Modulation of the Remote Heme Site Geometry of Recombinant Mouse Neuronal Nitric-oxide Synthase by the N-terminal Hook Region

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The role of two essential residues at the N-terminal hook region of neuronal nitric-oxide synthase (nNOS) in nitric-oxide synthase activity was investigated. Full-length mouse nNOS proteins containing single-point mutations of Thr-315 and Asp-314 to alanine were produced in the Escherichia coli and baculovirus-insect cell expression systems. The molecular properties of the mutant proteins were analyzed in detail by biochemical, optical, and electron paramagnetic resonance spectroscopic techniques and compared with those of the wild-type enzyme. Replacement of Asp-314 by Ala altered the geometry around the heme site and the substrate-binding pocket of the heme domain and abrogated the ability of nNOS to form catalytically active dimers. Replacement of Thr-315 by Ala reduced the protein stability and altered the geometry around the heme site, especially in the absence of bound (6R)-5,6,7,8-tetrahydro-L-biopterin cofactor. These results suggest that Asp-314 and Thr-315 both play critical structural roles in stabilizing the heme domain and subunit interactions in mouse nNOS.

Nitric-oxide synthase (NOS) is a complex flavo-hemoprotein that catalyzes the conversion of L-Arg to citrulline in the presence of oxygen, NADPH, (6R)-5,6,7,8-tetrahydro-L-biopterin (H₄BP), and Ca²⁺-calmodulin complex with concomitant production of nitric oxide in two stepwise monoxygenase reactions involving N₅-hydroxy-L-arginine as an intermediate (1–6). Studies on the heme centers of the NOS isoforms by absorption, electron paramagnetic resonance (EPR), resonance Raman, and magnetic circular dichroism spectroscopy, together with mutational analysis, have strongly suggested that an endogenous thiolate sulfur donor ligand is coordinated to the central heme iron, as in the cases of cytochrome P450 and chloroperoxidase (7–12). This was confirmed by the x-ray diffraction analysis of inducible NOS (iNOS) heme domain fragments (13, 14). A unique feature of the NOS isoforms is the requirement of dimerization, which is facilitated by binding of the pterin cofactor (5, 6, 15–27), for the citrulline- and NO-forming activity.

Oxygen activation in the regular cytochrome P450-type monooxygenase reaction requires a proton donor at the distal side of the heme center (11, 12, 28–35). In many regular cytochrome P450s, this proton donor is associated with a water molecule, which is usually hydrogen bonded to the conserved distal threonine residue or the adjacent conserved aspartate/glutamate residue located in the central helix I; whenever these conserved residues exist, they are arranged in a motif, (Asp/Glu)-Thr, positioned ~100 amino acid residues upstream of the cysteine ligand to the heme center (11, 29, 32, 33, 36–41). It was postulated, before the x-ray crystal structure determination of the dimeric iNOS heme domain by Crane et al. (14), that an analogous proton donor might exist in the NOS isoforms to facilitate the cytochrome P450-type monoxygenation reaction (42, 43). Amino acid sequence comparisons of NOS isoforms and cytochrome P450cam and P450BM-3 were made, with particular attention to the sequence motif, (Asp/Glu)-Thr, conserved in regular cytochrome P450s (Fig. 1). Among the 10 strictly conserved threonine residues in the heme domain of all NOS isoforms, a single site in the N-terminal region, positioned approximately 100 amino acid residues upstream of the cysteine ligand (Cys-415 in mouse nNOS), was found to have the conserved motif Asp-Thr (Asp-314-Thr-315 in mouse nNOS; see Fig. 1). Thus, Asp-314 and Thr-315 of mouse nNOS were considered as candidates for the putative distal proton donor. Site-directed mutagenesis studies were conducted with a heme domain fragment produced in an Escherichia coli expression system by us (42) and with full-length recombinant nNOS produced in a yeast expression system by others (43). Although the replacement of Asp-314 of the mouse nNOS heme domain fragment by Ala led to the formation of an inactive cytochrome P420-like species (42), the results of the latter studies with recombinant full-length nNOS led to the proposal that Asp-314 might serve as the putative distal proton donor by analogy with regular cytochrome P450s (43). This controversy remains to be resolved.

The recent x-ray crystal structural analysis of the H₄BP-bound dimeric iNOS heme domain fragment has shown unambiguously that neither Thr-315 nor Asp-314 is located at the distal substrate-binding pocket of iNOS, and that these residues are in fact located at the N-terminal hook region, far from the distal heme site (14). The N-terminal hook of the iNOS heme domain fragment is critically involved in the dimer in-

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‡ The glutathione S-transferase-heme domain fusion proteins of these mutant enzymes all contained a bound heme center as judged from the visible absorption spectra, indicating their ability to incorporate heme into the corresponding heme domain (T. Iwasaki, H. Hori, and T. Nishino, unpublished results).
terface and the binding of the pterin cofactor of the dimeric heme domain, in close proximity to the Hb-BP-binding site (14). In this study, we report a detailed biochemical and spectroscopic investigation of full-length recombinant Asp-314 → Ala and Thr-315 → Ala mutants of mouse nNOS produced using E. coli and baculovirus-insect cell (Sf9) expression systems either in the absence or presence of added Hb-BP. Our results strongly suggest that Asp-314 and Thr-315 both play critical structural roles in maintaining protein stability and the geometry around the heme site. These conclusions are consistent with inferences based on the recent x-ray structural analysis of the dimeric iNOS heme domain (14) and provide complementary evidence for the structural importance of the hairpin loop of the N-terminal hook of mouse nNOS. A part of the present work has been presented elsewhere (42).

**EXPERIMENTAL PROCEDURES**

**Materials**—Synthetic DNA oligomers were purchased from either SCI-MEDIA (Tokyo, Japan) or Nissinbo (Tokyo, Japan), and DNA modification enzymes and restriction enzymes were from either New England Biolabs or Takara Biomedicals (Otsu, Japan). 2,5-ADP-Sepharose 4B, Sephacryl S-200HR, DEAE-Sepharose Fast Flow, and Ampure SA were purchased from Amersham Pharmacia Biotech. Calmodulin, FDN, FAM, l-Arg, and l-citrulline were from Sigma, and 6(R)-5,6,7,8-tetrahydro-L-biotin (Hb-BP) was from the Schircks Laboratories (Jona, Switzerland). l-[14C]-UArg was obtained from NEN Life Science Products. Other chemicals used in this study were of analytical grade.

**DNA Manipulations**—The baculovirus-Spodoptera frugiperda (Sf9) insect cell expression system (44) and E. coli pcWori+ expression system (45–47) were employed for the expression of mouse full-length wild-type nNOS and the single-point mutant enzymes T315A, T315S, and D314A. Unless otherwise stated, vectors were constructed by utilizing E. coli HB101 as the host strain. The site-directed mutagenesis was conducted using a Muta-Gene Phagemid in vitro mutagenesis kit (United States Biochemical Rad) was transformed with pTZ19RNOS1, and this transformant in the E. coli Expression System—A heterologous expression system for E. coli in insect cell expression system (44, 45) and the chaperonin expression vectors and pKY206 were introduced by recombination using the Linear Transfection Module (Invitrogen), and the literature (44). The recombinant enzymes were produced in the presence of riboflavin, hemin, and sepiapterin as described in the literature (20, 51).

**Activity Measurement**—NOS activity was measured by monitoring the consumption of l-[14C]-UArg in the dimeric NOS heme domain of nNOS described previously (52). The standard assay was performed at 25 °C in assay mixture containing 16.7 mM HEPES-NaOH buffer, pH 7.4, 4.2 mM Tris-HCl buffer, pH 7.4, 667 μM EDTA, 167 μM EGTA, 667 μM dithiothreitol, 167 μM l-[14C]-UArg, 667 μM NADPH, 1.2 mM CaCl₂, 6.7 μg of calmodulin, 1.25 mM FAD, 1.25 mM FMN, 2.5 μM Hb-BP, and the enzyme, in a total volume of 30 μL. The specific activity of l-[14C]-UArg used in the assays was 11.84 GBq/nmol.

**Purification of Recombinant nNOS**—Purification of recombinant enzymes produced in E. coli strain BL21 was performed on ice or at 4 °C essentially as described in the literature (47), except that purification was conducted using a 2,5-ADP-Sepharose 4B column chromatography (Amersham Pharmacia Biotech), followed by Sephacryl S200HR and DEAE-Sepharose Fast Flow column chromatography (Amersham Pharmacia Biotech), and that the overnight dialysis step (47) was omitted. Hb-BP (10 μM) was supplied in the ultrasonification step, unless otherwise stated. The catalytic activity and the purity of the purified wild-type enzyme, nNOS1, were comparable to those previously reported for recombinant nNOS1 by others (47).

**Analytical Procedures**—Absorption spectra were recorded using a Hitachi U3210 spectrophotometer or a Beckman DU-7400 spectrophotometer. EPR measurements were carried out using a JEOL JEX-REIX spectrometer equipped with an Air Products model LTR-3 Heli-Tran cryostat system, in which the temperature was monitored with a Scientific Instruments digital temperature indicator/controller, as described previously (53). EPR spectra of several different batches of recombinant nNOS samples were also measured at JEOL Ltd. (Tokyo, Japan), using a JEOL JES-TE200 spectrometer equipped with an ESR470 Heli-Tran cryostat system, in which the temperature was monitored with a JEOL NMR field meter ES-FC5. All spectral data were processed using KaldeiaGraph software, version 3.05 (Abelbeck Software).

**RESULTS**

Among 10 conserved threonine residues in the heme domain of all NOS isoforms, only Thr-315 in the N-terminal region of mouse nNOS lies in an Asp-Thr motif (Asp-314-Thr-315 in mouse nNOS) (42, 43), resembling that found at the helix I region of many regular cytochrome P450s (29, 32) (Fig. 1). To investigate the possible function of these conserved residues, Asp-314 and Thr-315, at the N-terminal hook of mouse nNOS, previously (48), and the baculovirus transfer vector pVP10Z (49) kindly provided by Dr. S. Kawamoto (Yokohama City University) were used. The transfer vectors carrying the cDNA for the wild-type nNOS and the variants were individually constructed for recombination of Asp-314 and Thr-315 in Asp-314/T315A, Asp-314/T315S, and Thr-315/T315A. The DNA fragment coding the altered 5'-leader sequence and the N-terminal region of nNOS was exchanged for the corresponding region of the variants by utilizing two unique restriction enzyme sites, the Scl and Apal sites, which were obtained previously (pTZ19RNOS1/NheI, pTZ19RNOS1/T314A, pTZ19RNOS1/NheI/T315S) were excised by NheI and XbaI digestion, and the DNA fragment encoding the full-length nNOS gene was ligated into the NheI site of pVP10Z utilizing the compatibility between XbaI and NheI sites. The direction of the cDNA was identified by DNA sequencing.

Confinement with AcNPV DNA and constructed transfer vectors was conducted by using a Linear Transfection Module (Invitrogen), and the screening of the recombinant virus was carried out according to the manufacturer's manual, Max Bac Baculovirus Expression System Manual (Invitrogen), and the literature (44). The recombinant enzymes were produced in the presence of riboflavin, hemin, and sepiapterin as described in the literature (20, 51).
each of the two residues was subjected to single-point mutagenesis. The resultant full-length mutant enzymes were heterologously produced in *E. coli* and baculovirus-insect cell (Sf9) expression systems and characterized biochemically and spectroscopically as described below.

Expression and Biochemical Characterization of the Wild-type and Mutant Enzymes Produced Using the *E. coli* Expression System—The recombinant full-length wild-type and mutant enzymes, T315A, T315S, and D314A, were produced in an *E. coli* expression system co-producing *E. coli* chaperonin GroEL/S (46, 47). Fig. 2 shows the CO-reduced minus reduced difference spectra of the crude wild-type and mutant enzymes in the *E. coli* lysate prepared in the presence of added H4BP (10 μM). A cytochrome P450-like heme center was apparent in the difference spectra when the recombinant wild-type enzyme, T315A, or T315S was produced in *E. coli* but was virtually absent when D314A was produced (see Fig. 2). T315S was constructed because the serine residue has a hydroxyl group, as does the threonine residue, although essentially the same results were obtained for T315A and T315S. Parallel measurements of the citrulline-forming activity of the crude enzymes in the presence of 10 μM H4BP suggested that the recombinant wild-type enzyme and T315A were active, T315S was less active, and D314A was completely inactive (data not shown; see below).

The wild-type and T315A enzymes were purified to near electrophoretic homogeneity in the presence of added H4BP (10 μM) (see under “Experimental Procedures”). The purified T315A showed a single 160-kDa band in SDS-polyacrylamide gel electrophoresis (data not shown). The as-isolated mutant enzyme showed citrulline-forming activity of ~80 nmol/mg/min at 25 °C, which was lower than that of the wild-type enzyme (~220–280 nmol/mg/min at 25 °C; see Table I). This activity was greatly enhanced after preincubation of the recombinant enzyme in the presence of L-Arg and H4BP, when the purified T315A showed activity as high as ~390 nmol/mg/min at 25 °C with an apparent Kₘ for L-Arg of 1.1 μM, which is comparable to the values of the wild-type enzyme (~360–450 nmol/mg/min at 25 °C with an apparent Kₘ for L-Arg of 1.3 μM), as summarized in Table I.

Spectroscopic Characterization of the Wild-type and T315A Enzymes Purified in the Presence of H₄BP—Fig. 3 shows the visible absorption spectra of the wild-type and T315A enzymes purified in the presence of H₄BP (solid traces). The optical properties of the two are essentially identical in that the resting enzymes were predominantly high spin as isolated,
The properties of the ferriheme center of the purified enzymes were further investigated by EPR spectroscopy, which is a sensitive technique to detect minor changes at the ferriheme site (Fig. 4). The EPR spectra at 16 K of the ferriheme center of the resting wild-type enzyme showed a high spin component at $g = 7.68$, 4.07, and $-1.8$ as the predominant species (Fig. 4A). The rhombicity (defined as the ratio of the rhombic and axial zero field splitting parameters, $E/D$) of this high spin ferriheme species is 0.075, which is lower that of the hydroxyarginine-bound high spin ferriheme species ($E/D = 0.077$; data not shown). Incubation of the enzyme with 10 μM H$_4$BP in the absence of added l-Arg for 30 min did not cause any change of the apparent $g$ values of the high spin ferriheme; instead, a decrease of the relative intensity of the $g = 7.68$ signal was observed (Fig. 4B). This implies that the H$_4$BP cofactor may bind to substrate-free ferric nNOS with minimal disruption of the ligation geometry of the high spin heme site and/or may stabilize the ferrous state, although the details remain to be investigated.

The resting wild-type enzyme also contained at least two overlapping low spin ferriheme species at $g = 2.45–2.41$, 2.28, and 1.91–1.90, which are more clearly observed at $-20$–25 K (data not shown), in addition to a sharp flavin semiquinone radical at $g = 2.0$. The calculated crystal field parameters of tetragonality ($\Delta\lambda$) and rhombicity ($V/\Delta$) of the predominant low spin ferriheme species at $g = 2.41$ are consistent with the axial coordination of an oxygen ligand to the proximal cysteine thiolate ligand (56). Addition of 0.1 mM l-Arg to the full-length wild-type enzyme resulted in disappearance of the remaining $g = 2.41$ low spin ferriheme and the formation of a new high spin species at $g = 7.59$, 4.08, and 1.81 (Fig. 4C), and a decrease of the rhombicity $E/D$ of the high spin ferriheme ($E/D = 0.073$) was observed. This suggests that l-Arg binds to the wild-type enzyme without direct coordination to the ferriheme center.

The EPR spectrum of the ferriheme center of the resting mutant enzyme T315A purified in the presence of 10 μM H$_4$BP (Fig. 4D) showed a high spin species, but with slightly different $g$ values ($g = 7.62$, $-4.07$, and $-1.82$); its rhombicity $E/D$ of 0.074 is similar to that of the L-Arg-bound high spin ferriheme species at $g = 7.59$, 4.08, and 1.81 ($E/D = 0.073$). Its high spin ferriheme content was typically slightly lower by 10–20% than that of the wild-type enzyme, which may contribute to the stability of the enzyme.

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**Table 1: Citrulline-forming activity of recombinant enzymes produced using the E. coli expression system**

| Enzyme produced in E. coli | Citrulline-forming activity |
|---------------------------|-----------------------------|
| Wild-type nNOS$^a$         | 220–280 nmol/min/mg          |
|                           | (360–450 nmol/min/mg$^a$)    |
|                           | $K_m$ for l-Arg = 1.3 μM     |
|                           | $K_m$ for NADPH = 0.7 μM     |
| T315A$^a$                 | 80 nmol/min/mg               |
|                           | (390 nmol/min/mg$^a$)        |
|                           | $K_m$ for l-Arg = 1.1 μM     |
|                           | $K_m$ for NADPH = ~1–2 μM    |
| D314A$^a$                 | Not detectable              |

$^a$ The recombinant wild-type enzyme produced using the baculovirus-insect cell expression system had citrulline-forming activity and retained the intact 160-kDa subunit.

$^b$ The values in parentheses represent kinetic parameters obtained after preincubation of the enzyme in the presence of l-Arg and H$_4$BP.

$^c$ The recombinant mutant enzymes produced using the baculovirus-insect cell expression system showed no citrulline-forming activity and were heavily proteolyzed.

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**Fig. 3. Optical properties of wild-type nNOS and T315A mutant enzyme purified in the presence (solid trace) and absence (dashed trace) of H$_4$BP cofactor.**

A, visible absorption spectra of the resting forms of wild-type nNOS purified in the presence (solid trace) and absence (dashed trace) of H$_4$BP cofactor. B, visible absorption spectra of the resting forms of T315A mutant enzyme purified in the presence (solid trace) and absence (dashed trace) of H$_4$BP cofactor. C, the CO-reduced minus reduced difference spectra of purified wild-type nNOS (top) and T315A (bottom) purified in the presence (solid trace) and absence (dashed trace) of H$_4$BP cofactor.
lower citrulline-forming activity of T315A as isolated (prior to preincubation, see Table I). Nevertheless, addition of 0.1 mM L-Arg resulted in the conversion of the remaining low spin ferriheme of T315A to the high spin species (Fig. 4E), the apparent g values and rhombicity of which (E/D = 0.073) were essentially identical to those of the L-Arg-bound wild-type enzyme (Fig. 4C). These data suggest that the binding of L-Arg causes a local conformational change in both the wild-type and T315A enzymes that results in a substantial change in the geometry and spin-state of the ferriheme site. Thus, the EPR results confirm that the mutant enzyme T315A purified in the presence of H4BP is capable of binding L-Arg without direct coordination to the central heme iron and that the resultant H4BP- and L-Arg-bound high spin ferriheme is virtually identical to that of the wild-type enzyme.

Spectroscopic Characterization of D314A Purified in the Presence of H4BP—D314A partially purified in the presence of H4BP was very unstable even in the presence of added L-Arg and H4BP and exhibited no citrulline-forming activity (data not shown). Gel filtration analysis with a calibrated Tosoh G-3000SWXL column connected to a high performance liquid chromatography system suggested that purified D314A is predominantly multiligomeric, and not dimeric (Fig. 5, bottom trace). It has been reported previously that the multiligomeric form of NOS is inactive and exhibits only a monomer band (but no SDS-resistant dimer band) in low temperature SDS-polyacrylamide gel electrophoresis, indicating that it probably represents the multiligomeric state of monomer molecules (see Refs. 19, 20, and 57). Thus, replacement of Asp-314 by Ala affected the ability of mouse nNOS to form catalytically active dimers even in the presence of added pterin cofactor.

The visible absorption spectrum of D314A as isolated showed a Soret band at 418 nm, and split peaks at 421 and 446 nm were seen in the ferrous-CO state, indicating the heterogeneity of the ferrous-CO heme site (Fig. 6A). The CO-reduced minus reduced difference spectrum showed that the P450-like form of the ferrous-CO complex in the presence of L-Arg has a peak at 447 nm (Fig. 6B), which is red-shifted by 3 nm as compared with that of the wild-type enzyme (444 nm) (Fig. 3C).

The EPR spectrum at 7 K of D314A prepared in the presence of L-Arg and H4BP showed a high spin ferriheme component at g = 6.02 at 7 K (Fig. 7D), which is similar to the high spin ferriheme of horse metmyoglobin at g = 5.89 with axial coordination of water trans to the proximal histidine ligand (Fig. 7E). This signal is probably attributed to the cytochrome P420-like ferriheme species (see Fig. 6), which has axial coordination...
of a non-thiolate ligand (presumably histidine). It should be noted that no \( g = 7.59 \) EPR signal could be detected with D314A even in the presence of excess l-Arg (400 \( \mu \)M) (as opposed to the case of the wild-type enzyme; see Fig. 7B), indicating that the penta-coordinated ferric form is not formed at the substrate-binding pocket of the mutant enzyme. Instead, the EPR spectrum at 16 K of such preparations typically showed two distinctive, weak high spin ferriheme signals at \( g = 7.62 \) and 6.02 in the resting form (Fig. 7C), indicating that partial replacement of the proximal thiolate ligand coordinated to the central ferriheme iron by certain other amino acid residue. This was not observed with the wild-type enzyme purified under the same conditions (Figs. 3A, 3C, and 7A).

The partially purified, H4BP-free mutant enzyme D314A showed a single peak at 421 nm in the CO-reduced minus reduced difference spectrum, as observed with the E. coli lysate containing crude D314A mutant enzyme (Fig. 2). The EPR analysis confirmed the absence of any \( g = \approx 7.6 \) species (data not shown), suggesting that H4BP-free D314A exists predominantly in a cytochrome P420-like form.

Expression and Characterization of the Wild-type and Mutant Enzymes Using the Baculovirus-Insect Cell Expression System—Because of the absence of a biosynthetic pathway for the H4BP cofactor in the E. coli system (46, 47), we attempted to confirm the above results using the mutant proteins expressed in a baculovirus-insect cell (Sf9) expression system. Thus, recombinant full-length wild-type nNOS and the mutant enzymes T315A and D314A, were produced in the insect cells supplied with riboflavin, sepiapterin, and hemin as reported (20, 50, 51) and purified as described under “Experimental Procedures.” Although the wild-type enzyme showed an NADPH, \( \text{Cu}^{2+}\)-calmodulin-, and l-Arg-dependent citrulline-forming activity, the mutant enzymes T315A and D314A showed no enzymatic activity (summarized in Table I). Western blot analysis of the wild-type enzyme after SDS-polyacrylamide gel electrophoresis (using commercially available antibody raised against the C-terminal part of the P450 reductase domain of nNOS) suggested that the protein retained its mature size of 160 kDa. On the other hand, the mutant enzymes were extensively proteolyzed to five major polypeptide fragments with apparent sizes of 105, 90, 80, 60, and \( \approx 40 \) kDa (data not shown); they probably correspond to domains and subdomain fragments containing the C-terminal part of mouse nNOS, as judged from the proteolytic cleavage studies on nNOS by Lowe et al. (55). These results suggest that replacement of Thr-315 or Asp-314 by Ala modified the domain-domain and/or subunit-subunit interactions of recombinant nNOS, resulting in loss of the citrulline-forming activity and increased sensitivity to proteolysis (see Table I).
DISCUSSION

The present biochemical and spectroscopic analyses of two single-point mutant enzymes of mouse nNOS, T315A and D314A, produced in an E. coli expression system, were designed to explore the role of the strictly conserved Asp-314-Thr-315 array in the mouse nNOS heme domain (Fig. 1). Our results can be summarized as follows. (i) The Asp-314-Thr-315 array of mouse nNOS plays a critical structural role in stabilizing the domain-domain and/or subunit-subunit interaction (Table I). (ii) Replacement of Asp-314 by Ala abrogated the ability of nNOS to form catalytically active dimers and altered the geometry around the heme site and the substrate-binding pocket of the heme domain, resulting in a formation of an inactive P420-like species as the predominant species (Figs. 2, 5–7). (iii) Replacement of Thr-315 by Ala had little effect on the heme site or citrulline-forming activity of the H4BP-bound T315A mutant enzyme produced in E. coli (Figs. 2–4) but impaired the protein stability and altered the heme site geometry of the recombinant mutant enzyme purified in the absence of H4BP (Figs. 4, 5, and 7). These results could not be confirmed with mutant proteins produced in a baculovirus-insect cell (Sf9) expression system because of extensive proteolytic cleavage of the recombinant proteins. Nevertheless, the overall results strongly indicate a critical structural role for both Asp-314 and Thr-315 in stabilizing the heme domain and subunit interactions in mouse nNOS. The individual contributions of these residues are discussed below.

Critical Structural Role of Asp-314 of Mouse nNOS Heme Domain—The importance of the dimerization of NOS isoforms for citrulline and NO-forming activity is well known (5, 6, 15–27). The replacement of Asp-314 by Ala abrogated the ability of mouse nNOS to form catalytically active dimers and altered the geometry around the heme site and the substrate-binding pocket of the heme domain, resulting in a formation of an inactive P420-like species as the predominant species (Figs. 2, 5–7). The replacement of Thr-315 by Ala had little effect on the heme site or citrulline-forming activity of the H4BP-bound T315A mutant enzyme produced in E. coli (Figs. 2–4) but impaired the protein stability and altered the heme site geometry of the recombinant mutant enzyme purified in the absence of H4BP (Figs. 4, 5, and 7). These results could not be confirmed with mutant proteins produced in a baculovirus-insect cell (Sf9) expression system because of extensive proteolytic cleavage of the recombinant proteins. Nevertheless, the overall results strongly indicate a critical structural role for both Asp-314 and Thr-315 in stabilizing the heme domain and subunit interactions in mouse nNOS. The individual contributions of these residues are discussed below.
analysis of the cytochrome P420-like form of the H4BP-free nNOS and iNOS isoforms suggested partial conversion of the proximal thiolate ligand to a nitrogen-donor ligand (presumably histidine) in the ferrous-CO form (58). The present EPR analysis of the P420-like form of the D314A mutant enzyme also suggested that the P420-like form of ferric nNOS is predominantly high spin, having an axial ligand replaced by an unidentified non-thiolate ligand (presumably histidine, based on the spectral similarity to horse metmyoglobin; see Fig. 7). Moreover, the ferriheme center of the P450-like form of H4BP-supplemented D314A most likely has a hexa-coordinated low spin structure, even in the presence of excess L-Arg, which indicates that the substrate-binding pocket of this mutant enzyme is structurally modified.

On the basis of these results, we suggest that Asp-314 of mouse nNOS is critically involved in the dimerization of the enzyme, and its replacement with Ala affects the orientation and/or structure of other elements in the vicinity, leading to loss of citrulline-forming activity due to multiple structural distortions of the molecule. Indeed, the x-ray crystal structure of dimeric iNOS heme domain fragment Δ65 (residues 66–498) (14) has shown that Asp-92 and Thr-93 of mouse iNOS (corresponding to Asp-314 and Thr-315, respectively, of mouse nNOS; see Fig. 1) reside near the C-terminal end of the β2'-strand at the hairpin loop of the N-terminal hook (schematically illustrated in Fig. 8), which binds to the other subunit.

The N-terminal hook not only constitutes a part of the dimer interface of the dimeric iNOS heme domain fragment but also interacts with the N-terminal pterin-binding loop (residues 108–114), which is critically involved in binding of the H4BP cofactor (14) (see also Figs. 1 and 8). Thus, our results obtained with the D314A mutant enzyme of mouse nNOS are in line with the x-ray structural analysis of the iNOS heme domain fragment (14). On the other hand, they do not support the earlier hypothesis by Sagami and Shimizu (43) that Asp-314 might serve as a distal proton donor of the NOS isoform on the basis of activity measurements and the analogy with the regular cytochrome P450s (29, 32).

Cross-talk between the N-terminal Dimer-linking Region and the Remote Heme Site of nNOS—Thr-315 of mouse nNOS is also located at the N-terminal hook region (Figs. 1 and 8), although the effect of its replacement by Ala (or Ser) was substantially different from that in the case of the adjacent residue, Asp-314. Thus, although the H4BP-free T315A is a mixture of monomeric and multiligandemic forms and shows a tendency to form the inactive P420-like species, the H4BP-bound mutant enzyme is predominantly dimeric and active and has a heme site geometry that is indistinguishable from that of the wild-type enzyme, especially in the presence of bound L-Arg. A plausible interpretation of these results is that a structural bias at the remote heme site caused by replacement of Thr-315 by Ala is annullured upon binding of H4BP, which also facilitates dimerization and prevents the partial replacement of the axial ligand to the central heme iron.

Close inspection of the dimeric structure of the iNOS heme domain fragment (14) suggests that the N-terminal hook makes direct contact with the N-terminal pterin-binding loop and the substrate-binding helix α7a (residue nos. 370–378 in mouse iNOS) within the same subunit (Fig. 8), thereby constituting the dimer interface together with the adjacent helices α8, α9, α10, α11a, and α11b, and the β-strand β12a in the presence of bound H4BP. The substrate-binding helix α7a of the dimeric iNOS heme domain fragment contains several key residues involved in the binding of both the substrate and pterin cofactor, and directly spans above the substrate-binding distal heme pocket (14). These findings indicate a close structure-function relation among H4BP binding, dimerization, the shape of the substrate-binding distal heme pocket, and the citrulline-forming activity of the dimeric iNOS isoforms.

Unfortunately, the three-dimensional structure of the H4BP-free, monomeric NOS heme domain with the N-terminal hook region has not yet been determined, and it is currently not possible to predict the structural changes of the N-terminal hook and the neighboring regions associated with the monomer-dimer conversion of the NOS isoforms. Nevertheless, the replacement of either Asp-314 or Thr-315 at the N-terminal hook region of the H4BP-free mouse NOS by Ala resulted in marked modification of the geometry of the remote heme site and alteration of the oligomeric state of the mutant enzymes, which would not be expected if no structural interaction between the N-terminal hook and the remote heme site existed in H4BP-free nNOS. Thus, appropriate orientation and/or structure of the hairpin loop of the N-terminal hook are also required in H4BP-free mouse nNOS to prevent exchange of the proximal thiolate ligand and to maintain the geometry of the remote heme site.
In conclusion, our results provide evidence for a crucial structural role of the conserved Asp-314-Thr-315 array at the N-terminal hook region of mouse nNOS, in accordance with recent x-ray structural studies on iNOS heme domain fragments (14). In particular, our biochemical and spectroscopic analyses of the mutant enzymes T315A and D314A demonstrate that appropriate orientation and/or structure of the hairpin turn of the N-terminal hook are essential to maintain the integrity of the substrate-binding pocket at the distal side of the heme center and the geometry of the remote heme site (presumably via the substrate-binding helix α7a; see Ref. 14 and Fig. 8), as well as to facilitate formation of the active dimer. Thus, appropriate cross-talk between the N-terminal hook region and the substrate-binding distal pocket of nNOS is essential to facilitate the proper activation and regulation of the citrulline-NO-forming activity.

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