Zinc Content of Escherichia coli-expressed Constitutive Isoforms of Nitric-oxide Synthase

ENZYMATIC ACTIVITY AND EFFECT OF PTERIN*

(Received for publication, January 22, 1999, and in revised form, March 24, 1999)

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The constitutively expressed isoforms of nitric-oxide synthase (NOS),1 endothelial nitric-oxide synthase (eNOS; NOS III) and neuronal nitric-oxide synthase (nNOS; NOS I) catalyze the NADPH-dependent conversion of L-arginine to L-citrulline with the concomitant formation of nitric oxide (NO) (1–5). NOS isoforms are bidomain global structures in nature, being composed of a flavin-containing C-terminal reductase domain and an N-terminal oxygenase domain (6). The oxygenase domain contains iron protoporphyrin IX (7–10) and binding sites for tetrahydrobioppterin (BH4) and the substrate, L-arginine (11). All of the aforementioned cofactors are required for full enzymatic activity; thus cofactor binding would be expected to alter the enzyme function.

Site-directed mutagenesis studies have indicated that cysteine 99 of human eNOS is involved in BH4 binding to that isoform (12) and mutation of iNOS cysteine 109 leads to diminished BH4 binding (13). Additionally, deletion of the entire CXXXXC motif (Fig. 1) in bovine eNOS causes a dramatic loss of enzyme stability (14). To test the possibility of altered cofactor binding in nNOS, we mutated cysteine 331 (the homologous residue to cysteine 99 of human eNOS) to alanine and have shown that this mutation affects L-arginine binding and reductase-to-heme electron transfer (15, 16). Prolonged incubation of the nNOS C331A mutant with high concentrations of L-arginine is required for substrate binding to this mutant. However, once L-arginine binding is restored, BH4 can then bind, resulting in efficient flavoprotein-to-heme electron transfer. This reactivated mutant, with BH4 and L-arginine bound, possesses the same electron transfer properties and enzymatic activity as wild-type nNOS (16).

In addition to cysteine 331, cysteine 326 of nNOS is also phylogenetically conserved (Fig. 1). Recently, we have obtained x-ray crystallographic data showing the presence of a ZnS4 center in the trypsin-cleaved tetrahedral tetrathiolate zinc (ZnS4) center in the tryptsin-cleaved heme domain of eNOS (17) and also nNOS,2 which is formed by the CXXXXC motifs from two NOS subunits. Because full enzymatic activity of the C331A mutant can be achieved by prolonged incubation with L-arginine (16), we were interested in what role zinc plays in NOS structure/function. Therefore, considering that mutations within the CXXXXC motif should disrupt the ZnS4 center, we have determined the amount of zinc bound to wild-type forms of nNOS and eNOS, as well as to various constructs of each and the C331A-mutated nNOS. Furthermore, we have investigated the time course required for L-arginine activation of the C331A-mutated nNOS.

EXPERIMENTAL PROCEDURES

Chemicals—4-(2-Pyridylazo)resorcinol disodium salt (PAR), Trizma Base, nitriilotriacetic acid (NTA), EDTA, L-arginine, and 2-mercaptoethanol were obtained from Sigma (St. Louis, MO). Sodium chloride and glycerol were purchased from EM Science (Gibbstown, NJ). Chelex® 100 resin was purchased from Bio-Rad. Yeast extract and tryptone were from Difco Labs (Detroit, MI). Zinc chloride was from Aldrich. Guanidine HCl was from ICN Biomedicals (Aurora, OH).

nNOS, eNOS, and Constructs—The nNOS and the C331A-mutated nNOS were prepared as described by Roman et al. (18) and Martásek et al. (16), respectively, and portions of the preparations were incubated overnight either in the presence of 10 mM L-arginine or 10 mM L-arginine and 0.5 mM BH4, after affinity chromatography and before FPLC purification. Wild-type eNOS was prepared as described by Martásek et al. (19). Enzymes and constructs were purified by FPLC in Chelex® 100-treated buffer consisting of 50 mM Tris, 100 mM NaCl, 1 mM 2-mercaptoethanol, 0.5 mM L-arginine, and 10% glycerol, pH 7.8.

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Printed in U.S.A.
ETV-ICP-MS Analysis—All labware used for handling samples for metal determination was soaked overnight in 4 mM nitric acid and rinsed with Chelex®-treated water before use. Enzymes were purified by FPLC in EDTA-free Chelex®-treated buffer, concentrated, and subsequently passed over a 10-cm column of Chelex®-100 and reconcentrated. ETV-ICP-MS analysis was performed by Delony Langer and Dr. James Holcombe (Department of Chemistry and Biochemistry, University of Texas at Austin). Standard deviations were calculated from the uncertainties in the slopes of the calibration curves. All results were derived from three replicates, plotted, and analyzed using a least squares fit. Metal concentrations were corrected for the metal contribution of the buffer, which, as a percentage of signal, was 29% for zinc and 18% for copper.

Chelation Assay—Zinc content of the constitutive NOS isoforms and constructs was also measured by the PAR assay essentially as described by Crow et al. (20) with modifications. PAR has a low absorbance at 500 nm in the absence of Zn$^{2+}$ (Fig. 2). However, in the presence of Zn$^{2+}$, the absorbance at 500 nm increases dramatically as the PAR$\cdot$Zn$^{2+}$ complex is formed. Enzyme (1 nmol in a volume of 20 μl) was added to a rapidly stirred 3-ml cuvette containing 100 μM PAR in Chelex®-100-treated 50 mM Tris, 100 mM NaCl, pH 7.8, and, for some experiments, including Chelex®-100-treated 7 M guanidine HCl. In some assays, oxidants were added to facilitate Zn$^{2+}$ release. Assays were run at 23 °C in rapidly stirred cuvettes, and the total assay volume was 1.5 ml. Near the end of the assay, 1 mM EDTA was added. Under these assay conditions, NTA selectively chelates Zn$^{2+}$ from the PAR$\cdot$Zn$^{2+}$ complex and causes a decrease in absorbance at 500 nm, which is used to quantitate the amount of zinc that has been released from the protein. Following the NTA addition, 1 mM EDTA was added to confirm that no other metal was being chelated in addition to zinc. The extinction coefficient for PAR$\cdot$Zn$^{2+}$ was calculated, using a standard solution of PAR$\cdot$Zn$^{2+}$ to be ε77,700 M$^{-1}$ cm$^{-1}$ at pH 7.8. The results reported in Table I represent the means ± S.D. of three or four determinations except for those obtained with nNOS heme domain (nNOS-wt HD (BH$_4$)).

Activity Assay—NOS activity was measured using the hemoglobin capture assay in 50 mM Tris, 100 mM NaCl, pH 7.8. The rate of methemoglobin formation from oxyhemoglobin was measured as the difference in absorbance change between 401 and 411 nm/min as described by Sheta et al. (6) except that an extinction coefficient of 60 μM$^{-1}$ was used. All spectrophotometric assays were conducted on a Shimadzu 2101-PC dual-beam spectrophotometer.

Protein Determination—The protein concentration for the enzymes and constructs was determined on the basis of heme content by reduced CO difference spectra using an extinction coefficient of 100 nm$^{-1}$ cm$^{-1}$ for a Δε of 444–475 (21). This method of protein determination probably underestimates the actual protein concentration because apoprotein (minus heme) exists in all preparations to some extent. However, the average heme content of the NOS preparations used in this laboratory is ~80%.3

RESULTS AND DISCUSSION

With nNOS-wt holoenzyme, 90% of the zinc (based on 1 zinc atom/2 hemes) was released in the presence of 7 M guanidine HCl and 1 mM H$_2$O$_2$ (Table I). Results of the chelation assay were corroborated by ETV-ICP-MS analysis (Table II) and confirmed that zinc and not copper was bound to nNOS. Likewise, the trypsin-cleaved, dimeric heme domain of nNOS-wt contained a similar zinc content (89–92%). The zinc content (89–92%) of the trypsin-cleaved heme domain of nNOS, derived from the full-length nNOS holoenzyme, contrasts sharply to the zinc content of the heme domain construct (residues 1–714) of nNOS (~20%, data not shown) expressed in Escherichia coli. These observations are consistent with the results of Crane et al. (22), who crystallized the E. coli-expressed heme domain of iNOS (residues 66–498) and did not observe enzyme-bound zinc but rather identified a disulfide bond located in the analogous position where Raman et al. (17) found the ZnS$_4$ center in the eNOS heme domain. When the zinc content of the BH$_4$-free

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3 A.-L. Tsai, P. Martišek, L. J. Roman, and B. S. S. Masters, unpublished observations.
nNOS-wt holoenzyme was examined, a zinc content of only 66% was observed, suggesting that in the absence of bound BH₄, disruption or destabilization of the ZnS₄ center occurs. In contrast to the zinc contents observed with nNOS-wt holoenzyme and the trypsin-cleaved nNOS-wt heme domain, only 5 and 19% of the theoretical zinc content was obtained with the C331A nNOS holoenzyme mutant preincubated with L-arginine or preincubated with L-arginine and BH₄, respectively. These data strongly suggest that mutation of cysteine 331 of nNOS results in disruption of the ZnS₄ center and prevents stoichiometric zinc binding. However, because prolonged incubation of the nNOS C331A mutant as isolated with high concentrations of L-arginine is sufficient to restore enzymatic activity (16), these findings strongly implicate zinc in a structural rather than a catalytic role.

The nNOS C331A mutant is inactive as isolated, but activity can be restored by prolonged incubation with L-arginine. Fig. 3 shows the time course for activation of the nNOS C331A mutant upon exposure to 10 mM L-arginine. The mutant enzyme progressively became more active with time. However, maximal activity (255 nmol/min/mg) was not reached for 6–8 h following exposure to L-arginine.

In contrast to the recent findings of Perry and Marletta (23), which showed that copper is bound to E. coli-expressed nNOS-wt holoenzyme as isolated, we found no significant amount of copper in our preparations (copper to heme ratios: nNOS 0.01 and eNOS 0.03; Table II) and feel that zinc is the endogenous metal. Therefore, we do not feel that iron is the endogenous metal in NOS expressed in mammalian cells. We are currently investigating the identity of the metal bound to NOS expressed in mammalian cells.

The nNOS C331A mutant is inactive (as isolated), but activity can be restored by prolonged incubation with L-arginine. Fig. 3 shows the time course for activation of the nNOS C331A mutant upon exposure to 10 mM L-arginine. The mutant enzyme progressively became more active with time. However, maximal activity (255 nmol/min/mg) was not reached for 6–8 h following exposure to L-arginine.

The C331A-mutated nNOS is inactive (as isolated), but activity can be restored by prolonged incubation with L-arginine. Fig. 3 shows the time course for activation of the nNOS C331A mutant upon exposure to 10 mM L-arginine. The mutant enzyme progressively became more active with time. However, maximal activity (255 nmol/min/mg) was not reached for 6–8 h following exposure to L-arginine.
Therefore, it is clear that zinc-mediated stabilization of the bottom of the dimer interface is key for catalytic activity, albeit an indirect effect. The comparison of other ZnS₄ proteins with the NOS isoforms suggests that the ZnS₄ center facilitates protein-protein interaction(s). In NOS, the ZnS₄ center stabilizes the dimer interface and/or the flavoprotein-heme domain interface (17). Zinc is not needed for folding because L-arginine is sufficient to restore enzymatically active protein. If zinc were only essential for folding, then the enzyme should function perfectly in its absence. Without preincubation in the presence of high concentrations of L-arginine, this is definitely not the case. Preincubation with L-arginine must stabilize an active conformation. However, it does not restore zinc binding. Therefore, zinc facilitates but is not absolutely required for activity. The present study substantiates that zinc content is influenced profoundly by pterin and confirms a structural, rather than catalytic, role for zinc in maintaining enzymatically active constitutive nitric-oxide synthases. Furthermore, we demonstrate the one zinc/two heme stoichiometry in the dimers of the constitutive NOS isoforms isolated from E. coli expression systems.

Note Added in Proof—In support of our finding of zinc in the E. coli-expressed nitric-oxide synthases, Fischmann and colleagues (30) have reported the crystal structures of human inducible and endothelial nitric-oxide synthase domains in which they find a zinc tetrathiolate center at the dimer interface.

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