Communication between the Zinc and Tetrahydrobiopterin Binding Sites in Nitric Oxide Synthase

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ABSTRACT: The nitric oxide synthase (NOS) dimer is stabilized by a Zn$^{2+}$ ion coordinated to four symmetry-related Cys residues exactly along the dimer 2-fold axis. Each of the two essential tetrahydrobiopterin (H$_4$B) molecules in the dimer interacts directly with the heme, and each H$_4$B molecule is ∼15 Å from the Zn$^{2+}$. We have determined the crystal structures of the bovine endothelial NOS dimer oxygenase domain bound to three different pterin analogues, which reveal an intimate structural communication between the H$_4$B and Zn$^{2+}$ sites. The binding of one of these compounds, 6-acetyl-2-amino-7,7-dimethyl-7,8-dihydro-4(3$H$)-pteridine-7-one (1), to the pterin site and Zn$^{2+}$ binding are mutually exclusive. Compound 1 both directly and indirectly disrupts hydrogen bonding between key residues in the Zn$^{2+}$ binding motif, resulting in destabilization of the dimer and a complete disruption of the Zn$^{2+}$ site. Addition of excess Zn$^{2+}$ stabilizes the Zn$^{2+}$ site at the expense of weakened binding of 1. The unique structural features of 1 that disrupt the dimer interface are extra methyl groups that extend into the dimer interface and force a slight opening of the dimer, thus resulting in disruption of the Zn$^{2+}$ site. These results illustrate a very delicate balance of forces and structure at the dimer interface that must be maintained to properly form the Zn$^{2+}$, pterin, and substrate binding sites.

Mammalian nitric oxide synthases (NOSs) require the cofactor (6$R$)-5,6,7,8-tetrahydrobiopterin (H$_4$B) to convert l-arginine to l-citrulline and nitric oxide, an important second-messenger molecule in neural and cardiovascular systems. The mammalian NOS enzyme family consists of three isoforms, neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). Each isoform is active only as a homodimer because the pterin binding site is located right at the dimer interface and monomeric NOS does not bind H$_4$B or the substrate. The dimer interface is formed between two N-terminal heme binding oxygenase domains that is further stabilized by the coordination of a Zn$^{2+}$ ion ligated to two cysteine thioles from each subunit (ZnS$_4$) (Figure 1). H$_4$B plays the role of a redox active one-electron donor that activates the heme-bound O$_2$, resulting in the formation of an H$_4$B radical. With l-Arg as the substrate, this radical is then reduced by obtaining an electron from the ferrous NO complex generated at the end of the catalytic reaction, thus allowing the release of NO from the ferric heme. All NOS isoforms share a strikingly similar pterin binding pocket with comparable H$_4$B binding affinities, and cofactor and substrate binding events have been shown to synergistically stabilize the NOS dimer. Low-temperature sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and urea dissociation studies indicate that the relative dimer strengths of the three mammalian NOS isoforms decrease from eNOS to nNOS to iNOS and that the role of H$_4$B in dimer stability is less critical in eNOS. The structural basis for this disparity, however, is not yet fully understood.

One way of exploring the relationship between dimer stability and H$_4$B binding is to investigate the pterin binding...
pocket using various pterin analogues. Moreover, inactive pterin analogues could potentially serve as NOS inhibitors and have proven to be useful in probing the function of H4B. In this study, we have determined the crystal structures of three novel pterin compounds (Figure 2) analogous to H4B bound to eNOS, which has unexpectedly provided important insights into the intimate connection among the Zn2+, pterin, and substrate binding sites.

## MATERIALS AND METHODS

### Protein Expression and Purification

The bovine holo-eNOS pCWori construct containing an AmpR gene and an N-terminal six-His tag was expressed in Escherichia coli BL21-(DE3) cells already containing the calmodulin plasmid (with a ChlR gene), and the cells were then plated on LB agar with ampicillin (100 μg/mL) and chloramphenicol (35 μg/mL) to ensure that eNOS would remain free of H4B. A single colony was used to inoculate each 5 mL of LB starter culture (100 μg/mL ampicillin and 35 μg/mL chloramphenicol). The culture was incubated for 8 h at 37 °C with agitation at 220 rpm and 4 °C with agitation at 220 rpm until the OD (600 nm) reached 1.0. The soluble fraction was isolated by centrifugation at 17000 × g for 30 min. The following protease inhibitors were added to buffer A before being loaded with the crude extract: trypsin inhibitor (5 μg/mL), pepstatin A (1 μg/mL), and leupeptin (1 μg/mL). Cells were lysed by being passed through a microfluidizer at 18K psi (Microfluidics International Co.). The soluble fraction was isolated by centrifugation at 17000 rpm and 4 °C for 1 h. The crude extract was then loaded onto a Ni2+-nitrilotriacetate column pre-equilibrated with 10 bed volumes of buffer A. After being loaded with the crude extract, the column was washed with 10 bed volumes of 10 mM imidazole in buffer A before being eluted with a 10 to 200 mM imidazole linear gradient in buffer A. Colored fractions were pooled and loaded onto a 2′S,5′-ADP Sepharose column pre-equilibrated with buffer B [50 mM Tris-HCl (pH 7.8), 10% glycerol, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 0.5 mM L-Arg, and 200 mM NaCl]. The column was then washed with 10 bed volumes of buffer B and eluted with 10 mM NADP+ in buffer B. Colored fractions were pooled and concentrated in a 30000 molecular weight cutoff (MWCO) Amicon concentrator at 4 °C. The eNOS heme domain used for crystallization was generated by limited trypsinolysis: a 20:1 eNOS:trypsin weight ratio was used for a 1 h incubation at 25 °C. The digested sample was then loaded onto a Superdex 200 column (HiLoad 26/60, GE Healthcare) controlled by an FPLC system and pre-equilibrated with buffer B to separate the heme domain and flavin-containing fragment generated by the trypsin digest. Fractions were pooled according to an A280/A395 spectral ratio of <1.7, and sample homogeneity was determined by SDS-PAGE.

### Synthesis of H2B Analogues

The preparation of pterin compounds 6-acetyl-2-amino-7,7-dimethyl-7,8-dihydro-4(3H)-pteridinone (1 in Figure 2), 2-amino-9a-methyl-6,7,8,9,9a,10-hexahydrobenzo[g]pteridin-4(3H)-one (2), and 2-amino-9a-methyl-8,9,9a,10-tetrahydrobenzo[g]pteridine-4,6(3H,7H)-dione (3) used in this study has been previously described.

### Crystal Preparation

All eNOS heme domain samples were prepared for crystallization by being concentrated to 12 mg/mL in buffer B using a 30000 MWCO Amicon concentrator. Cocrystallization was conducted by combining protein with 5 mM cofactor and 5 mM L-arginine. Crystals were grown at 4 °C in 18–20% PEG 3350 (w/v), 250 mM magnesium acetate, 100 mM cacodylate (pH 6.25), and 5 mM tris(2-carboxyethyl)-phosphine (TCEP) in a sitting-drop vapor diffusion setup. Freshly grown crystals were passed stepwise through a cryoprotectant solution containing 20% PEG 3350, 10% (w/v) glycerol, 10% (w/v) trehalose, 5% (w/v) sucrose, 5% (w/v) glycerol.
mannitol, 10 mM cofactor, and 5−10 mM L-Arg for 4−6 h at 4 °C before being flash-cooled with liquid nitrogen.

X-ray Diffraction Data Collection, Processing, and Structure Refinement. Cryogenic (100 K) X-ray diffraction data were collected remotely at Stanford Synchrotron Radiation Lightsource (SSRL) using the data collection control software Blu-Ice and a crystal mounting robot. An ADSC Q315r CCD detector at beamline 7-1 or a Mar325 CCD detector at beamline 9-2 was used for data collection. Raw data frames were indexed, integrated, and scaled using HKL2000. The binding of H4B cofactors was detected by the initial difference Fourier maps calculated with REFMAC. The pterin molecules were then modeled in COOT and reﬁned using REFMAC. Water molecules were added in REFMAC and

Table 1. Crystallographic Data and Refinement Statistics

|                | 1                     | 2                     | 3                     | 4 (with 50 μM Zn acetate) |
|----------------|-----------------------|-----------------------|-----------------------|--------------------------|
| PDB entry      | 4CUL                  | 4CUM                  | 4CUN                  | 4CVG                     |
| radiation source| SSRL BL 7-1           | SSRL BL 7-1           | SSRL BL 7-1           | SSRL BL 9-2              |
| space group    | P2₁2₁2₁               | P2₁2₁2₁               | P2₁2₁2₁               | P2₁2₁2₁                  |
| unit cell dimensions a, b, c (Å) | 57.09, 105.47, 158.25 | 58.01, 106.49, 156.48 | 58.87, 106.18, 156.74 | 57.41, 105.96, 156.53    |
| data resolution (Å) (highest-resolution shell) | 50.0−2.23 (2.31−2.23) | 88.04−2.33 (2.41−2.33) | 87.9−2.48 (2.57−2.48) | 50.0−2.31 (2.39−2.31) |
| X-ray wavelength (Å) | 1.13          | 1.13                  | 1.13                  | 0.98                     |
| total no. of observations | 208127          | 184003                | 140206                | 164961                   |
| no. of unique reflections (highest-resolution shell) | 47234 (4613) | 42159 (4091) | 35505 (3482) | 42009 (3784) |
| completeness (%) (highest-resolution shell) | 99.77 (98.99) | 99.53 (97.87) | 99.47 (99.63) | 97.91 (90.01) |
| Rmerge (highest-resolution shell) | 0.08 (0.884) | 0.08 (0.862) | 0.078 (0.689) | 0.06 (0.690) |
| I/σ (highest-resolution shell) | 4.4 (4.4) | 4.4 (4.4) | 3.9 (3.8) | 4.0 (3.9) |
| B factor, Wilson plot (Å²) | 38.83 | 44.96 | 56.81 | 43.54 |
| no. of protein atoms | 6141          | 6446                  | 6446                  | 6400                     |
| no. of heteroatoms | 158           | 169                   | 173                   | 131                      |
| no. of waters | 279           | 214                   | 51                    | 231                      |
| disordered residues | 40−66, 91−120 (A) | 40−66, 110−120 (A) | 40−66, 110−120 (A) | 40−66, 108−120 (A) |
| Rmerge/Rfree | 0.165/0.209 | 0.170/0.227 | 0.184/0.242 | 0.155/0.214 |
| root-mean-square deviation for bond lengths (Å) | 1.47 | 1.69 | 1.97 | 1.83 |

Figure 3. (A) Active site of bovine eNOS in complex with pterin analogue 1 with the 2Fo − Fc electron density map contoured at 1.0σ. The strong density supports binding to the pterin site even though the compound lacks the ability to form a hydrogen bond with Ser104 of chain A. (B and C) Active site of bovine eNOS in complex with 2 and 3, respectively, with the 2Fo − Fc density map contoured at 1.0σ. The density for 2 is not as strong as that for analogue 1 but supports binding of 2 with the extra methyl facing F462, while the density for 3 is weaker than that for 1 or 2 yet strong enough to support binding of 3 in the shown orientation. (D) Active site of bovine eNOS in complex with pterin analogue 1 as shown in panel A but overlaid with the 2Fo − Fc map (1.0σ) calculated using the data collected with a crystal supplemented with 50 μM Zn acetate during the cryoprotectant soaks. The poorly deﬁned pterin and substrate density at best supports partial occupancy. The color scheme for this figure and Figure 4 is as follows: Chain A is colored green, chain B yellow, pterin blue, substrate L-arginine cyan, and the heme orange.
checked by COOT. The TLS\textsuperscript{30} protocol was implemented in the final stage of refinements with each subunit as one TLS group. The refined structures were validated in COOT before being deposited in the Protein Data Bank. The crystallographic data collection and structure refinement statistics are listed in Table 1 with Protein Data Bank (PDB) entry codes included.

■ RESULTS AND DISCUSSION

Structural Characterization of the H\textsubscript{4}B Binding Pocket. As shown in Figure 2, the three dihydropterin analogues retain the ring structure of H\textsubscript{4}B and introduce variations only in the side chain. Figure 3 shows the electron density of the three dihydropterin analogues bound to eNOS. Compound 1 (Figure 3A) exhibits the strongest and most well-defined electron density. As expected from its structural similarity to H\textsubscript{4}B, compound 1 fits into the pterin binding pocket quite well, maintaining most of the interactions found with H\textsubscript{4}B: the π–π stacking with W449, the H-bonds from its 2-aminopyrimidine nitrogens to the heme propionate A, the H-bond from O4 to R367, and the van der Waals contacts with 2-aminopyrimidine nitrogens to the heme propionate A, the H-bond from O4 to R367, and the van der Waals contacts with aromatic residues of the other subunits (W76 and F462). Therefore, it is not surprising that the tetrahydro form of 1 can support the conversion of L-arginine to NO in nNOS.\textsuperscript{31} From kinetic studies, the estimated $K_D$ for 1 is 115 μM compared to a value of 1.1 μM for H\textsubscript{4}B.\textsuperscript{31}

However, the two additional H-bonds from the dihydroxypropyl side chain of H\textsubscript{4}B to the carbonyls of S104 and F462 are lost in 1. Unique to 1 is the close contact from one of its methyl groups at the C7 position to the W447 side chain of the other subunit. Both 2 and 3 in the dihydro oxidation state introduce a third cyclohexane ring to replace the H\textsubscript{4}B side chain. Although the third ring is tolerated by the pterin binding pocket, the extra methyl group at C7 may generate steric clashes with the protein. As a result, 2 (Figure 3B) and 3 (Figure 3C) exhibit weaker electron density. Crystals of the eNOS–3 complex diffract poorly, which we have found correlates well with poor ligand binding. Because 3 is structurally similar to 2 and differs only in the carbonyl O atom on the cyclohexane ring, the additional steric crowding of this oxygen in 3 very likely accounts for why this pterin analogue binds more poorly. Overall, the inability of the three pterin analogues to form hydrogen bonds with S104 and F462 and the steric clashes from their protruding methyl groups may be attributed to a binding affinity that is poorer than that of the native pterin, H\textsubscript{4}B.

Disruption of the Zn\textsuperscript{2+} Binding Site. As shown in Figure 4, a single Zn\textsuperscript{2+} ion is situated at the dimer interface where it is tetrahedrally coordinated by symmetry-related Cys residues along the dimer axis. The Zn\textsuperscript{2+} is ~15 Å from the center of the pterin binding pocket in both subunits A and B of the dimer. Quite unexpectedly, we found that the binding of 1 completely disrupts the Zn\textsuperscript{2+} binding region, as evidenced by a total lack of electron density for residues 91–109 and the Zn\textsuperscript{2+} (Figure 4A). It has been known for some time that both Zn\textsuperscript{2+} and H\textsubscript{4}B contribute to dimer stability,\textsuperscript{8,32,33} but this is the first indication that there is a relatively long-range communication between these two sites. To probe whether the binding of 1 and the binding of Zn\textsuperscript{2+} are mutually exclusive, we soaked crystals of the eNOS–1 complex in a cryoprotectant solution supplemented with 50 μM Zn acetate. The crystal structure shows that Zn\textsuperscript{2+} binding is restored (Figure 4D), while the electron density for 1 and the substrate, L-Arg, are poorly defined (Figure 3D). We next soaked crystals at a more moderate Zn acetate concentration of 20 μM. In this case, 1 and L-Arg bind well but the Zn\textsuperscript{2+} site is disordered (data not shown). In contrast, binding of 2 and binding of 3 do not interfere with Zn\textsuperscript{2+} binding (Figure 4B,C). The interdependence of pterin and L-Arg binding is well-known and is very likely mediated by the fact H\textsubscript{4}B H-bonds to the same heme propionate as the ω-amino group of L-Arg (Figure 3).\textsuperscript{12–14}

Figure 4. (A) 2$F_o$ – $F_c$ electron density map (at the 1.0σ contour level) from the eNOS–1 complex structure overlaid on a reference bovine eNOS structure with bound H\textsubscript{4}B and an ordered Zn\textsuperscript{2+} site (PDB entry 9NSE). This is to illustrate the disordered Zn\textsuperscript{2+} site resulting from binding of compound 1 to the pterin pocket. The lack of electron density spans residues 91–109 in chain A and residues 91–111 in chain B. (B and C) Zn\textsuperscript{2+} binding site of bovine eNOS in complex with 2 and 3, respectively. The 2$F_o$ – $F_c$ density map is at a contour level of 1.0σ and shows a fully ordered Zn\textsuperscript{2+} site, supporting undisrupted Zn\textsuperscript{2+} binding. (D) 2$F_o$ – $F_c$ density map for the Zn\textsuperscript{2+} binding site at a contour level of 1.0σ derived from the same eNOS–1 structure showing poor pterin and substrate density in Figure 3D. The crystal was soaked in a cryoprotectant solution supplemented with 50 μM Zn acetate. The Zn\textsuperscript{2+} site is fully ordered, while compound 1 is disordered in structure.

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superposed on the basis of only chain A with an H4B-bound − square deviations (rmsds) of chains A and B for residues 121 further quantify the observation, we calculated the root-mean-backbone of chain B and observed a similar shift in chain A. To α For the sake of consistency, we superposed the cases, chain B does not move significantly relative to chain A. W447 is not merely absorbed locally, but instead substituted forces W447 of chain B to shift away from chain A. The side eNOS Dimer? These results reveal that the eNOS dimer is able to loosen up and expand to accommodate 1. The side effect is that all hydrogen bonding interactions that stabilize the Zn2+ binding site, mainly the two between N468 and C101, are weakened or lost, as shown in Figure 6. This effect being compounded on both chains causes a complete disruption of Zn2+ binding and destabilization of the dimer.

What Does This Communication Reveal about the NOS Dimer? These results reveal that the eNOS dimer is able to loosen up and expand to accommodate 1. The side effect is that all hydrogen bonding interactions that stabilize the Zn2+ binding site, mainly the two between N468 and C101, are weakened or lost, as shown in Figure 6. This effect being compounded on both chains causes a complete disruption of Zn2+ binding and destabilization of the dimer.

Previous studies have shown that eNOS has the most stable dimer compared to nNOS and iNOS. To obtain NOS—pterin

Table 2. Calculation of Root-Mean-Square Deviations of α-Carbons

| structure                          | PDB entry | root-mean-square deviation (Å) |
|------------------------------------|-----------|--------------------------------|
| H4B-free                           | 5NSE      | 0.165                           |
| compound 1                         | 4CUL      | 0.250                           |
| compound 2                         | 4CUM      | 0.220                           |
| compound 3                         | 4CUN      | 0.309                           |
| compound 1 (with 50 μM Zn acetate) | 4CVG      | 0.218                           |

*Chain A of each structure was superposed with chain A of H4B-bound eNOS (PDB entry 9NSE), and all rmsds were calculated using LSQMAN (http://xray.bmc.uu.se/usf/)."
complexes, it is necessary to purify NOS in the absence of H4B and only eNOS, not nNOS, is stable enough without H4B during purification and the proteolysis required to generate the heme domain for crystallization. Two attempts were made to purify nNOS with pterin-free buffer or the buffer supplemented with 1, but the protein denatured completely upon trypsinolysis required for generating the heme domain. This very likely reflects the fact that the eNOS dimer is more stable than nNOS: it can survive purification without H4B bound and can bind 1 without disruption of the dimer. This also suggests that the combination of intersubunit contacts attributed to the greater dimer strength of eNOS allows it to survive disruption of the Zn2+ site without complete disruption of the dimer.

SUMMARY

Even though the importance of H4B in stabilizing the NOS dimer has been known for some time, this study provides the structural basis for the intimate structural communication among H4B, Zn2+, substrate binding, and dimer stability. It is important to note that zinc-free NOS retains near full catalytic activity, although the dimer is substantially less stable. A number of biochemical studies have shown that the main role of the zinc site is to promote H4B binding, which in turn increases the affinity for the substrate, L-Arg. The study presented here provides a structural basis for this interdependence. We were fortunate that the additional stability of the eNOS dimer allowed us to probe perturbations at the dimer interface without totally disrupting the dimer, which would preclude any detailed crystallographic analysis as in the case of nNOS. It is remarkable that the mere addition of the methyl groups in 1 can have such a dramatic effect on the Zn2+ site. This underscores the exquisite fine-tuning of interactions that stabilize the NOS dimer and the close interdependence of the Zn2+, pterin, and substrate binding sites.

ASSOCIATED CONTENT

Accession Codes

Coordinates and structure factors have been deposited in the Protein Data Base as entries 4CUL, 4CUM, 4CUN, and 4CVG.
Biochemistry

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Notes
The authors declare no competing financial interest.

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