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Role of substrate functional groups in binding to nitric oxide synthase

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ABSTRACT

The interactions between the heme CO ligand in the oxygenase domain of nitric oxide synthase and a set of substrate analogues were determined by measuring the resonance Raman spectra of the Fe–C–O vibrational modes. Substrates were selected that have variations in all the functional units: the guanidino group, the amino acid site and the number of methylene units connecting the two ends. In comparison to the substrate free form of the enzyme, Interactions of the analogues with the CO moiety caused the Fe–CO stretching and the Fe–C–O bending modes to shift in frequency due to the electrostatic environment. An unmodified guanidino group interacted with the CO in a similar fashion despite changes in the amino acid end. However, an unmodified amino acid end is required for catalysis owing to the H-bonding network involving the substrate, the heme and the pterin cofactor.

Nitric oxide synthase (NOS) catalyzes the synthesis of NO in a two-step mechanism in which the reaction of L-Arg with oxygen forms *N*-hydroxy-L-Arginine (NOHA) which in turn reacts with another molecule of oxygen to yield NO and citrulline. In addition to L-Arg and NOHA, only two other L-Arg derivatives (NMA and homoarginine) have been shown to be substrates for NOS [1–3]. Three major mammalian isoforms of the enzyme have been identified, two constitutive forms from brain (nNOS) and endothelium cells (eNOS), and one inducible form (iNOS), from marcrophage [4,5]. The functional form of all three isoforms are homodimeric with a heme-containing oxygenase domain incorporating a tetrahydrobiopterin (H4B) cofactor binding site, and a reductase domain that has the site for the electron donor, NADPH. The electron transfer to the oxygenase domain is enabled by the binding of Ca-calmodulin.

The NOS substrates possess multiple points for interaction with the enzyme. Both the positively charged guanidino group and the amino acid end interact directly with either amino acid residues or prosthetic groups in the active site as revealed by crystal structure studies [6,7]. The length of the intervening methylene groups also affects the binding and activity [8]. The similarity of the substrate binding sites in each of the isoforms has made the development of isoform-specific inhibitors a challenging task. A major advance in selective inhibitor design was made recently by constructing inhibitors that can bind in the substrate site but also have bulky tails that can bind in remote sites [9]. These remote sites differ among the isoforms so outstanding selectivity can be achieved. As these inhibitors need structures to interact both with the normal substrate site as well as the remote site a clear understanding of the interactions in the substrate binding site are of crucial importance for efficient inhibitor design.

To identify substrate-enzyme interactions in NOS, resonance Raman spectroscopy coupled with small molecular probes has proven to be a powerful tool as it reflects the position of the substrate or substrate analogue with the bound ligand [10,11]. Well before the determination of NOS structures, it was established that substrates strongly interact with heme bound ligands and the interactions are dependent on the nature of bound substrate [10]. Also, structural differences among the three NOS isoforms were identified in which the interaction of the substrate with the ligand was the same in iNOS and eNOS and distinct from that in nNOS [12]. This work reports the studies of the interaction of a bound-CO ligand of $iNOS_{oxy}$ with substrates and analogues with variations in all the functional units: the guanidino group, the number of methylene groups and the amino acid site (Fig. 1).

Materials and methods

L-thiocitrulline and *S*-methyl-thiocitrulline were obtained from Calbiochem (La Jolla, CA) and all of the others substrates and

Abbreviations: HomoArg, L-homoarginine; NOHA, N^{ω}-hydroxy-L-arginine; NMA, N^{ω}-methyl-L-arginine; ArgA, argininic acid; Agmt, agmatine; AGPA, L-2-amino-3-guanidino-propionic acid; L-NIO, L-*N*5-(I-iminoethyl)-ornithine; Cit, L-citrulline; ThioCit, L-thiocitrulline; SMTC, *S*-methyl-thiocitrulline; $iNOS_{oxy}$, $nNOS_{oxy}$ and $eNOS_{oxy}$, oxygenase domains of the inducible, neuronal and endothelial isoforms of nitric oxide synthase; BH4, tetrahydrobioptopterin; bsNOS, Bacillus subtilis nitric oxide synthase

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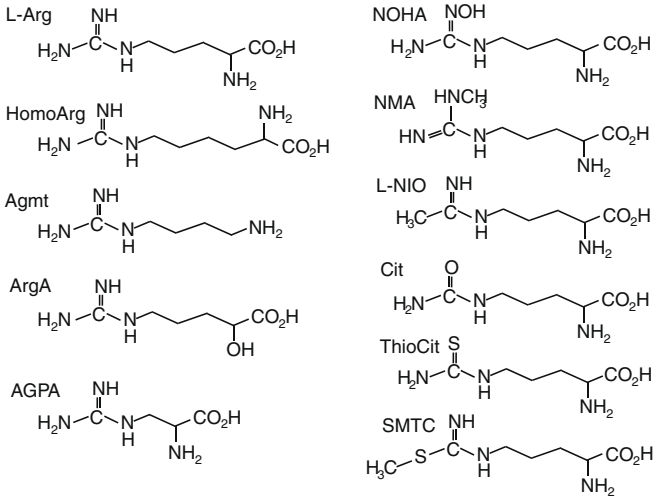

Fig. 1. Structures of the NOS substrate analogs used in this study.

analogues were purchased from Sigma (St. Louis, MO). Carbon monoxide was purchased from Matheson (CP grade, 99.5%).

The expression and reconstitution of $iNOS_{oxy}$ was described previously [13]. Samples were buffered (pH 7.6, 40 mM Bis–Tris with 1 mM of dithiothreitol) and mixed with substrates or inhibitors. The final heme and substrate analog concentrations were 30 μM and 3 mM, respectively. Ferrous CO-adducts of the enzyme were prepared by reducing the sample in a sealed Raman cell with 5-fold excess of sodium dithionite, followed by exposure to the CO gas and monitored by optical absorption. To avoid any spectral complications from H4B [14], the pterin was not introduced in any of the samples.

Resonance Raman measurements were performed with a 1.25 m polychromator (Spex) equipped with a CCD detector (Princeton Instruments). The signal was collected in a right angle geometry with a 100 micron entrance slit. Excitation at 441.6 nm, provided by a He–Cd laser (Liconix), was used to selectively enhance the active form of the CO-bound enzyme which has an absorption maximum at ~445 nm [14]. Spectra were calibrated with lines from toluene and an aqueous solution of sodium ferrocyanide. Data processing and analysis were performed with routines provided by Grams386 (Galactic Industries Corp.).

Results

Arginine analogs were introduced into the CO-bound complex of $iNOS_{oxy}$ and the resonance Raman spectra were measured. The orientation of the substrate with respect to the CO, and also the bound oxygen during catalytic activity, is determined by the H-bonding interactions between the substrate, or analogue, and the residues in the catalytic site. The binding of native substrates, such as L-Arg, involves H-bonding interactions to both the guanidino end and the amino acid end of the molecule as shown in Fig. 2 for the oxygenase domain of nNOS. The guanidino end is held in place by Glu-592 (the nNOS sequence numbering is used throughout) and by an H-bond to the polypeptide carbonyl group of Trp-587. The carboxylate group of the amino acid end of L-Arg is H-bonded to Tyr-588 and the amino group is H-bonded to Glu-592 and to one of the carboxylate oxygen atoms of a heme propionate group. The other oxygen atom of the propionate group is H-bonded to the pterin cofactor. As shown in Fig. 2A, when CO is present there is an H-bonding interaction to it from the NH group of the L-Arg and from a water molecule coordinated in the distal pocket. The distance of each of these groups to the oxygen atom of the CO is ~3 Å.

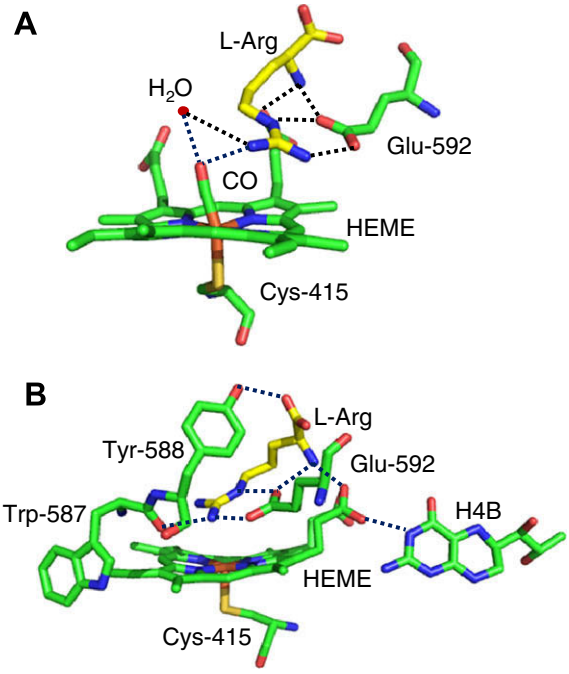

Fig. 2. The structure of the L-arginine-bound $nNOS_{oxy}$ catalytic site. The models were depicted with PyMOL from the structure (2G6M) reported by Li et al. [7]. (A) The interactions between the CO heme-ligand and the L-Arg and water molecule in the distal pocket. (B) The H-bonding interaction that stabilize the L-Arg substrate in the catalytic site.

To determine the affect of these two ends of the Arg and its analogues on their juxtaposition with respect to the heme ligand, the selected analogues are divided into two groups: those in which the native guanidino group is present and those in which the amino acid end of the molecule has its native substituents (Fig. 1). By this organization, the influence of each end of the analogues on the interactions at the heme catalytic site can be evaluated. For the native guanidino group, HomoArg and AGPA represent cases in which the methylene spacing between the two ends is either longer or shorter, respectively, than that in L-Arg. Agmt and ArgA have modified amino acid ends. For those cases in which the amino acid terminal is intact some of the molecules have derivatized NH groups in the guanidino end, such as NOHA, a native substrate, and NMA. All of the others, including the final product, Cit have one of the terminal NH groups replaced by a different atom or atoms.

To measure the interactions between each of the analogues and the catalytic site, the vibrational modes of heme-bound CO, an oxygen surrogate, were examined. The Fe–CO vibrational modes are very sensitive to the electrostatic environment owing to changes in the back-bonding between the heme and the CO as reflected in the relationship below relating the limiting case structures [15].

$$Fe^{\delta-} - C \equiv O^{\delta-}(I) \leftrightarrow Fe{=}C{=}O(II)$$

A positively charged distal environment shifts the equilibrium toward structure II, thereby increasing the Fe–CO bond strength and hence its stretching mode frequency and concomitantly decreasing the C–O bond strength and its associated stretching mode frequency as compared to structure I.

Several prior studies of the effect of substrates and their analogues on the FeCO modes in the NOS isoforms have been reported and have revealed important properties of the heme active site [10,11,14]. In the absence of substrates and the pterin cofactor the Fe–CO stretching mode is broad and can be deconvoluted into two or three different components reflecting alternate conformations in the distal pocket accessible in the absence of the substrates