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A Structural Role for Tryptophan 188 of Inducible Nitric Oxide Synthase

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All nitric oxide synthase (NOS) isotypes bear a conserved tryptophan that stacks against the proximal face of the heme cofactor. Recently two hyperactive variants of neuronal NOS were reported in which this residue (W409) was replaced by phenylalanine or tyrosine. We find that mutation of the same residue in the oxygenase domain of inducible NOS (W188) to phenylalanine causes severe destabilization of heme binding. W188F is isolated in a predominantly heme-free state, and axial thiolate ligation to the residual bound heme is unstable. However, W188F is soluble and is expressed at levels comparable to wild type. While circular dichroism spectroscopy demonstrates the loss of some secondary structure, the protein chain is not completely denatured and it retains much of its fold between pH 7.5 and 4. This proximal tryptophan of NOS represents a case where a residue is conserved within an enzyme family but for distinct purposes that are isotype-dependent. **© 2001 Academic Press**

Key Words: **nitric oxide synthase; site-directed mutagenesis; circular dichroism spectroscopy.**

Nitric oxide (NO) is a versatile molecule that can be used for both cell signaling and host defense, depending on the context of its production (2). These diverse requirements are met by the nitric oxide synthase family of enzymes which, while alike in structure and catalytic mechanism, differ in subtle ways that tailor the control and extent of NO production to the desired ends. All isotypes share a multidomain structure, in which a carboxy-terminal flavin-binding reductase domain is linked to an amino-terminal heme and pterin-binding oxygenase domain by means of an intervening calmodulin binding site.

Catalytic activity resides in the oxygenase domain, and mutagenic studies on the residues that constitute its cofactor and substrate binding sites are important in the quest to relate NOS structure to enzyme function. For example, while NO production in neuronal NOS is controlled in part by reversible feedback inhibition due to binding of NO to the heme center, the variants W409F and W409Y relieve this inhibition and are hyperactive (1, 3, 4). Neuronal NOS residue W409 is conserved in endothelial (W180) and inducible (W188) isotypes and it makes two important contributions to the heme binding site. The side chain stacks against the proximal face of the heme cofactor in a coplanar manner, while the indole nitrogen participates in a hydrogen bond to the sulfur of the conserved cysteine residue that provides the axial ligand to the heme (5–7) The loss of this hydrogen bond in the W409F and W409Y variants of neuronal NOS may be the reason for their enhanced activity owing to a destabilized heme–NO complex (4).

We have been studying an analogous W188F variant in the oxygenase domain of inducible NOS. In contrast to the findings on neuronal NOS, we note that this variant is expressed in a largely heme-free state, although circular dichroism spectroscopy indicates the protein retains a folded structure.

MATERIALS AND METHODS

Molecular biology and protein expression. The coding region of the core oxygenase domain of mouse inducible nitric oxide synthase in the *pCRII Script* vector was used as the template for mutagenesis (8). Site-directed mutagenesis was done using an ExSite kit from Stratagene (La Jolla, CA) using the following back-to-back oligonucleotides:

Sense (mutated codon underlined):
TTTAGGAATGCCCCTCGCTGCATCGGC
Antisense: GGCCATCTTGGTGGCAAAGATGAGC.

Clones of *Escherichia coli* strain DH5α transformed with the product of the mutagenesis reaction were screened by DNA sequencing for the desired mutation, and the veracity of the rest of the sequence was confirmed. The coding region was excised with restriction enzymes *Nde*I and *Xho*I and was ligated to the expression vector pET23a (Novagen, Madison, WI) that had been linearized with the same restriction enzymes. The resulting expression vector was used to transform *E. coli* LE392 (λDE3), and the expression and purification of the protein were performed as previously described (8). Protein concentrations of the purified oxygenase domain were determined by Bradford assay using bovine serum albumin standards.

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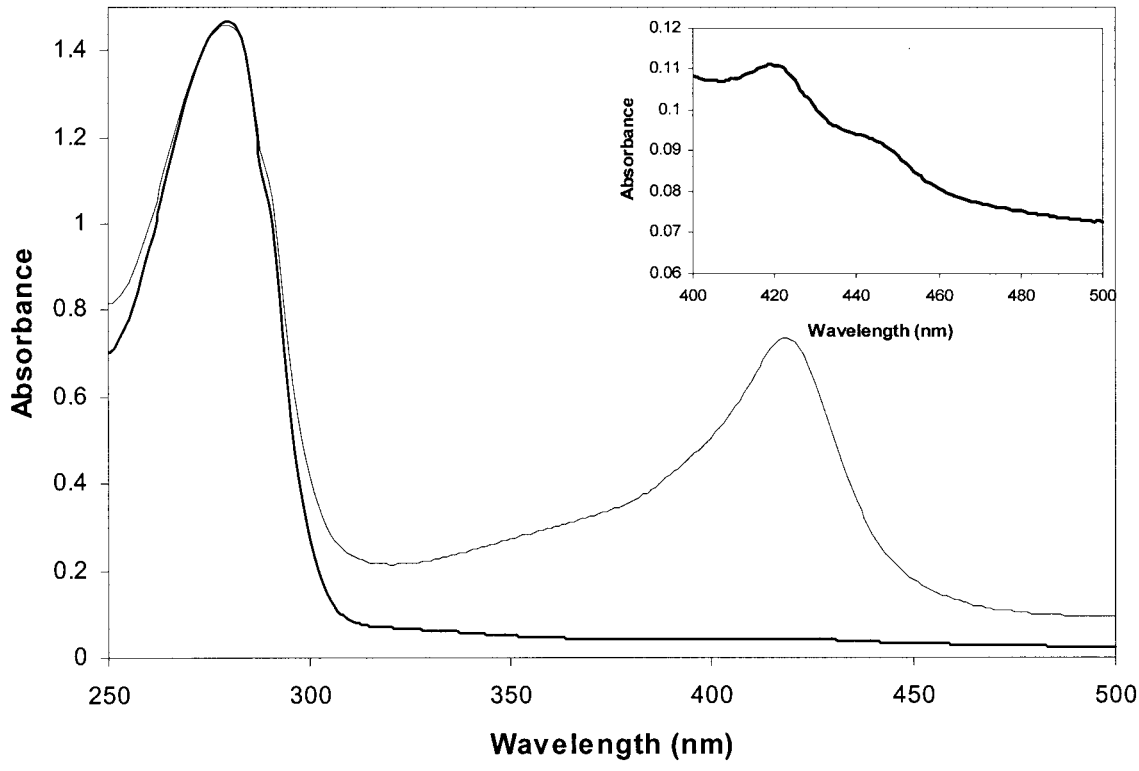

FIG. 1. UV-visible spectra of inducible NOS oxygenase domains, recorded in 50 mM Tris–HCl, pH 7.5. Heavy line W188F; thin line, wild type. The spectra have been normalized to give the same value at 280 nm. (Inset) Spectrum of the reduced, carbon monoxide form of W188F recorded immediately after reduction with dithionite.

Spectroscopic measurements. For UV-visible spectroscopy, a Cary 450 spectrophotometer was used. To record the spectrum of the reduced protein in the presence of carbon monoxide, solutions of protein were flushed with CO gas for 5 min, several milligrams of sodium dithionite were added, and the spectrum was promptly recorded. Circular dichroism spectroscopy was performed using a JASCO 700 instrument in the laboratory of Dr. Guy Guillemette at the University of Waterloo. Spectra were recorded between 250 and 180 nm and each spectrum was the average of 10 scans. Samples were prepared in 50 mM Tris, pH 7.5, or, for scans at low pH, in deionized distilled water with the pH adjusted with dilute hydrochloric acid. All measurements were made at 25°C.

RESULTS AND DISCUSSION

The residues that define the cofactor binding pocket of heme proteins tend to be well conserved, with the consequences of their alteration ranging in severity from altered spectroscopic and catalytic properties to cofactor loss (9–11). In the case of the proximal tryptophan of NOS, the consequences of mutation to phenylalanine are strongly dependent on the isotype under study. In neuronal NOS the W409F variant is nearly three times more active than the wild-type enzyme (3, 4). On this basis it has been proposed that this residue has been conserved to act as a modulator of enzyme activity by stabilizing the enzyme in a catalytically inert Fe(II)–NO state; this is converted back to an active Fe(III) state by reaction with molecular oxygen, thereby tailoring NO production to oxygen availability. Recent work on inducible NOS indicates that it too binds NO generated during catalysis, but as both Fe(II) and Fe(III) complexes, with the latter prevalent at concentrations of NO near 1 μM or higher (12). A proximal tryptophan residue (W188) is also conserved within this isotype; apart from its possible role in the regulation of catalysis, our findings indicate that W188 is maintained in inducible NOS for its role in stabilizing a viable heme-binding pocket.

Heme incorporation was severely impaired in the W188F variant of the inducible NOS oxygenase domain (Fig. 1), although expression levels were comparable to that of the wild type protein. Expression of the wild type protein in *E. coli* gives protein which is replete in heme; moreover, pellets and lysates from cells expressing the wild type oxygenase domain are dark brown, in contrast to those from cells expressing the mutant, which yield a buff-colored pellet and a straw-colored lysate.

The axial ligand to heme in the native enzyme is a thiolate group provided by cysteine-184, which gives a diagnostic Soret band at 444 nm in the dithionite-reduced, CO-bound state. Although severely lacking in heme, a small residual Soret band was detected in the UV-visible spectrum of W188F. Immediately after di-

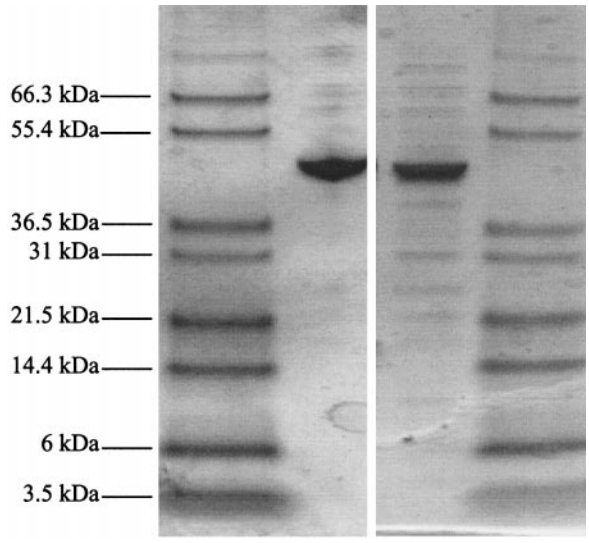

FIG. 2. SDS–PAGE of inducible NOS oxygenase domains purified by Ni–NTA chromatography. Lanes 1 and 4, size markers; lane 2, wild type; lane 3, W188F.

thionite reduction in the presence of carbon monoxide the spectrum of the variant has a band at 420 nm and a small shoulder at 444 nm, consistent with unstable thiolate ligation to the heme group (Fig. 1, inset). In contrast, native inducible NOS oxygenase domain replete with heme, tetrahydrobiopterin and arginine gives a stable peak at 444 nm, while native protein without tetrahydrobiopterin and arginine slowly loses its peak at 444 nm over the course of 1 h (8).

As the heme cofactor is usually critical for maintaining the folded conformation of a heme protein, the lack of heme incorporation could possibly render the protein chain more susceptible to proteolysis during expression or purification. The protein chain of the W188F variant is largely intact, and runs at the same apparent size as wild type by denaturing SDS–polyacrylamide gel electrophoresis (Fig. 2).

Circular dichroism spectroscopy between 250 and 180 nm indicates the loss of some secondary structure in W188F (Fig. 3A). On the other hand the CD spectrum of this variant exhibits a pH-dependence similar to the wild-type inducible NOS oxygenase domain. For both mutant and wild type there is little difference in their spectra at pH 7.5 and pH 4.0, indicating that the fold remains intact in this pH interval (Figs. 3B and 3C). In both cases significant changes occur in the spectrum below pH 2 that are consistent with the loss of alpha helical content of the protein. Thus, although heme loss in the W188F variant destabilizes the folded state of the protein, it does not lead to complete denaturation.

That a mutation in the heme binding pocket would destabilize the binding of this cofactor and the folded state of the protein is not unique, as the W409L variant of neuronal NOS is also impaired in heme binding (13). What is surprising is that the same mutation at a conserved residue in two isotypes of the same enzyme has such a pronounced difference in consequence. In neuronal NOS, the replacement of W409 by phenylalanine has no apparent effect on heme binding, or on the spectrum of the protein, and it enhances enzyme activity; this isotype is likewise tolerant toward substitution with tyrosine and histidine at position 409 (3, 13). The reason why the analogous mutation in the induc-

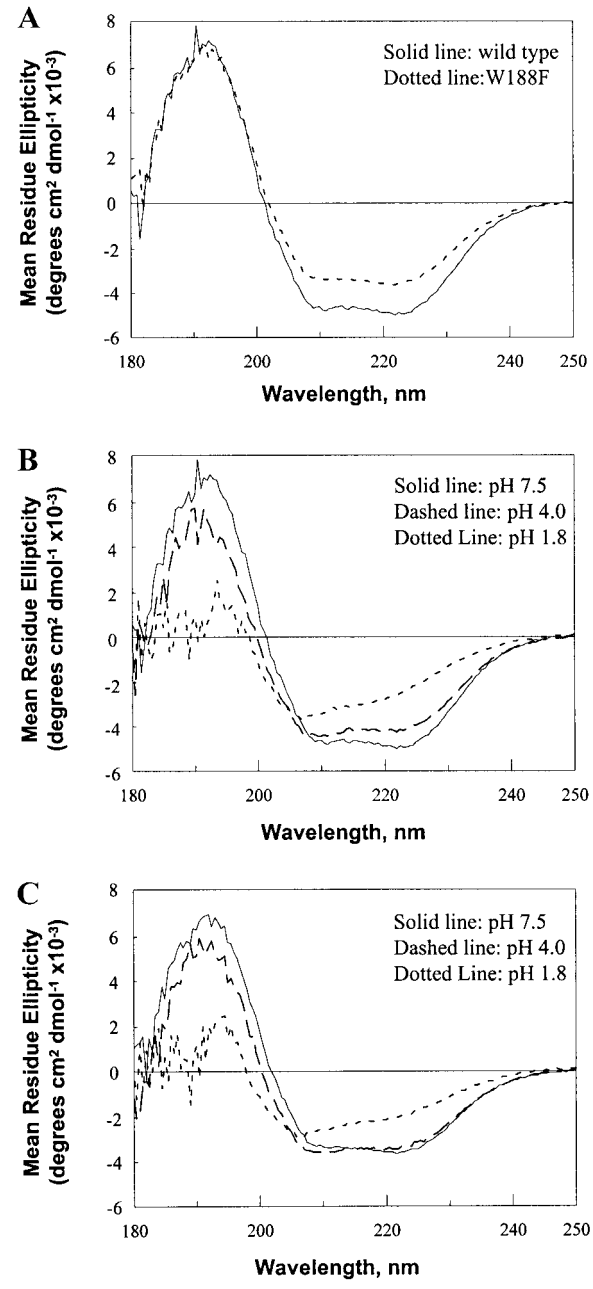

FIG. 3. CD Spectroscopy of the inducible NOS oxygenase domain. The concentration of protein in each spectrum was 0.22 ± 0.01 mg/mL and the path length was 0.2 cm. (A) Wild type and W188F at pH 7.5; (B) wild-type pH dependence; (C) W188F pH dependence.

ible isotype is incompatible with heme binding is unknown. However, it has been established that the NOS isotypes differ with respect to the relative stability of their dimeric structures and the factors that contribute to dimer stabilization. For example, endothelial NOS can form stable dimers in the absence of tetrahydrobiopterin while inducible NOS requires this cofactor to shift the monomer/dimer equilibrium to favor dimerization (14, 15). Dimers of neuronal and endothelial NOS can be detected by low-temperature SDS–PAGE while monomeric species only are detected for inducible NOS (16). It appears that inducible NOS has a built-in instability which is also reflected in its inability to tolerate mutations that are functional in other isotypes. The converse is also true, and it is possible that some of the mutations that render inducible NOS non-functional may not be as severe if introduced into the other isotypes.

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