Characterization by Electron Paramagnetic Resonance of the Interactions of L-Arginine and L-Thiocitrulline with the Heme Cofactor Region of Nitric Oxide Synthase*

(Received for publication, June 5, 1995, and in revised form, August 14, 1995)

John C. Salerno, Christopher Frey§, Kirk McMillant, Robert F. Williams, Bettie Sue Siler Masters¶, and Owen W. Griffith§**

From the DDepartment of Biology and Center for Biochemistry and Biophysics, Rensselaer Polytechnic Institute, Troy, New York 12180, the EDepartment of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin 53226, the FDepartment of Biochemistry and the IResearch Imaging Center, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284-7760

Nitric oxide synthase (NOS) catalyzes sequential NADPH- and O2-dependent mono-oxygenase reactions converting L-arginine to NO−-hydroxy-L-arginine and NO−-hydroxy-L-arginine to citrulline and nitric oxide. The homodimeric enzyme contains one heme/monomer, and that cofactor is thought to mediate both partial reactions. Here we show by electron paramagnetic resonance spectroscopy that binding of substrate L-arginine to neuronal NOS perturbs the heme cofactor binding pocket without directly interacting as a sixth axial ligand; heme iron is exclusively high spin. In contrast, binding of L-thiocitrulline, a NOS inhibitor, produces both high and low spin iron spectra; L-thiocitrulline sulfur is a sixth axial heme ligand in one, but not all, of the low spin forms. The high spin forms of the L-thiocitrulline/NOS complex display a distortion in the opposite direction to that caused by L-arginine binding. The findings elucidate the binding interactions of L-arginine and L-thiocitrulline to neuronal NOS and demonstrate that each causes a unique perturbation to the heme cofactor pocket of NOS.

Three isoforms of nitric oxide synthase (NOS)1 have been identified in mammals; all catalyze the NADPH- and O2-dependent oxidation of L-arginine to citrulline and nitric oxide (NO) (1–3). Neuronal NOS (nNOS) and endothelial NOS are constitutive. Ca2+/calmodulin-dependent isoforms that, when activated, produce NO in low (i.e. nanomolar) concentrations as part of signal transduction pathways involved in neurotransmission (4) and blood pressure regulation (5, 6), respectively. A distinct NOS isoform, not regulated by changes in intracellular Ca2+ levels, is expressed in many cell types in response to inflammatory cytokines. Nitric oxide produced by this isoform can reach levels (10–100 μM) that are disruptive to processes normally controlled by endothelial NOS or nNOS. Such levels are also potentially toxic to inducible NOS-containing cells and adjacent cells as well as to viruses and microbial pathogens within those cells (7, 8).

The overall NOS reaction proceeds via two mono-oxygenations; the first forms NO−-hydroxy-L-arginine (NOH-Arg) from L-arginine, and the second converts that tightly bound intermediate to citrulline and NO (9). Both mono-oxygenations are reminiscent of reactions catalyzed by the cytochrome P-450 system. Consistent with this view, all NOS isoforms contain a C-terminal reductase domain having significant (39%) sequence homology to cytochrome P-450 reductase; putative binding sites for NADPH as well as for FAD and FMN, tightly bound cofactors, are identified in this domain (3, 10). The NOS N-terminal oxygenase domain shows little or no sequence homology to known cytochrome P-450 but does contain a heme ligand sequence homology to cytochrome P-450 (3, 10). Recent studies with the isolated oxygenase domain establish that this region also contains binding sites for substrate L-arginine in addition to tetrahydrobiopterin, a cofactor of poorly characterized function (14–16). The oxygenase and reductase domains are connected by a calmodulin-binding region (10). For nNOS and endothelial NOS, binding of Ca2+/calmodulin to this region activates electron transfer between the reductase and oxygenase domains and allows electron flow from NADPH, through the flavins, to the heme cofactor (17). Dioxygen can then be bound to and activated by the heme cofactor, and a guanidino nitrogen of L-arginine is oxidized first to NOH-Arg and then to NO. Inducible NOS, calmodulin is tightly and permanently bound, and the enzyme is fully active at basal Ca2+ levels (18).

Detailed chemical mechanisms for the NOS reaction have been proposed (1, 2, 19, 20–22) (e.g. see Fig. 5 in Ref. 1). Central to all of the proposed schemes is the close physical approximation of heme iron and the reactive guanidino nitrogen of substrate L-arginine. In previous studies, we have probed possible interactions between these groups using substrate and inhibitor perturbation optical difference spectroscopy (23, 24). In the present studies, we have used electron paramagnetic resonance (EPR) spectra of the heme ferric iron to further elucidate the degree and nature of heme and heme pocket interaction with the substrate, L-arginine, and with the putative heme-binding inhibitory citrulline analog, L-thiocitrulline. EPR spectra of the ferriheme and flavosemiquinone groups of native NOS have previously been reported by Stuehr and Ikeda-Saito (25).

EXPERIMENTAL PROCEDURES

Materials—L-Thiocitrulline (γ-thiourea-L-norvaline) and L-homothiocitrulline (ε-thiourea-L-norleucine) were prepared as described previously (26). Other biochemical reagents were obtained from Sigma.
Enzyme Purification—Rat nNOS was purified from stably transfected kidney 293 cells essentially as described previously (23). A modification of the reported method was made in that enzyme was eluted from the 2′,5′-ADP Sepharose using 2′-AMP instead of NADPH (5 mM 2′-AMP, 0.4 mM NaCl, 50 mM Tris-HCl buffer, pH 7.5). Purified enzyme was obtained in 50 mM Tris-HCl buffer, 10% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.5. Enzyme preparations exhibited specific activities of ~400 nmol/min/mg when assayed by measuring the conversion of L-[14C]arginine to [14C]l-citrulline as described previously (23).

Sample Preparation and Spectroscopy—Enzyme samples were incubated with arginine or inhibitors at 0 °C in a final volume of 0.25 ml. After 5 min, enzyme samples were flash-frozen in quartz EPR tubes (using 1:1 isopentane/cyclohexane cooled with liquid nitrogen) and were held in liquid nitrogen prior to spectroscopic measurements. Enzyme concentrations are expressed on the basis of heme concentration. Spectra were obtained using a Bruker 300 EPR spectrometer and an Oxford optical cryostat. The addition of substrate or inhibitor resulted in <2% change in sample volume. Spectra were acquired at 9.445 GHz using the following instrument parameters: microwave power, 20 mW; modulation frequency, 100 KHz; and modulation amplitude, 14.3 G.

Interpretation of EPR Spectra—For a high spin ferrihemoprotein, the two numerically largest principle values of the g tensor correspond to orthogonal directions in the heme plane and are designated g<sub>x</sub> and g<sub>y</sub>, by convention; g<sub>z</sub> corresponds to molecules with the heme normal along the laboratory magnetic field direction. The positions of g<sub>x</sub>, g<sub>y</sub>, features are primarily determined by E/D, the ratio of the rhombic and axial zero field splitting parameters associated with the high spin state (see "Discussion") (27, 28).

The low spin features associated with Zeeman field orientation in the heme plane are the two numerically smallest values and correspond to g<sub>x</sub> and g<sub>y</sub>; the largest value of g lies approximately along the heme normal. The positions of these features depend directly on the relative energies of the iron t<sub>2g</sub>, d orbitals (29–31).

Simulations of EPR spectra for quantitation were performed using previously described programs (32). Double integration of high spin spectra. EPR, the intensity factor of Aasa and Vaangard (33). Ligand field splittings of low spin ferrihemes were calculated from g tensors as described by Peisach et al. (29).

RESULTS

Binding of L-Arginine to nNOS—As isolated, nNOS contains primarily high spin heme (23). Binding of L-arginine to nNOS causes small changes in the Soret region of the optical spectrum consistent with conversion of the 10–15% of enzyme that is initially low spin to the high spin state (23). Because the optical spectral perturbations mainly reflect changes in spin state equilibrium and are insensitive to small changes in heme iron ligand field geometry, further studies were carried out using EPR, the intensity factor of Aasa and Vaangard (33). Ligand field splittings of low spin ferrihemes were calculated from g tensors as described by Peisach et al. (29).

Fig. 1 (line B) shows the X-band EPR spectrum at 11 °K of nNOS as isolated. Both the high and low spin components of the heme state equilibrium are visible and are completely resolved. Features from the high spin state of the ferric heme are visible near g = 7.65, 4.04, and 1.89. The g = 4.04 feature is overlapped with the g = 4.3 signal from the middle Kramers doublet of adventitious rhombic high spin ferric iron. Because of the high transition probability and nearly isotropic nature of this species, this intense feature represents only a small concentration of contaminating iron in the enzyme preparation or buffer. Partly saturated signals from the low spin state of native nNOS can be observed near g = 2.43, 2.28, and 1.89; the sharp signal at g = 2 is contributed by a flavin radical, probably FMNHR (25). The broad signal near g = 2 is contributed by impurities in the cavity and Dewar assembly.

Addition of L-arginine to nNOS alters the spin state equilibrium in favor of the high spin form; no low spin form remains detectable either at 11 °K (Fig. 1, line A) or at 21 °K (not shown). The amplitude of the low field peak of the high spin form (g = 7.56) increases by ~15% following L-arginine addition. Integration of this slightly narrowed peak indicates that the high spin fraction has increased by 10–12%, indicating that the high spin to low spin ratio for nNOS in the absence of L-arginine is ~90/10. A similar conclusion has been reached through simulation studies comparing the high spin component of nNOS measured without L-arginine and the 100% low spin EPR signal seen when nNOS is saturated with imidazole, a heme-binding ligand. The temperature dependence of the EPR signals could be fit by Curie Law behavior for the low spin features up to 40 °K, where signal to noise problems became significant. For high spin signals, a higher temperature decreases the distribution within the S = 5/2 sextet with D = -3.8 cm<sup>-1</sup> produced a good fit. Although future studies might provide a more accurate measurement of D, the current result indicates that there is no temperature dependence in the spin state equilibrium below 25 °K.

It is apparent from results shown in Fig. 1 that the high spin species observed in the presence and absence of L-arginine are similar but not identical. In Fig. 2, the region between 0.07 and 0.21 tesla is displayed for the purpose of more detailed comparison. The calculated g values of the high spin features of the enzyme as isolated are 7.65 and 4.04 (line B); the latter g value is difficult to measure precisely due to the overlap with the rhombic ferric iron contaminant. The small shoulder on the high field side of the g = 7.65 peak suggests that the enzyme as isolated also contains a minority high spin species. In the presence of L-arginine, the corresponding high spin g values are 7.56 and 4.09 (line A). The narrowing of the EPR signals on L-arginine binding is even more pronounced in the g = 4 region than near g = 7.6. Note also that comparison of line A and line B shows that virtually none (<10%) of the original high spin species remains after addition of L-arginine, a finding indicating complete conversion of the original high and low spin species to a distinct high spin enzyme-arginine complex (i.e. L-arginine binds to both the low and high spin forms of nNOS).

Fig. 2 (line A-B) shows the difference spectrum of the L-arginine-saturated enzyme versus the enzyme as isolated. The
The low field peak has a value of 7.33. The difference spectrum for \( \text{L-arginine} \) was of the resting enzyme, with the difference (A-B) shown as the final spectrum of the figure.

The high spin features of thenNOS-thiocitrulline complex in the presence of \( \text{L-thiocitrulline} \) (Fig. 4) shows two new low spin species with principal \( g \) values of 2.47, 2.27, and 1.89 (line B), the nNOS-thiocitrulline complex shows two new low spin species with principal \( g \) values of 2.47, 2.27, and 1.89 (line B). Assignment of the last \( g \) value in each set was made on the basis of crystal field theory; the assignment given produced an orbital reduction factor closer to 1 for both species than the reverse assignment.

It is notable that the percentage of ferriheme in the high spin state in the presence of \( \text{L-thiocitrulline} \) as measured by EPR at 11 °K increases slightly in comparison with the enzyme as isolated. This can be appreciated from the decrease in the low spin signals shown in Fig. 4 and also from the slight increase in the high spin signals seen following \( \text{L-thiocitrulline} \) binding in Fig. 3 (compare line A with line C or note the relatively larger size of the positive lobes in the difference spectrum, line A-C). This finding is in contrast to the increase in low spin form following \( \text{L-thiocitrulline} \) binding at room temperature deduced from changes in the optical spectrum in the Soret region (24) and is reminiscent of other temperature-sensitive spin state equilibria (34).

The high spin features of the nNOS-thiocitrulline complex in the \( g = 4 \) region are more complex. Two components of approximately equal amplitude can be resolved at \( g \) values of about 3.98 and 3.83. Neither of these features is associated with the species responsible for the minority line at \( g = 7.33 \); the amplitudes are mismatched, and the position of the peaks in the \( g = 4 \) region is inappropriate (see "Discussion"). They must therefore be the \( g \) features of two high spin components, which are unresolved at \( g_x = 7.7 \). The difference spectrum for \( \text{L-thiocitrulline} \) binding is shown as line A-C in Fig. 3. The relative positions of the positive and negative lobes illustrate that the general effect of \( \text{L-thiocitrulline} \) binding is an increase in the rhombicity of the high spin state.

The low spin state species associated with nNOS as isolated and nNOS in the presence of \( \text{L-thiocitrulline} \) are compared in Fig. 4. Whereas the native enzyme shows a low spin species with features at 2.43, 2.28, and 1.89 (line B), the nNOS-thiocitrulline complex shows two new low spin species with principal \( g \) values of 2.47, 2.27, and 1.89, and 2.39, 2.27, and 1.90 (line C). Assignment of the last \( g \) value in each set was made on the basis of crystal field theory; the assignment given produced an orbital reduction factor closer to 1 for both species than the reverse assignment.

It is notable that the percentage of ferriheme in the high spin state in the presence of \( \text{L-thiocitrulline} \) as measured by EPR at 11 °K increases slightly in comparison with the enzyme as isolated. This can be appreciated from the decrease in the low spin signals shown in Fig. 4 and also from the slight increase in the high spin signals seen following \( \text{L-thiocitrulline} \) binding in Fig. 3 (compare line A with line C or note the relatively larger size of the positive lobes in the difference spectrum, line A-C). This finding is in contrast to the increase in low spin form following \( \text{L-thiocitrulline} \) binding at room temperature deduced from changes in the optical spectrum in the Soret region (24) and is reminiscent of other temperature-sensitive spin state equilibria (34).
L-thiocitrulline (line A). A minority peak is resolved at g = 7.33; this species represents a larger fraction of the total high spin content (~10%) than the similar species observed in the presence of L-thiocitrulline. The corresponding minority g_z feature is now resolved at g = 4.46.

Direct comparison of the spectral features of the nNOS-thiocitrulline and nNOS-homothiocitrulline spectra (Fig. 3, lines A and B) shows that the majority L-homothiocitrulline complex is intermediate in character between the two unresolved major L-thiocitrulline complexes. This is most readily apparent from the g_y feature of the majority L-homothiocitrulline complex (g = 3.9), which lies between the two L-thiocitrulline-induced g_y features (g = 3.98 and 3.83). The shifts in the positions of these high spin peaks and the shift in the spin state equilibrium toward the high spin state upon L-homothiocitrulline binding to nNOS are confirmed in the difference spectrum (Fig. 3, line B-C). Only weak low spin ferriheme features could be observed for L-homothiocitrulline at temperatures between 7 and 25 °C, consistent with the type I spectral changes induced at room temperature (data not shown).

**DISCUSSION**

In previous studies, we have used substrate- or inhibitor-perturbation optical difference spectroscopy to establish that L-arginine, NOH-Arg (23), L-thiocitrulline, and L-homothiocitrulline (24) alter the spin state equilibria of the heme cofactor of nNOS. Such studies were not able, however, to distinguish or structurally characterize specific high or low spin enzyme-ligand complexes. In the present studies, we show that EPR spectroscopy can be used not only to confirm the central findings of the optical studies but also to provide for each substrate or inhibitor a spectroscopic "fingerprint" characteristic of its interaction with the heme and heme-binding pocket.

Table I summarizes the findings with respect to the species detected. In addition, the sensitivity of EPR spectra to ligand structure provides structural information on the binding of heme to nNOS and establishes a powerful probe of the interactions between the O_2-binding position of heme and the L-arginine-binding site.

The power of EPR spectroscopy for these purposes derives from the exquisite sensitivity of the iron d orbitals in ferriheme to both the nature and number of axial ligands (1 or 2) and to subtle, heme-binding pocket-induced perturbations of the iron ligation geometry. More specifically, the features of the high spin nNOS EPR spectra near g = 7.6, 4, and 1.8 are associated with transitions within the lowest Kramer’s doublet of the S = 5/2 sextet of high spin ferriheme. The g_δ = 7.6 and g_ε = 4 features derive from enzyme molecules in which the plane of the heme lies in the x and y direction of the Zeeman magnetic field; the z direction of the field (normal to the heme) gives rise to a transition near g_z = 1.8 (Table I). As long as intermixing of the Kramer’s doublets by Zeeman terms (~0.3 cm⁻¹) is small, the spectra are controlled by the ratio of E/D, where E is the rhombic zero field splitting parameter and D is the axial zero field splitting parameter (27, 28). Terms in E mix the three S = 5/2 Kramer’s doublets, splitting g_y and g_z about an axial value of ~6. These considerations were used to help assign the features shown in Figs. 1–4 (eg. in Fig. 3, line B the g_y = 7.33 peak could only be matched by a corresponding g_x feature near 4.35).

Because E and D are determined by the ligand-induced splittings of the iron d orbitals, they reflect both the identity of the axial ligands and the geometry of the heme site. For high spin nNOS, the only axial heme ligand is provided by an nNOS amino acid residue; in all cases E/D (Table I) was in the range characteristic of enzymes and model complexes having thiolate ligands (29, 35). This finding directly confirms predictions of cysteine residue participation based on sequence analysis (10, 12), spectral studies (11, 36), and work using mutational analysis (13, 16).

Our data indicate that an enzyme thiolate-heme axial ligand bond similar to that of native nNOS persists in the complexes of nNOS with L-arginine, L-thiocitrulline, and L-homothiocitrulline, but it is notable that each of these ligands causes a characteristic shift in the EPR spectrum reflecting changes in E/D. Such changes indicate that binding of substrate or inhibitors induces a (probably local) conformational change in the enzyme that is reflected in a change in the geometry of the heme site. Note that none of the changes seen in the high spin spectral features are attributable to ligand-iron bond formation; if L-arginine contributed a sixth axial heme ligand, it would drive the heme low spin. This is not seen with the nNOS-arginine complex, a finding consistent with the fact that CO or O_2 can still bind to the heme of such a complex in the ferrous state and that CN⁻ can bind to the ferric complexes. The fact that native nNOS shows none of the ferriheme EPR spectral features of the nNOS-arginine complex indicates that nNOS as isolated does not contain L-arginine or any intermediates or substrate analogs that would distort the heme-binding pocket as L-arginine does. On the other hand, the finding that nNOS as isolated has at least 10% low spin heme suggests that in this fraction of the enzyme water or an adjacent residue in the active site is serving as a sixth axial heme ligand. The g values of the native nNOS low spin species indicate that the
that in normal catalysis, heme-bound oxygen first reacts with a nitrogenous sixth ligand, probably derived from an amino acid side chain. Whether these species represent alternative ligand structures is unclear. We note that the iron signal for nNOS as isolated (HS); majority 5/2 7.65 4.04 1.8 0.079 5.66 1.9
nNOS as isolated (LS) 1/2 1.89 2.28 2.43 0.075
nNOS-arginine (HS); majority 5/2 7.56 4.09 1.8 0.081
nNOS-thiocitrulline (HSA); major 5/2 7.68 3.98 1.8 0.087
nNOS-thiocitrulline (HSb); major 5/2 7.76 3.83 1.8 0.087
nNOS-thiocitrulline (LSa) 1/2 1.87 2.27 2.47 5.46 2.02
nNOS-thiocitrulline (LSb) 1/2 1.9 2.27 2.39 5.96 1.83
nNOS-homothiocitrulline (HS); majority 5/2 7.75 3.9 1.8 0.085
nNOS-homothiocitrulline (HS) 5/2 7.33 4.46 (1.83) 0.063

The results shown in Fig. 3 demonstrate that both L-thiocitrulline and L-homothiocitrulline produce changes in the geometry of the heme site detectable by EPR spectroscopy. As indicated by E/D values of 0.081 and 0.087 (Table I), the two major high spin components seen in the presence of L-thiocitrulline are both more rhombic than the high spin form of nNOS as isolated (E/D = 0.079). That is, although the energies of the d_{xz} and d_{yz} orbitals are more nearly equivalent in the arginine complex than in the enzyme as isolated, the inhibitors both appear to distort the heme iron ligation geometry in a way that increases the d_{xz} - d_{yz} splitting. We do not believe the E/D = 0.075 species of the nNOS-thiocitrulline complex is a remnant of the unliganded native state, which it closely resembles, for two reasons. First, the concentration of L-thiocitrulline used (100 μM) is >1000-fold the K_i (24) and presumably saturating. Second, the g_x peak is shifted far enough to low field by L-thiocitrulline that there does not appear to be enough intensity at g values below 7.6 to account for the large component visible at g = -4. The improved resolution of the g = -4 feature may result in part from a small upfield shift, separating it somewhat from the rhombic iron signal.

The minor component of the high spin L-thiocitrulline spectra with g_x = -7.33 has a significantly smaller rhombicity than even the L-arginine complex; E/D is about 0.06 (not listed in Table I). Although the E/D value is low, the enzymatic thiolate ligand probably remains in place even in this complex. In the presence of L-homothiocitrulline, a similar complex (E/D = 0.063) is easily detected and is in sufficient concentration to resolve both the g_x and g_y signals (Fig. 3 and Table I). Detection of multiple species suggests that in contrast to L-arginine, L-thiocitrulline and L-homothiocitrulline share an ability to bind in several different modes giving distinct high spin species. Whether these species represent alternative ligand structures (e.g. thione versus thiol tautomers) or alternative conformational adjustments in the binding site is unclear. We note that in normal catalysis, heme-bound oxygen first reacts with a guanidino nitrogen of L-arginine and that a second heme-bound oxygen then reacts with the guanidino carbon of NOH-Arg. In order to do this, the substrate-binding site may accommodate some slippage. The enzyme may have evolved to bind its normal substrate, L-arginine, uniquely, but L-thiocitrulline and L-homothiocitrulline may bind in either an L-arginine or a NOH-Arg mode. It is worth noting that although binding of L-thiocitrulline leads to the formation of two high spin species with increased rhombicity (compared with the enzyme as isolated and the arginine complex), the longer side chain of L-homothiocitrulline permits the formation of only one such species. The increased formation of the lower rhombicity (g_x = 7.33) species suggests that this represents a binding mode/conformational state in which the extra length of the side chain affects the electronic properties. Whereas the nNOS-arginine (23) and nNOS-homothiocitrulline (24) complexes are exclusively high spin, nNOS forms two distinct minority low spin complexes with L-thiocitrulline (24). As shown in Fig. 4 and summarized in Table I, neither of the nNOS-thiocitrulline low spin species can be attributed to the minority low spin species of nNOS as isolated; in all cases the g values are distinct. Furthermore, crystal field calculations indicate that the values of the axial and rhombic ligand field splittings Δ and V of the t_{2g} set of iron d orbitals for the low spin state of nNOS as isolated are, respectively, about 5.66 and 1.9 (in units of spin orbit coupling) using the axis system introduced originally by Pechs et al. (29). The corresponding parameters for the L-thiocitrulline complexes are about 5.46 and 2.02 for the species with g_x = 2.47 and about 5.96 and 1.83 for the species with g_x = 2.39. If we refer these values to the Blumberg and Pechs crystal field diagrams (29), we find the species fall in region P. This region contains low spin species with thiolate ligands; Δ and V for nNOS low spin species are comparable with ferriceme model complexes with a thiolate ligand described by several groups.

All three low spin species appear to retain the endogenous axial thiolate ligand. The g_x = 2.39 nNOS-thiocitrulline species is similar to model complexes with g_x between 2.33 and 2.42 in which both axial ligands are thiolate sulfurs (35). This supports a model for L-thiocitrulline binding in which the inhibitor is close enough to the ferriheme to form a true coordination complex. Note, however, that this is a minority species; as shown previously in optical studies (24), most L-thiocitrulline is bound without direct sulfur-ferriheme coordination. The identity of the sixth heme ligand in the other two low spin complexes is less certain. Comparison with model complexes suggests that the sixth ligand may be oxygen in both cases. This is almost certainly the case for the native nNOS species with g_x = -2.43, but whether the sixth ligand is derived from solvent or the side chain of an NOS amino acid is unclear. The difference between low spin complex and the g_x = 2.47 complex of L-thiocitrulline could be the result of a small distortion of the original ligand geometry rather than ligand replacement. We note that the g_x = 2.47 peak seen with L-thiocitrulline is also consistent with a nitrogen ligand as might be expected if the inhibitor bound with the terminal thiourea nitrogen rather
than the sulfur near heme. L-Citrulline, a product of NOS, does not inhibit \((K_i > 10 \text{ mM})\), although it could presumably bind similarly (i.e. with the thione nitrogen in the heme pocket). The present results, taken with previous kinetic and optical spectroscopy studies (24), demonstrate that L-arginine, L-thiocitrulline, and L-homothiocitrulline all bind tightly \((K_m \text{ and } K_i < 0.06-10 \mu M)\) but distinctly to nNOS. Because binding is in all cases enantiomer-specific, the enzyme must associate similarly with the \(\alpha\)-amino and carboxylate groups of the three species. On the other hand, the L-arginine side chain clearly adopts a high spin binding mode that is distinct from the multiple high spin binding modes seen with L-thiocitrulline and L-homothiocitrulline. The guanidino group of L-arginine does not contribute a sixth axial ligand to the heme cofactor or cause dissociation of the thiolate ligand but nonetheless converts the high spin state associated with nNOS as isolated to a high spin state with different properties.

Because E and D, and hence the EPR spectra, are primarily a function of the ligand field splittings of the iron \(d\) orbitals, binding of arginine (and arginine analogs) must perturb the ligation geometry by more subtle interactions in the heme pocket. It does this presumably by directly interacting with and perturbing the heme porphyrin ring or by interacting with nNOS residues forming the heme pocket. This could transmit strain to the iron through interactions between the polypeptide and the porphyrin periphery or through the axial thiolate ligand. Whereas the high spin nNOS-arginine complex is less rhombic (lower \(g_x\)) than the high spin form of nNOS as isolated, the majority high spin forms of nNOS-thiocitrulline and nNOS-homothiocitrulline are more rhombic (higher \(g_x\)). These species, in which the ligand does not directly coordinate heme iron, must perturb the heme porphyrin or its binding pocket differently than does L-arginine.

Although nNOS may have evolved to bind L-arginine uniquely, these synthetic inhibitors are bound in multiple modes with at least three high spin and two low spin states for L-thiocitrulline. It seems clear that arginine analogs with various substituents binding in the site normally occupied by the reactive guanidino nitrogen produce characteristic modifications of ligand-heme pocket interactions, which are reflected in characteristic spectroscopic signatures for each complex. Because this group is the initial hydroxylation site, it is apparent that only short range interactions need be invoked and that this part of the substrate-binding site interacts intimately with the heme. It will be interesting to explore the extent to which various EPR-detectable heme pocket perturbations are induced by ligand binding as specific structural changes are made to substrates and to inhibitors such as L-thiocitrulline.

REFERENCES

1. Griffith, O. W., and Stuehr, D. J. (1995) Annu. Rev. Physiol. 57, 707–736
2. Masters, B. S. S. (1994) Annu. Rev. Nutr. 14, 131–145
3. Sessa, W. C. (1994) J. Vasc. Res. 31, 131–143
4. Brett, D. S., and Snyder, S. H. (1994) Annu. Rev. Biochem. 63, 175–195
5. Alisa, K., Groes, S. S., Griffith, O. W., and Levi, R. (1989) Biochem. Biophys. Res. Commun. 160, 881–886
6. Rees, D. D., Palmer, R. M. J., and Moncada, S. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3375–3378
7. Nathan, C. F., and Hibiis, J. B. J. (1991) Curr. Opin. Immunol. 3, 65–70
8. Nathan, C., and Xie, Q. (1994) Cell 78, 915–918
9. Stuehr, D. J., Kwon, N. S., Nathan, C. F., Griffith, O. W., Feldman, P. L., and Wiseman, J. (1991) J. Biol. Chem. 266, 6259–6263
10. Brett, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C. E., Reed, R. R., and Snyder, S. H. (1991) Science 251, 714–718
11. McMillan, K., Brett, D. S., Hirsch, D. J., Snyder, S. H., Clark, J. E., and Masters, B. S. S. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11141–11145
12. Renard, J. P., Boucher, J. L., Vadon, S., Delaforgue, M., and Mansuy, D. (1993) Biochem. Biophys. Res. Commun. 197, 53–60
13. Chen, P. F., Tsai, A. L., and Wu, K. K. (1994) J. Biol. Chem. 269, 25062–25066
14. Sheha, E. A., McMillan, K., and Masters, B. S. S. (1994) J. Biol. Chem. 269, 15147–15153
15. Ghosh, D. K., and Stuehr, D. J. (1995) Biochemistry 34, 801–807
16. McMillan, K., and Masters, B. S. S. (1995) Biochemistry 34, 3686–3693
17. Abu-Soud, H. M., and Stuehr, D. J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10769–10772
18. Cho, H. J., Xie, Q., Calaycay, J., Mumford, R. A., Swidersk, K. M., Lee, T. D., Nathan, C. (1992) J. Exp. Med. 176, 599–604
19. Stuehr, D. J., and Griffith, O. W. (1992) Adv. Enzymol. Relat. Areas Mol. Biol. 65, 287–346
20. Feldman, P. L., Griffith, O. W., and Stuehr, D. J. (1993) Chem. Eng. News 71 (51), 26–38
21. Marletta, M. A. (1993) J. Biol. Chem. 268, 12231–12234
22. Klatt, P., Schmidt, K., Uray, G., and Mayer, B. (1993) J. Biol. Chem. 268, 14781–14787
23. McMillan, K., and Masters, B. S. S. (1993) Biochemistry 32, 9875–9880
24. Frey, C., Narayan, K., McMillan, K., Spack, L., Gross, S. S., Masters, B. S., and Griffith, D. W. (1994) J. Biol. Chem. 269, 26383–26391
25. Stuehr, D. J., and Ikeda-Saito, M. (1992) J. Biol. Chem. 267, 20547–20550
26. Narayan, K., and Griffith, O. W. (1994) J. Med. Chem. 37, 885–887
27. Eisenberger, P., and Pershans, P. S. J. (1966) J. Chem. Phys. 45, 2832–2835
28. Slappendel, S., Veldink, G. A., Vliegenthart, J. F. G., Aasa, R., and Malmstrom, B. (1981) Biochim. Biophys. Acta 667, 77–86
29. Pelsach, J., Blumberg, W. E., and Adler, A. (1973) Annu. N. Y. Acad. Sci. 206, 310–327
30. Bohan, T. (1977) J. Magn. Reson. 26, 109–118
31. Taylor, C. P. S. (1977) Biochim. Biophys. Acta 491, 137–149
32. Salerno, J. C., Lim, J., King, T. E., Blum, H., and Ohnishi, T. (1979) J. Biol. Chem. 254, 4628–4635
33. Aasa, R., and Vaangard, T. (1975) J. Magn. Reson. 19, 308–315
34. Vaangard, T. (1985) Biochem. Soc. Trans. 13, 619–622
35. Soo, M., and Dawson, J. H. (1982) J. Biol. Chem. 257, 5496–5502
36. Wang, J., Stuehr, D. J., Ikeda-Saito, M., and Rousseau, D. L. (1993) J. Biol. Chem. 268, 22255–22258
37. Kotani, M. (1969) Annu. N. Y. Acad. Sci. 158, 20–49
Characterization by Electron Paramagnetic Resonance of the Interactions of L-Arginine and L-Thiocitrulline with the Heme Cofactor Region of Nitric Oxide Synthase
John C. Salerno, Christopher Frey, Kirk McMillan, Robert F. Williams, Bettie Sue Siler Masters and Owen W. Griffith

J. Biol. Chem. 1995, 270:27423-27428.
doi: 10.1074/jbc.270.46.27423

Access the most updated version of this article at http://www.jbc.org/content/270/46/27423

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 37 references, 14 of which can be accessed free at http://www.jbc.org/content/270/46/27423.full.html#ref-list-1