superose 6 column. Iron, zinc, and copper were detected in rat nNOS expressed in baculovirus-infected Sf9 cells and in NOS isolated from pig brain (Fig. 1). The samples were also monitored for cobalt, manganese, molybdenum, nickel, and vanadium, but none of these metals were detected. The pig brain NOS contained 0.60 mol of zinc and 0.35 mol of copper/mol of NOS; the baculovirus-expressed rat nNOS contained 0.49 ± 0.02 mol of zinc released per NOS subunit (mean ± S.E. of three preparations). We also measured release of zinc from NOS by PMPS using on-line gel-filtration/ICP-MS. Three-quarters of the bound zinc was released (from 0.28 ± 0.03 to 0.07 ± 0.01 mol of zinc/mol of NOS; means ± S.E. of three preparations). Activity of Zinc-depleted NOS—Thiol-free nNOS had a specific activity of 460 ± 20 nmol of citrulline·mg⁻¹·min⁻¹. PMPS-treated nNOS was strongly inactivated (residual activity 47 ± 22 nmol of citrulline·mg⁻¹·min⁻¹) and was nearly zinc-free (Fig. 3B). When DTPA was present during the reaction with PMPS and the enzyme was subsequently reduced by l-cysteine, reincorporation of zinc could be limited to 43 ± 16% of the original value (Fig. 3C), or 0.12 ± 0.05 mol per NOS subunit. The reduced enzyme regained 93 ± 10% (426 ± 46 nmol of citrulline·mg⁻¹·min⁻¹) of the activity of non-PMPS-treated controls.

Effects of PMPS on Dimerization of nNOS—We examined the effects of PMPS on dimerization of nNOS by gel filtration. Thiol-free nNOS treated with PMPS underwent substantial dissociation, from 83 ± 3% to 40 ± 3% dimer (means ± S.E. of three preparations) (Fig. 4, A and B). The monomers generated by PMPS treatment exhibited a peak in the 398-nm trace (dotted line in Fig. 4B). Addition of l-cysteine to the PMPS-oxidized enzyme resulted in a significant reversal of the dissociation, reaching a final value of 50 ± 4% dimer. l-Cysteine alone was found to cause a slight dissociation (to 65 ± 1% dimer).

Because H₄biopterin has been shown to stabilize NOS dimers, this experiment was repeated with nNOS that had been preincubated with a saturating concentration of H₄biopterin before adding PMPS, and using an elution buffer supplemented with H₄biopterin (Fig. 4, C and D). Under these conditions, PMPS treatment caused no significant dissociation (81 ± 3% to 79 ± 3% dimer). In the PAR assay, preincubation of thiol-free nNOS with H₄biopterin did not affect the extent of zinc release. However, preincubation with H₄biopterin did not prevent inactivation of the enzyme (to 7.6 ± 3.6% of controls; mean ± S.E. of three preparations).

Redox and PMPS Effects on Formation of SDS-resistant NOS Dimer—The ability of thiol-free nNOS to form SDS-resistant dimers was examined by low temperature SDS-PAGE (Fig. 5). Samples that had been preincubated with or without H₄biopterin and/or PMPS were split and subjected to electrophoresis under reducing (5% 2-mercaptoethanol (0.6 M) in the sample buffer) or non-reducing (thiol-free sample buffer) con-

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**TABLE I**

| Metal per NOS subunit | Iron | Zinc | Copper |
|-----------------------|------|------|--------|
| Treatment             | mol  | mol  | mol    |
| No chelators added    | 0.79 ± 0.07 | 0.50 ± 0.11 | 2.00 ± 0.58 |
| Column purged with DTPA | 1.01 ± 0.19 | 0.50 ± 0.04 | 1.23 ± 0.08 |
| DTPA added to samples | ND* | 0.28 ± 0.07 | 0.97 ± 0.24 |
| DTPA added to thiol-free samples | 0.76 ± 0.11 | 0.28 ± 0.03 | 0.02 |

* ND, not determined.
conditions. On reducing gels, control samples to which no additions had been made contained a small proportion of SDS-resistant dimer (8.2 ± 3% (mean ± S.E. of three preparations)), which increased significantly in the samples preincubated with H$_4$biopterin (to 38 ± 5%). This is the familiar behavior observed in earlier studies (8). The same samples applied to non-reducing gels exhibited the same pattern, except that the basal formation of SDS-resistant dimer without added H$_4$biopterin was more pronounced (20 ± 2%), rising to 38 ± 2% on preincubation with H$_4$biopterin.

Essentially the same behavior was observed for thiol-free nNOS. On reducing gels, H$_4$biopterin increased the fraction of dimer present from 8.4 ± 2% to 41 ± 2%, and on non-reducing gels from 9.6 ± 5% to 23 ± 7%. However, on the non-reducing gels an additional band was observed above the “normal” SDS-resistant dimer. This species was resistant to heating at 95 °C (“boiled” sample on non-reducing gel) but disappeared when the same samples were electrophoresed in the presence of 2-mercaptoethanol.

When thiol-free nNOS that had been preincubated with H$_4$biopterin was treated with PMPS, the “normal” SDS-resistant dimer band disappeared completely. The additional band of lower mobility was not affected. When a PMPS-treated sample was applied to a reducing gel, the SDS-resistant dimer content recovered to 23 ± 9%. “Normal” nNOS, which already contained 12 mM 2-mercaptoethanol, was not affected by PMPS. Thus, as observed under native conditions, the effect of PMPS on SDS-resistant dimerization was antagonized by excess thiol.

**DISCUSSION**

Occurrence of Bound Zinc in nNOS—NO synthase has long been known to contain iron (28–30); interest in other metals has been more recent. Several groups have found zinc in NOS (as isolated, in eNOS or nNOS (3, 16, 19) or inserted in vitro into iNOS (11, 12, 31)). The present results show that zinc is bound to baculovirus-expressed rat nNOS and, for the first time, NOS isolated from a natural tissue (pig brain).

The amount of zinc bound to NOS was substantially reduced by flushing the chromatography system with DTPA and adding DTPA to the NOS samples. The latter condition being more stringent, we suppose that the lower zinc values most accurately represent the zinc bound to high affinity sites such as that linking the subunits. The results obtained without this precaution suggest that NOS (unsurprisingly for a large protein) can also bind metals adventitiously. A comparison of Fig. 1 with Fig. 3 also suggests that the more stringent conditions removed nearly all metal from the NOS monomers (the sample in Fig. 3A contained about 30% monomer). The Fe-Zn ratio using the more stringent method was 2.7:1, suggesting that three-quarters of the intact nNOS dimers with bound heme also contained zinc.
Similarly, the large and variable amount of copper found in our initial experiments may well have resulted from the NOS picking up copper on its way through the chromatography system. Use of DTPA gave a value close to 1 mol/mol. Exposure of the NOS to slightly oxidizing conditions (removal of 2-mercaptoethanol) caused complete loss of copper. Perry and Marletta (17) also found close to 1 mol of copper/mol of NOS, which was easily removed.

**Release of Zinc by PMPS**—PMPS is a mercurial reagent that can reversibly oxidize thiol groups to mercaptides, in the process releasing metals bound to the thiols (24, 25, 32). The amounts of zinc it released from NOS, as detected by the PAR assay (−0.5 mol/mol of NOS), were greater than those detected by gel filtration/ICP-MS of thiol-free NOS samples (−0.3 mol/mol of NOS). A component of the PAR signal seems to be zinc that is not tightly bound to NOS and separates from the enzyme on the column. Thus caution is needed in interpreting the PAR results in terms of occupancy of the intersubunit site.

When we used NTA to mask the zinc signal in the PAR assay, we never detected any release of copper, agreeing with the ICP-MS result that the copper was completely lost from the thiol-free enzyme.

**Zinc Depletion and NOS Activity**—The inactivation of the enzyme by PMPS was not surprising, because the stabilizing effect of reduced thiols on NOS activity, most easily explained by an essential role of one or more reduced cysteines, is well known (33–35). NOS contains several cysteines besides the zinc ligands, so that the inactivation is not necessarily related to the zinc site. Having found that excess reduced thiol restored virtually full activity to the PMPS-modified enzyme, we looked for conditions that would keep the zinc out of its binding site despite the reversal of the PMPS modification. We found that the presence of DTPA was sufficient to limit the zinc binding to the re-reduced enzyme to 43 ± 16% of the starting value. The resulting enzyme regained 93 ± 10% of its original activity (before addition of PMPS), reaching a specific activity of over 10% of its original activity (18 M).

The samples used for enzyme assays were diluted from those used for ICP-MS, and it could be objected that this might tilt the conditions toward greater zinc binding in the NOS assay than was measured by ICP-MS. To exclude this, we raised the molar ratio of DTPA to NOS from 400:1 in the samples analyzed for zinc to 2000:1 in the diluted stocks and the enzyme assays. DTPA is compatible with the NOS assay because its affinity for Zn²⁺ is about 10⁸-fold higher than for Ca²⁺ (36). Thus we could provide sufficient free Ca²⁺ to support NOS activity without impairing the ability of the DTPA to bind zinc. We also used the highest practicable NOS concentration (~0.1 µM) in the assays, to maximize the ratio of NOS to possibly available zinc.

The only previous experiment to test whether zinc is needed for NOS activity was the reactivation of the zinc-deficient C331A mutant by extended preincubation with L-Arg, reported by Miller et al. (16). In that case, the zinc content of the enzyme was, apparently, only determined before the preincubation, and no evidence was shown to exclude that zinc bound to the enzyme during the preincubation and assay. It is difficult to reduce background zinc to levels below the usual enzyme concentrations used in NOS assays (typically 5–20 nM). Thus the interpretation of this result as evidence against an essential role of zinc seems to rest on the assumption that the mutant enzyme would not bind zinc. Although this seems fairly plausible, the affinity of the wild-type zinc site is such that it can compete partially with a 400-fold excess of DTPA (the dissociation constant for the DTPA-zinc complex is around 10⁻¹⁸ M), and even the mutated site with only two ligands might still have considerable affinity. Thus we find that our use of a protein-chemical approach with the wild-type enzyme is an important complement to this result.

**Effects of PMPS on Dimerization of NOS**—In parallel with the release of zinc, PMPS caused substantial dissociation of the NOS dimer, which could be reversed by adding reduced thiol. We have not shown definitively that this effect is due to the disruption and reconstitution of the zinc site. However, it is not obvious what other kind of dimer-stabilizing interaction except for a metal site could depend on keeping a protein thiol in a reduced state. In NOS there is one other such site, namely, the proximal cysteine thiolate ligand of the heme. PMPS did not affect the intensity of the heme Soret band (data not shown), nor did it cause any significant loss of iron (measured by ICP-MS). Zinc release thus appears to be the most likely explanation for PMPS effects on dimerization.

The PMPS-induced dissociation is unusual for nNOS in two respects. First, the monomers formed in this fashion clearly retain some heme. Before now, we generally found that monomers of nNOS were heme-free, and that heme binding implied dimerization (20, 37). Second, the dissociation was prevented by H₄biopterin. Previously, we have not observed large effects of H₂biopterin on the net dimerization of nNOS, rather, the predominant effect was a stabilization of already existing dimer. In contrast, for iNOS both heme-containing monomers and pteridine-dependent conversion of monomers to dimers are well documented (9, 38–40). Interestingly, iNOS, as isolated by Perry and Marletta (17) also found close to 1 mol of copper/mol of NOS, which was easily removed.

![Scheme 1. Summary of NOS dimerization.](image-url)
to depend on pteridine binding (8, 19, 41, 42). The present result suggests that, in addition to bound H$_4$biopterin, an intact zinc bridge between the subunits is essential to make the dimer resistant to dissociation by SDS. Indeed, this type of linkage would fulfill exactly the known characteristics of the dimer observed in low temperature SDS-PAGE, namely, resistance to SDS (the zinc effectively forms a covalent bridge) and to 2-mercaptoethanol (which would disrupt disulfide linkages, but stabilize the zinc site by keeping the cysteines reduced). Why should such a structure require additional stabilization through H$_4$biopterin binding? The key might be an effect of H$_4$biopterin on the degree of exposure of the zinc site to solvent. Simply by excluding solvent from its own binding site, H$_4$biopterin might significantly protect the zinc site from oxidants or from potential chelators. This could reconcile the observed dependence of SDS resistance on H$_4$biopterin with the finding that pteridine binding does not involve large conformational changes of the protein (3).

We have attempted to integrate these and previous findings on NOS dimerization in Scheme 1. We previously studied heme-dependent dimerization of heme-free nNOS (species I in Scheme 1) (20, 37); based on the present results and on the positive effect of dithiothreitol on heme-reconstituted NOS (11), we now suspect that this process may have been assisted by binding of background zinc. Species III is fully active, native NOS. Removal of thiol from the medium and treatment with PMPS leads to the inactive, PMPS-substituted enzyme. Inhibitors of thiol (such as PMPS) are permitted to cause zinc release, is an interesting question, particularly in two respects. First, NOS itself is suspected to contribute to oxidative stress in a variety of ischemic or inflammatory states, and thus the release of zinc could offer a last-resort mechanism to destabilize and ultimately inactivate the enzyme. Second, it is tempting to link the lack of bound zinc in nNOS to the fact that this isozyme must routinely operate under relatively oxidizing conditions, for example in activated macrophages.