NO Synthase Isozymes Have Distinct Substrate Binding Sites†

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ABSTRACT: The resonance Raman spectra of the carbon monoxide (CO) derivatives of nitric oxide synthases (NOSs), in which CO coordinates to the heme at the site occupied by oxygen under physiological conditions, are very sensitive to the presence of substrates and inhibitors. Significant differences in the modes associated with the bound CO are now found to depend on the isoenzyme. In the presence of L-arginine, the physiological substrate, the frequencies of the Fe−CO stretching mode and the C−O stretching mode in nNOS, the brain enzyme, are detected at 503 and 1929 cm^{-1}, respectively; whereas in iNOS, the inducible enzyme from macrophage, the modes are detected at 512 and 1906 cm^{-1}, respectively. The frequencies in eNOS, the endothelial isozyme, are similar to those of iNOS. These results indicate that nNOS has a much more open substrate-binding pocket than iNOS and eNOS. A theoretical simulation based on the interaction between the CO and a positively charged guanidino group on the arginine indicates that the polar environment of the CO differs markedly between the isozymes. This may be accounted for either by an arginine−CO distance that is as much as 1 Å greater in nNOS than in iNOS and eNOS or by a substantial shielding of the charge on the arginine in nNOS as compared to the other isozymes. This is the first reported detection of a structural difference of the substrate binding sites between the isozymes and serves as an initial step in a rational drug design for NOS.

Nitric oxide (NO), a small gaseous molecule, has been found to play many diverse roles in physiological and pathophysiological processes (Forstermann et al., 1994). NO is made from a homodimeric family of enzymes termed nitric oxide synthases (NOSs)[1] (Griffith & Stuehr, 1995). There are three NOS isozymes: nNOS, first isolated from neurons in the brain; eNOS, initially detected in endothelial cells; and iNOS, an inducible NOS, first found in macrophages. The enzymes oxidize arginine to NO and citrulline in a stepwise process. All three NOS isozymes contain FAD, FMN, tetrahydrobiopterin, and iron protoporphyrin IX prosthetic groups (Marletta, 1994). The flavins facilitate the electron transfer from NADPH to the heme iron, the catalytic center. In the constitutive isozymes, nNOS and eNOS, electron transfer only occurs when calmodulin binds to the protein in response to an influx of calcium. In iNOS, the calmodulin is tightly coupled to the protein so there is no requirement for calcium. For nNOS, NO production has been related to synaptic plasticity in the central nervous systems, but overproduction of NO can result in neuroinjury (Dawson & Dawson, 1996). NO formed in the vascular system by eNOS regulates blood pressure by dilating blood vessels through the activation of soluble guanylyl cyclase which increases cGMP in smooth muscle (Dinerman et al., 1993); inhibition of the enzyme results in vasoconstriction and elevated blood pressure. The iNOS isozyme is induced in macrophage and other cells by cytokines. It produces NO that can bind to iron centers of different enzymes in the invading pathogens to regulate their activity, but overproduction of NO can lead to circulatory shock and inflammation (Szabo, 1996).

The need for selective inhibitors of NOS isozymes is clear in order to be able to control the production of NO in certain cells but not in others. Owing to the complexity of the enzyme there are many targeted sites for inhibition, including coordination to the heme iron, blockage of the electron transfer pathway, and competitive coordination to the substrate binding site. The latter is the most attractive point of inhibition; indeed, many arginine analogues bind in the substrate binding site and show a varying degree of isozyme specificity. For example *S*-alkylthiocitrulline (Furfine et al., 1994) and cyclopropyl-L-arginine (Lambert et al., 1992) selectively inhibit nNOS; L-*N*-(1-aminoethyl)lysine (Connor et al., 1995), certain guanidines (Misco et al., 1993), and isothiourea derivatives (Garvey et al., 1994) selectively inhibit iNOS. The mechanisms of the selective inhibition are unknown although they must be consequences of differences in the structures of the substrate binding sites. For a quantitative understanding of the differences, a probe of the structure of the substrate binding site is needed. Unfortunately, studies by other techniques on the complex between NOS isozymes and their native substrate, L-arginine,

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[1] Abbreviations: BH_4, tetrahydrobiopterin; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; ν_{Fe-CO}, the iron−carbon monoxide stretching mode; δ_{Fe-C-O}, the iron−carbon−oxygen bending mode; ν_{C-O}, the carbon−oxygen stretching mode.

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have shown no differences. However, although small differences were found in electron paramagnetic resonance spectroscopy studies on NOSs bound by various substrate analogues (Salerno et al., 1995, 1996a,b; Tsai et al., 1996), no structural basis for the observations could be determined (Salerno et al., 1996b).

Resonance Raman spectra of CO-coordinated ferrous hemes have been very useful for the understanding of the properties of the distal pockets of heme proteins since CO is a mimetic of its physiological counterpart, oxygen, and its heme derivatives have high sensitivity to the environment (Yu & Kerr, 1988). CO forms a very stable adduct and both the Fe−CO and the C−O stretching modes (ν_{Fe-CO} and ν_{C-O}, respectively) as well as the Fe−C−O bending mode (δ_{Fe-C-O}) can be detected. Moreover, CO is very sensitive to the structure of the distal pocket. Studies of the resonance Raman spectra of cytochrome P-450 have demonstrated that the frequency of ν_{Fe-CO} varies with the structure of the molecule occupying the substrate binding site in this class of enzymes. When the substrate binding site is vacant, a low frequency for ν_{Fe-CO} is found ($\sim$464 cm^{-1}). However, when it is occupied by various substrates and inhibitors, the frequencies in cytochrome P-450 range from 473 to 485 cm^{-1} (Uno et al., 1985). Corresponding changes are detected in ν_{C-O} as well which have been found to correlate with changes in the spin equilibrium in the ferric derivatives brought about by the same inhibitors (Jung et al., 1996).

Prior studies of the CO adducts of nNOS in our laboratory have established a similar high sensitivity of the CO associated modes in the resonance Raman spectra to the presence of substrate and the properties of the distal environment (Wang et al., 1993, 1997). In this study, we extend the technique to characterize the structural differences among the three NOS isozymes, in an effort to provide a structural basis for differences in their functional properties.

EXPERIMENTAL PROCEDURES

The procedures for the purification of rat nNOS, murine iNOS, and bovine eNOS used in this study have been described previously (Stuehr & Ikeda-Saito, 1992; Wu et al., 1996). The nNOS is the principal full-length transcript of the enzyme (Elliasson et al., 1997). The eNOS was a generous gift from Dr. K. K. Wu. L-Arginine was purchased from Sigma Chemicals and used without further purification. Natural abundance carbon monoxide was purchased from Matheson Inc. (CP grade, 99.5%). Isotopically labeled $^{13}C^{18}O$ (99% ^{13}C, 99% ^{18}O) was purchased from ICON, Inc.

For the resonance Raman measurements, samples were dissolved in 40 mM Bis-Tris Buffer at pH 7.6 containing 1 mM dithiothreitol (DTT). The enzyme concentration was maintained at about 30 μM for all the measurements. CO adducts of the enzyme were generated by first reducing the enzyme using sodium dithionite under anaerobic conditions, followed by introducing either naturally abundant or isotopically labeled CO gas into the sealed Raman cell. The conversion from the ferrous form to the CO bound form was confirmed by measuring the optical absorption spectra.

UV−vis absorption was monitored using a Shimadzu UV2100 recording spectrophotometer. Resonance Raman measurements were performed with previously described instrumentation (Wang et al., 1996) in which the scattered light is dispersed by a 1.25 m polychrometer (Spex) and detected by a liquid nitrogen cooled CCD camera (Princeton Instruments). In the absence of L-arginine and tetrahydrobiopterin, small portions of the CO-bound enzymes exist in inactive forms with an absorption maximum at $\sim$420 nm (Wang et al., 1995). An excitation wavelength of 441.6 nm provided from a Liconix He-Cd laser was used to selectively enhance the active form of the enzyme which has an absorption maximum at 444 nm. Low laser power was used ($<$1 mW) to avoid possible CO photolysis. Raman frequencies were calibrated using lines from indene, toluene, and ferrocyanide. The Raman data were baseline corrected but not smoothed. Spectral analysis was performed using routines provided by GRAMS386 (Galactic). In the curve fitting procedures, no peak parameters (band width, position, or shape) were fixed.

RESULTS

Resonance Raman studies of the CO adducts of nNOS have established a sensitivity of the CO-associated modes to the presence of substrate and the properties of the distal environment (Wang et al., 1997). In the absence of substrate but in the presence of tetrahydrobiopterin (BH_4), a broad line in the ν_{Fe-CO} region centered at 491 cm^{-1} was observed for nNOS. It could be deconvoluted into three components: 487, 501, and 512 cm^{-1}, the first two of which were assigned as components of the ν_{Fe-CO} mode and the last as a porphyrin mode. The δ_{Fe-C-O} mode was located at 562 cm^{-1}. When either L-arginine or *N*-hydroxy-L-arginine was added to the enzyme, ν_{Fe-CO} displayed only a single line at 503 cm^{-1} and δ_{Fe-C-O} was detected at 566 cm^{-1}. Corresponding behavior was seen for ν_{C-O} with a doublet at 1949 and 1930 cm^{-1} in the absence of substrate and only a single line at 1929 cm^{-1} in the presence of either L-arginine or *N*-hydroxy-L-arginine (Wang et al., 1997). This experiment was repeated in the present study to ensure a direct comparison between the isozymes under exactly the same conditions (see Figure 1, spectrum A).

In the absence of both arginine and BH_4, the ν_{Fe-CO} mode of iNOS is also broad but is found at slightly lower frequency (487 cm^{-1}) (Figure 1, spectrum F) and could be deconvoluted into two lines (479 and 499 cm^{-1}). A much weaker line, δ_{Fe-C-O}, is observed at 560 cm^{-1}, similar to that observed in nNOS. The ν_{C-O} mode was observed as a broad band centered at 1945 cm^{-1} (Figure 2, spectra A−C). Introducing BH_4 causes changes in the ν_{Fe-CO} mode region, primarily by increasing a shoulder at 512 cm^{-1} (data not shown). The frequencies obtained after deconvolution are listed in Table 1. The effects of BH_4 in the absence of arginine are very complex and are discussed in a separate article (J. Wang, et al., submitted for publication). The assignment of these vibrational modes involving the bound CO were confirmed by isotopic substitution experiments (See Figure 1 and 2).

Substrate binding causes a much larger change in the frequency of ν_{Fe-CO} in iNOS than in nNOS; lines are detected at 512 and 568 cm^{-1} in iNOS (Figure 1, spectrum C). The data are summarized in Table 1. Upon changing from $^{12}C^{16}O$ to $^{13}C^{18}O$, these lines shift to 499 and 551 cm^{-1}, respectively (Figure 1, spectrum D and difference spectrum E) confirming their assignment to ν_{Fe-CO} and δ_{Fe-C-O}, respectively. The corresponding modes are found at nearly the same frequencies in eNOS (Figure 1, spectrum B). Thus, in the presence of substrate for both iNOS and eNOS, ν_{Fe-CO} is at a much higher frequency than in nNOS in which it was detected at

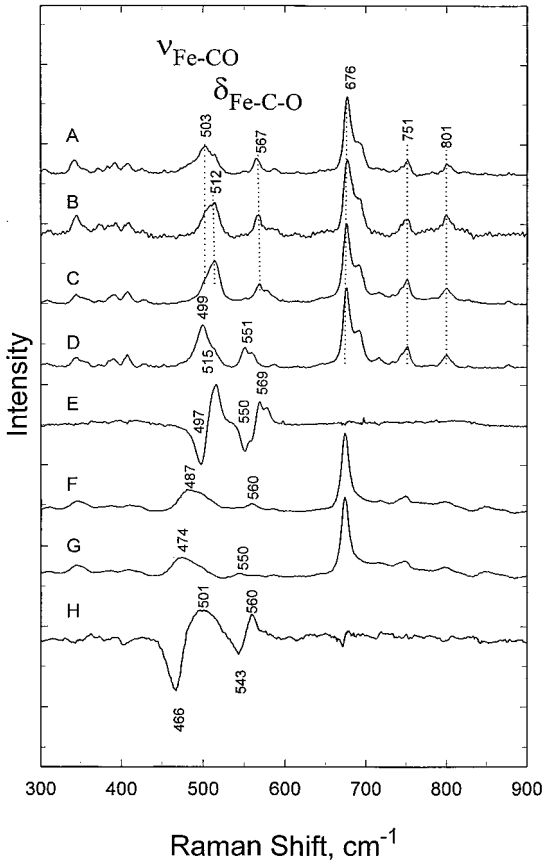


FIGURE 1: Low frequency resonance Raman spectra of the CO-bound ferrous NOS isozymes. (A) $^{12}C^{16}O$-bound nNOS (30 μM) in the presence of L-arginine (5 mM). (B) $^{12}C^{16}O$-bound eNOS (30 μM) in the presence of L-arginine (5 mM). (C) $^{12}C^{16}O$-bound iNOS (30 μM) in the presence of L-arginine (5 mM). (D) $^{13}C^{18}O$-bound iNOS (30 μM) in the presence of L-arginine (5 mM). (E) Difference spectrum (trace C − trace D) to show the isotope effect on CO-bound iNOS in the presence of L-arginine. (F) Arginine-free, $^{12}C^{16}O$-bound iNOS. (G) Arginine-free, $^{13}C^{18}O$-bound iNOS. (H) Difference spectrum (trace F − trace G) to show the isotope effect on L-arginine-free CO-bound iNOS. The laser excitation wavelength was 441.6 nm. Each spectrum was signal averaged for 15 min and then baseline corrected.

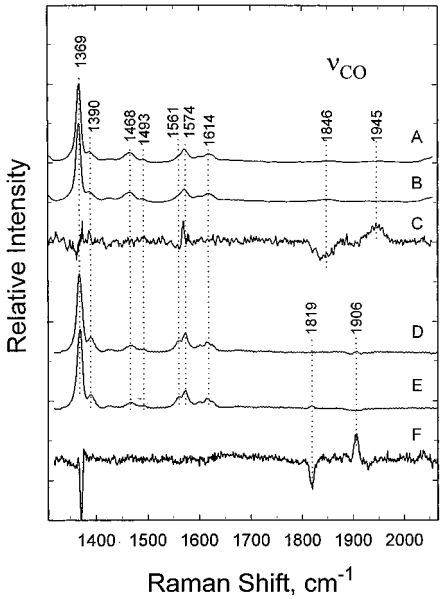


FIGURE 2: High frequency resonance Raman spectra of iNOS. The experimental conditions were the same as those in Figure 1. (A) Arginine-free, $^{12}C^{16}O$-bound iNOS. (B) Arginine-free, $^{13}C^{18}O$-bound iNOS. (C) Difference spectrum (trace A − trace B) to show the isotope effect on L-arginine-free, CO-bound iNOS. (D) $^{12}C^{16}O$-bound iNOS (30 μM) in the presence of L-arginine (5 mM). (E) $^{13}C^{18}O$-bound iNOS (30 μM) in the presence of L-arginine (5 mM). (F) Difference spectrum (trace D − trace E) to show the isotope effect on CO-bound iNOS in the presence of L-arginine.

503 cm^{-1}. For the arginine-bound isozymes in the high frequency region (Figure 2), ν_{C-O} is detected at 1906 cm^{-1} for iNOS but at 1929 cm^{-1} for nNOS. Despite the large differences in the ν_{Fe-CO} and ν_{C-O} modes, a very similar frequency for δ_{Fe-C-O} was observed for all the three isozymes at 566−567 cm^{-1}. The large difference in the CO associated vibrational modes is the first direct observation of structural differences in the substrate binding sphere in the NOS isozymes. The magnitude of the frequency differences are indicative of very large structural differences in the substrate binding pockets.

DISCUSSION

The resonance Raman spectra of the substrate-free forms of the CO-bound enzyme shows complicated features making a clear comparison between isozymes difficult. For all of the isozymes, three lines are present in the ν_{Fe-CO} mode region, one of which originates from a porphyrin mode. The porphyrin mode couples to the ν_{Fe-CO} modes, making analysis of the data difficult. However, it is clear that differences exist between isozymes in the substrate free form as indicated by the positions of the deconvoluted components of the ν_{Fe-CO} mode (See Table 1). Specifically, the lowest frequency component in this region after the deconvolution appears at 487 cm^{-1} in nNOS and at 479 cm^{-1} in iNOS. These differences clearly indicate that the heme pockets are different between these isozymes. How these differences impact on function are best understood by consideration of the substrate-bound forms of the enzymes.

The substrate-bound forms have much simpler spectral features than the substrate-free forms and allow for a clear distinction between the isozymes. We consider two possible origins for the differences observed in the resonance Raman spectra in the substrate-bound forms of the NOS isozymes. The differences could originate from either an intrinsic difference in the heme ligation structure or different interactions between the bound ligand and its environment in the distal pocket. If there were differences in the heme ligation structure, we would have expected to see differences in the resonance Raman spectra of the heme macrocycle modes. We find no systematic differences in the heme vibrational modes in the comparison among the isozymes. In EPR studies on different isozymes, the arginine-bound nNOS and iNOS show similar corresponding g values, indicating a similar rombicity and heme coordination geometry (Galli et al., 1996; Salerno et al., 1995, 1996a,b; Tsai et al., 1996). Confirmation that the differences between the isozymes do not result from differences in the ligation structure will come from the identification of the Fe−S stretching mode in the Raman spectrum which is currently in progress. Thus, we can attribute the differences observed in this study to a variation in the interaction between the heme-bound CO and its distal environment; the effect becomes most pronounced when arginine is present. This conclusion is consistent with a large variation in the frequencies of the Fe−C−O modes found in a series of arginine analogue derivatives of iNOS

Table 1: Summary of Resonance Raman Frequencies of the Three NOS Isozymes[a]

isoform	frequency (cm^{-1}) ν_{Fe-CO}	δ_{Fe-C-O}	ν_{CO}	reference
nNOS				
L-arginine-free	491 (487/501/513)	562	1936 (1949/1930)	Wang et al. (1997)
L-arginine-present	503	566	1929	Wang et al. (1997)
iNOS				
L-arginine-free	487 (479/499/512)	560	1945 (broad)	this work
L-arginine-present	512	567	1906	this work
eNOS				
L-arginine-present	512	567	ND[b]	this work

[a] All of the data were obtained from samples in the presence of tetrahydrobiopterin. The deconvolution of the broad lines into two or three components are included in parentheses. [b] ND, not determined.

(B. Fan, D. J. Stuehr, and D. L. Rousseau, unpublished results).

The understanding of the nature of the interactions between the bound CO and its environment has been greatly advanced over the past few years. It has been reported in a series of studies of myoglobin site-directed mutants and model compounds that the electrostatic potential near the CO plays a dominant role in the determination of the ν_{Fe-CO} and ν_{C-O} frequencies (Li & Spiro, 1988; Ray et al., 1994). Placement of positive charge near the CO decreases the C−O bond order and increases the Fe−CO bond order, i.e., the positive charge causes movement along the well established ν_{Fe-CO} versus ν_{C-O} inverse correlation curve. Related effects were studied in cytochrome P-450 (Jung et al., 1996). Although the structure of cytochrome P-450 is complicated by the presence of water molecules in the distal pocket, a frequency increase in ν_{Fe-CO} upon coordination of camphor in the substrate binding site was accounted for by movement of the CO toward a polar residue. This interpretation is consistent with the crystal structure which shows that the camphor causes a 14° tilt of the CO toward the hydroxyl group of Thr-352 (Raag & Poulos, 1989).

A qualitative description of the differences between the isozymes emerges from the data. The polar interactions between CO and the substrate are weaker in nNOS than they are in iNOS or eNOS. *Thus, in nNOS the substrate binding site is more open than in either iNOS or eNOS.* Theoretical studies by Stavrov and co-workers have placed the influence of polar effects on the vibrational modes on a quantitative basis (Kushkuley & Stavrov, 1996). When the CO unit is placed in a positively charged potential, a frequency increase of the ν_{Fe-CO} and a decrease of ν_{CO} is predicted but the frequency shifts are dependent on both the magnitudes and the positions of the charges. Drawing on their analyses, we consider two possible extreme cases to interpret the data from NOS. In the first scenario the substrate is held at a significantly different distance from the CO in the two isozymes, whereas in the second case, the position of the substrate is the same but the charge on the substrate is shielded by a different degree in the isozymes because of either sequence or structural differences in the distal pocket.

The change in the vibrational frequencies of the Fe−C−O modes are related quantitatively to the polarity of the environment surrounding the bound CO. Under the assumption that the charges are identical in the isozymes but the distance between the CO moiety and the substrate are different, the bond index difference (ΔB) is correlated with the difference in the distance between the CO and any charged residues in its environment as reported by Kushkuley and Stavrov (1996). In turn, the change in bond index is related to the change in vibrational frequency ($\Delta\nu$) as follows:

$$\frac{\Delta\nu}{\nu_0} = 0.5\frac{\Delta B}{B_0}$$

where ν_0 and B_0 are the frequency and bond index for the mode of interest when the charge and the CO have infinite separation along the axis of the Fe−C−O to the porphyrin normal. A similar relationship was reported when orientations other than co-axial were studied. Since in all the NOS isozymes, the lowest ν_{Fe-CO} frequency observed is that for the lower frequency component of the substrate-free form of iNOS (479 cm^{-1}), this is taken as the ν_0. B_0 (1.122) is taken from Stavrov's original work (S. S. Stavrov, personal communication). According to the observed ν_{Fe-CO} values, 503 cm^{-1} for nNOS and 512 cm^{-1} for iNOS and eNOS, the above semiquantitative calculations show that the observed differences between nNOS and iNOS correspond to a difference in separation between CO and the positive charge of as large as 1 Å. This model places the substrate in a position much more distant from the CO in nNOS than in iNOS. A similar difference is anticipated for the relationship between the substrate and the bound dioxygen under catalytic conditions.

The other model we used to account for the spectral differences originates from a different degree of shielding between the bound CO and the positive charge of arginine's guanidino group. Using similar calculations as discussed above, if we assume a 3−4 Å separation between the CO and a unit charge, a difference of 0.5−0.25 charge units, respectively, is projected. Similar results are obtained if the separation between the CO and the polar group is assumed to be larger. It has been established that polar residues are present near the substrate binding site. Mutation of a negatively charged residue, Glu-371, to a neutral alanine residue, leads to the complete abolishment of arginine binding (Gachhui et al., 1997). This was confirmed by resonance Raman experiments on the CO complex in which the spectral perturbations caused by arginine binding in the wild-type enzyme are not observed upon addition of arginine to the E371A mutant. Instead, a spectrum which is similar to that of the wild-type substrate-free enzyme is observed, confirming the integrity of the mutant protein and the absence of arginine coordination (data not shown). Mutation of Glu-361, the analogous residue in human eNOS, also selectively abolished the arginine binding (Chen et al., 1997). These experiments demonstrate that the interaction of the substrate with negatively charged residues in the ligation vicinity play

key role(s) in determining the ligand binding properties. Thus, additional groups or changes in the positioning of key residues could cause the changes detected here. If shielding changes are the origin of our observations, it indicates that the isozymes have structural differences in their distal pockets which could influence many of the catalytic and ligand binding properties. In addition, differences in the charge distribution at the catalytic site can be exploited to generate isozyme selective inhibitors.

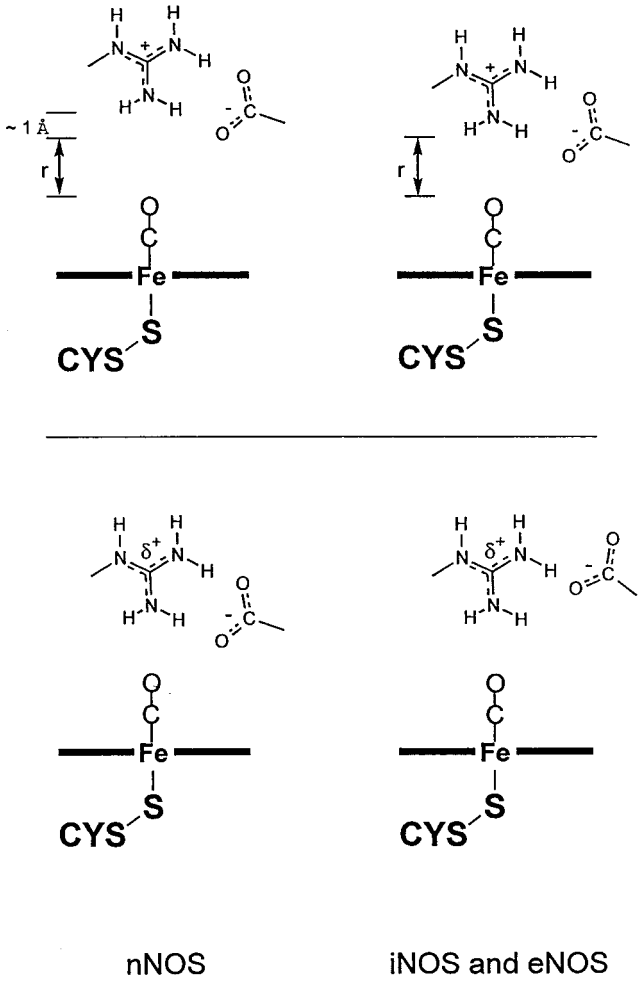
Experiments are currently in progress to distinguish between the two possibilities presented here. Although the structural basis of the arginine binding is still not clear, the differences we detect indicate that the interaction of L-arginine with the NOS isozymes are significantly different and the quantitative description supports the qualitative observation that nNOS has a more open substrate binding pocket as compared to iNOS or eNOS. The more open pocket may allow more flexibility for bound substrate and/or amino acid residues. Thus, the L-arginine may not be positioned as close to the CO in nNOS as in iNOS or eNOS, or a negatively charged amino acid may be free to form a strong electrostatic interaction with the positively charged L-arginine and shield its charge from the CO. These possible consequences of an open pocket would lead to a smaller shift in the CO stretching frequencies upon binding L-arginine. These effects are illustrated in Scheme 1.

The differences we detect in the CO-associated frequencies clearly indicate that the active sites of the isozymes are different, and as discussed below, this would be expected to give rise to functional differences. However, since the substrate binding has an impact on the Fe−C−O structure, this raises the question as to whether or not, under physiological conditions, the structure of the bound O_2 also is different for each isozyme. Carbon monoxide preferentially adopts a linear structure perpendicular to the heme plane when coordinated to the iron, whereas O_2 adopts a bent configuration. Thus, the influence of residues near the ligand binding site need not have equivalent effects on the two ligands as recently pointed out (Hirota et al., 1996).

A large difference in active site structure among the isozymes would be expected to have several manifestations. First, the reaction rates may be significantly impacted; second, substrate positioning could affect the coordination of ligands to the heme such as oxygen or NO; third, the release of ligands from the heme may be influenced by the substrate; fourth, altered substrate positioning with respect to the catalytic site makes the possibility of finding sterically selective isozyme sensitive substrate analogues attractive.

Evidence of a more open distal pocket in nNOS is consistent with inhibition studies. Most heme based inhibitors show a competitive mechanism toward arginine. Thus, the inhibitory efficiency is an indication of the binding affinity. In a comparative study on the three NOS isozymes, a bulky inhibitor (N^G-cyclopropyl-L-arginine) shows a much greater efficiency toward nNOS than toward the other isozymes (IC_{50} values of 0.55, 184, and 258 for nNOS, iNOS, and eNOS, respectively) (Lambert et al., 1992), indicating that nNOS can accommodate bulkier substrates. Evidence supporting the observed size difference among the three isozymes is also found in the inhibitory studies with imidazole, which coordinates to the heme at the same site at which the CO binds. Competitive inhibition of arginine versus imidazole indicates that the substrate and the imidazole cannot simultaneously be present whereas noncompetitive inhibition indicates a more open substrate binding pocket. The trends from the imidazole binding studies are consistent with the observations reported here. In a study with rat nNOS, imidazole has been demonstrated to be a noncompetitive inhibitor toward arginine (Wolff et al., 1993). The noncompetitive behavior suggests that imidazole is not displaced by the binding of arginine, and thus, an open pocket is suggested. In contrast, studies on eNOS (Wolff et al., 1994) reveal that imidazole is a competitive inhibitor toward arginine binding, suggesting a more closed pocket. However, the behavior of iNOS is more complex. In recombinant human iNOS (Chabin et al., 1996), imidazole coordination is competitive with arginine whereas in murine iNOS, imidazole is a noncompetitive inhibitor, indicating that significant species differences exist. The consistency in nNOS and eNOS with our results and the complicated behavior observed in iNOS suggests that the substrate binding pocket in iNOS may be intermediate between the other two isozymes. Furthermore, in some cases the cross species differences may be as large as the isozyme differences. The need to carry out the same type of CO measurements reported here on different species of the isozymes is clearly indicated. However, despite these questions, the data suggest that the size of the pocket in iNOS

Scheme 1. Illustration of the Difference in the Ligand Binding Site of the NOS Isozymes[a]



[a] For this purpose, iNOS and eNOS are taken as being identical. Top: change in distance between the CO and the arginine. Bottom: change in shielding of the charge on the arginine.

is intermediate between nNOS and eNOS. Such a relationship in which a decrease of the size of the ceiling forming the heme active site in the order nNOS > iNOS > eNOS was observed by Gerber et al. (1997) using phenyl−iron complex formation rate as a measure of the size of the site. Each of these techniques are probing different regions of the substrate binding site. A full picture will only be available after several such diverse measurements on many different isozymes from different species are integrated.

It is surprising that large differences between the two constitutive isozymes (nNOS and eNOS) exist, whereas the inducible isozyme (iNOS) is very similar to one of the constitutive isozymes (eNOS), in view of the differences in their functionality. Although the physiological consequence of this observation is not clear at present, the results presented here show for the first time that there are identifiable structural differences. These data provide a starting point for determining a structural basis for the observed functional differences between the isozymes and an approach for studying possible differences among isozymes of different species. In addition, the results offer a strategy for directing the search for isozyme-selective inhibitors and thereby should facilitate drug design. Future experiments with a variety of inhibitors will allow the differences in the distal pockets to be mapped out leading to new understanding of the catalytic process and a structural basis for isozyme selectivity.

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