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Role of an isoform-specific substrate access channel residue in CO ligand accessibilities of neuronal and inducible nitric oxide synthase isoforms

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Abstract

The rates of the bimolecular CO rebinding to the oxygenase domains of inducible and neuronal NOS proteins (iNOSoxy and nNOSoxy, respectively) after photolytic dissociation have been determined by laser flash photolysis. The following mutants at the isoform-specific sites (murine iNOSoxy N115L and rat nNOSoxy L337N, L337F) have been constructed to investigate role of the residues in the CO ligand accessibilities of the NOS isoforms. These residues are in the NOS distal substrate access channel. The effect of the (6R)-5,6,7,8-tetrahydrobiopterin (H₄B) cofactor and L-arginine (Arg) substrate on the rates of CO rebinding have also been assessed. Addition of L-Arg to the iNOSoxy N115L mutant results in much faster CO rebinding rates, compared to the wild type. The results indicate that modifications to the iNOS channel in which the hydrophilic residue N115 is replaced by leucine (to resemble its nNOS cognate) open the channel somewhat, thereby improving access to the axial heme ligand binding position. On the other hand, introduction of a hydrophilic residue (L337N) or a bulky rigid aromatic residue (L337F) in the nNOS isoform does not significantly affect the kinetics profile, suggesting that the geometry of the substrate access pocket is not greatly altered. The bimolecular CO rebinding rate data indicate that the opening of the substrate access channel in the iNOS N115L mutant may be due to more widespread structural alterations induced by the mutation.

Keywords

Nitric oxide synthase; Kinetics; Mutation; Mechanism; Ligand binding

1. Introduction

Nitric oxide (NO), synthesized by NO synthase (NOS) enzyme (EC 1.14.13.39), is a ubiquitous signaling molecule (at low nM concentrations) and a cytotoxin (at higher μM concentrations) [1,2]. Mammalian NOS catalyzes the oxidation of L-arginine (Arg) to NO and L-citrulline with NADPH and O₂ as co-substrates [3]. It is a homodimeric protein

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comprised of an N-terminal oxygenase domain (containing a catalytic heme active site) and a C-terminal reductase domain, with an intervening calmodulin (CaM) binding region between the two domains [4,5]. Three mammalian NOS isoforms are found: endothelial, neuronal and inducible NOS (eNOS, nNOS and iNOS, respectively). Under physiological conditions, NO produced by eNOS regulates vascular tone, insulin secretion, and smooth muscle tension, and NO produced by nNOS functions as a neurotransmitter [6]. These 'constitutive' isoforms (cNOSs) are Ca^{2+} /CaM regulated [7], generating NO as an intercellular messenger [2]. On the other hand, iNOS binds CaM irreversibly and is transcriptionally regulated.

Aberrant NO synthesis by NOS is associated with an increasing number of human pathologies, including stroke, inflammation, arthritis, and cancer [2,8]. Selective NOS modulators are required for therapeutic intervention because of the ubiquitous nature of NO in mammalian physiology, and the fact that multiple NOS isoforms are each capable of producing NO *in vivo*. The three NOS isoforms function differently in human health and disease. For example, in acute ischemic stroke, NO produced by the neuronal NOS or inducible NOS (nNOS or iNOS) is detrimental, whereas NO derived from the endothelial NOS (eNOS) and the accompanying dilation are beneficial [9]. Recent advances in understanding the structural and functional mechanisms of this enzyme class have led to identification of agents that are designed to selectively modulate the various NOS isoforms [10]. It is of current interest to develop new selective NOS inhibitors by using a combination of crystallography, computational methods, and site-directed mutagenesis [11–14]. This is challenging because of the high level of amino acid conservation and striking structural similarity in the immediate vicinity of the substrate binding sites of the heme active sites of the three NOS isoforms [15].

One of the targets for developing new selective NOS inhibitors is the substrate access channel [16] that has distinct sequence differences among the NOS isoforms. Inhibition or activation of NO formation could be based on fine-tuned conformational control by these unique residues in the substrate channel. Indeed, recent structural studies on an nNOS-specific inhibitor AR-R17447 bound to nNOS and iNOS oxygenase domains (nNOSoxy and iNOSoxy, respectively) have suggested a promising source of the isoform selectivity provided by the isoform-specific residues in the NOS substrate access channel [17]. In that work analysis of the AR-R17447 bound crystal structures [17] suggests that the structural basis of isoform-specific binding of AR-R17477 is the interaction of the inhibitor with the substrate access channel residues L337 and N115 in nNOS and iNOS, respectively (Figure 1a); note that these residues are isoform-specific (Figure 1b).

In the present study we have performed laser flash photolysis experiments to investigate the roles of the unique substrate access channel residues (i.e. rat nNOS L337 and murine iNOS N115) in bimolecular CO rebinding kinetics of the nNOS and iNOS isoforms. This process, although non-physiological, can serve as a probe of the access of small molecules in the solvent medium to the heme iron. First, we have swapped these residues in rat nNOS and murine iNOS, creating the mutants rat nNOSoxy L337N and murine iNOSoxy N115L. We have also introduced the equivalent human eNOS residue F105 to nNOS, and generated rat nNOSoxy L337F. The observed differences provide new insights into the important role of the unique substrate access channel residues in heme ligand accessibility.

2. Experimental procedures

2.1. Expression and purification of NOS oxygenase constructs and mutants

The oxygenase domains of murine iNOS (residues 65–498) and rat nNOS (residues 291–722) were cloned, mutagenized, expressed, and purified as described [17–19]. The

construction, expression and purification of the NOS mutants (rat nNOSoxy L337N and L337F, and murine iNOSoxy N115L) were also conducted as described [17]. All materials were of the highest purity available. The Advantage cDNA PCR kit was obtained from Clontech (Palo Alto, CA). Primers were synthesized at Duke University Core facility. Site-specific oligonucleotide-directed mutagenesis was performed utilizing the QuickChange mutagenesis system (Stratagene) and confirmed by DNA sequencing.

2.2. Laser flash photolysis

Laser flash photolysis experiments were performed anaerobically on 0.30 ml solutions containing 5-deazariboflavin (dRF) and 0.1 mM EDTA as the sacrificial reductant [20,21]. The laser apparatus and associated visible absorbance detection system have been extensively described [22]. Briefly, a N₂ laser (Photochemical Research Associates (PRA), London, Ontario, Canada) was used to pump a dye laser (450 nm wavelength maximum; PRA), which was focused onto the sample cell, and used to trigger the reactions. The dRF solution was de-aerated by vigorously bubbling with H₂O-saturated O₂-free CO for ~ 1 hour, and the absorption change at 510 nm due to dRFH[•] formation and disappearance was monitored to check the degassing efficiency, prior to addition of microliter volumes of concentrated protein. CO was purged over the surface of the protein solution to remove traces of O₂ before mixing the protein droplet into the bulk solution. The protein was photoreduced in the presence of dRF and CO, and then flashed with a 450 nm laser pulse. All experiments were performed at room temperature and at saturating CO concentrations. Generally, data from six to twelve laser flashes were averaged. Transient absorbance changes were analyzed using OriginPro 8.0 (OriginLab Co.).

3. Results and Discussion

3.1. CO rebinding kinetics in wild type nNOSoxy and iNOSoxy proteins

The NOSoxy proteins can be readily reduced to the [Fe(II)–CO] form by illumination in the presence of dRF and CO, as shown by the difference spectra (Figure S1, Supplementary material). The CO ligand was then flashed off from the heme iron center by a 450 nm laser pulse, and the CO rebinding kinetics can be determined by following absorption changes at selected wavelengths. Flash-induced difference spectra (Figure S2) show that the CO rebinding process can be best monitored at 407 nm and 445 nm.

Figures 2a and 2b show the 450 nm laser flash induced absorption change of the [Fe(II)–CO] form of Arg-H₄B-free (–/–) iNOSoxy at 407 nm and 455 nm, respectively; 455 nm was chosen for the sample so as to obtain a strong signal without overloading the oscilloscope. The traces can be best fitted by a double exponential model: $1226 \pm 128 \text{ s}^{-1}$ (74%) and $352 \pm 46 \text{ s}^{-1}$ (26%); the relative amplitude of each phase is indicated in the parentheses. Note that the time scale is in the 0 – 0.02 sec range, which monitors the bimolecular CO rebinding process [23]. This process is appropriate for looking into the role of *distal* residues in ligand accessibility from the solvent medium, as opposed to the much faster geminate recombination process that is controlled by the immediate environment of the heme group prior to CO escape from the binding pocket. The CO rebinding rates are in good agreement with the literature values [23]. The heterogeneous kinetics reflects the complexity of the CO rebinding process in NOS proteins. This has been attributed to multiple conformation states of the NOS binding pocket [24]. Note that we collected traces at the two wavelengths (i.e. 407 and 455 nm) to confirm that the fast phase is not due to the laser artifact; this is because the laser artifact should always cause a positive transient spike in the traces, while the fast phases in the traces at 407 and 455 nm occur in opposite directions (also see the difference spectra in Figure S2). The rate constants for the bimolecular rebinding of CO to the NOSoxy proteins are listed in Table 1.

Addition of L-Arg to the Arg-H₄B-free iNOSoxy protein results in a 20- and 50-fold decrease in the fast and slow rates of the bimolecular rebinding of CO to the heme iron after photolysis, respectively (Figure 3; note the significant difference in the time scales of Figures 2 and 3). On the other hand, the presence of H₄B in the iNOS active site completely abolishes the fast phase of the CO rebinding process, and significantly decreases the slow phase by an order of magnitude (Table 1). These results are consistent with the reported effects of murine iNOS heme domain ligands on the kinetics of CO rebinding to the heme [23]. Binding of Arg/H₄B to nNOSoxy also significantly alter the CO rebinding kinetic profile by completely abolishing the fast phase (Table 1). It is generally believed [23,24] that in the absence of L-Arg and H₄B a relatively open ligand-binding pocket allows CO to bind unhindered to the heme in the active site of murine iNOS, whereas in the presence of H₄B/L-Arg the enzyme adopts a more closed structure that can significantly decrease ligand access to the heme iron.

3.2. CO rebinding kinetics in murine iNOSoxy N115L and rat nNOSoxy L337N and L337F mutants

In the absence of L-Arg and H₄B, the bimolecular CO rebinding to the iNOSoxy N115L mutant does not change significantly, compared to the wild type iNOSoxy, while in the presence of H₄B, the slow CO rebinding rate in the N115L mutant is only slightly decreased compared to wild type (Table 1). On the other hand, L-Arg binding to the N115L mutant results in distinct CO rebinding kinetics (Figure 3): 104 ± 13 (44%) and 33 ± 5 (56%) s⁻¹ for the N115L mutant, and 67 ± 10 (82%) and 6.8 ± 53 (18%) s⁻¹ for the wild type; note that the rate of the slow phase in the N115L mutant is increased by 4-fold. These results indicate that modifications to the iNOS channel in which the hydrophilic residue N115 is replaced by leucine (to resemble its nNOS cognate) somewhat open the channel, thereby improving access to axial heme ligand binding position. This suggests that interactions with the hydrophobic leucine residue in the N115L mutant may force the channel to adopt a conformation in which the side chains are more compactly arranged, whereas in the wild type iNOS the N115 residue and its neighbors are more extended due to their ability to interact favorably with solvent water.

As for the nNOSoxy mutants, in the absence of L-Arg and H₄B, the bimolecular CO rebinding processes to rat nNOS L337N and L337F are biphasic, similar to the wild type, although the fast phase rates are decreased by nearly 55% (Table 1). Moreover, in the presence of H₄B, L337N possesses biphasic kinetics (87 ± 6 and 21 ± 1 s⁻¹), whereas the CO rebinding to the wild type and L337F are similar and monophasic (23 ± 1 s⁻¹). On the other hand, in the presence of L-Arg, the CO rebinding kinetics of the L337N and L337F mutants are monophasic, and the rates are around 25 s⁻¹, which are similar to the wild type (Table 1). These results show that introduction of either a hydrophilic residue (L337N) or a bulky rigid aromatic residue (L337F) in nNOS does not significantly affect the kinetics profile, suggesting that the geometry of the substrate access pocket is not altered greatly.

In conclusion, the bimolecular CO rebinding rate data indicate that the opening of the substrate access channel in the iNOS N115L mutant may be due to more widespread structural alterations induced by the mutation. Our findings are consistent with a recent study in which L337 is part of a hot spot for designing new selective NOS inhibitors [25].

Research Highlights

- The rates of bimolecular CO rebinding to the nNOS and iNOS oxygenase proteins were determined.

- L-Arg binding to murine iNOS N115L mutant gives much faster CO rebinding rate, compared to wild type.
- Rat nNOS mutants at the equivalent site (L337N and L337F) have similar kinetics profile as wild type.
- The distal isoform-specific residue N115 in murine iNOS may control heme ligand accessibility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

NO	nitric oxide
NOS	nitric oxide synthase
iNOS	inducible NOS
nNOS	neuronal NOS
NOSoxy	the oxygenase domain of nitric oxide synthase
CaM	calmodulin
CO	carbon monoxide
Fe^{II}	ferrous heme species
Fe^{II}CO	ferrous CO species
dRF	5-deazariboflavin
dRFH[•]	5-deazariboflavin semiquinone
H4B	6R-5,6,7,8-tetrahydrobiopterin
Arg	L-arginine

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(a)

(b)

NOS1_RAT	LKVKNWETDVVLDTLHLKSTLETGCTEHICMGSIM	L PSQHTRKPE-DVRTKDQLFPLAK	359
NOS2_MOUSE	VRIKNWGSGEILHDTLHHKATSDFTCKSKSCLGSI	N PKSLTRGPRDKPTPLEELLPHAI	138
NOS3_HUMAN	PRVKNWEVGSITYDTLSAQAQQDGFCTPRRCLGSLV	F PRKLQGRPSPGPPAPEQLLSQAR	128

Figure 1.

(a) The isoform-unique L337 residue in distal site of substrate access channel of rat nNOSoxy (pdb ID 1ZVL). The substrate L-Arg is shown in yellow. (b) Alignment of the sequences of rat nNOS, murine iNOS and human eNOS; note that the equivalent residues of the rat nNOS L337 in murine iNOS and human eNOS are N115 and F105, respectively (as highlighted in red).

Figure 2.

Transient traces at (a) 407 nm and (b) 455 nm obtained for the [Fe(II)–CO] form of Arg-H₄B-free (–/–) iNOSoxy flashed by 450 nm laser excitation; note the existence of a fast phase in both traces. Plots of residuals from fitting to a double exponential function are shown in the bottom panel; note that the fittings give excellent normal distributions of the residuals. Anaerobic solutions contained 4 μ M iNOS, $\sim$ 100 μ M dRF and 0.5 mM EDTA in pH 7.6 buffer (40 mM bis-Tris propane, 200 mM NaCl and 10% glycerol). The sample was well degassed by CO before reducing the protein to ferrous-CO by illumination (see Experimental procedures).

Figure 3.

Transient traces at 446 nm nm at 0 – 0.2 s obtained for the [Fe(II)–CO] form of L-Arg-bound wild type (black) and N115L (red) iNOSoxy flashed by 450 nm laser excitation. The traces can be fitted by a double exponential function, and the rate constants are listed in Table 1 (as “+Arg”). Note that the kinetics are distinctly different. Final concentration of L-Arg is 2 mM; other experimental conditions are the same as Figure 2.

Table 1

Observed rates of bimolecular rebinding of CO to the nNOS and iNOS oxygenase

Proteins		k_1 (s ⁻¹)	k_2 (s ⁻¹)
wild type iNOSoxy	-/-	1226 ± 128 (74%)	352 ± 46 (26%)
	+ Arg	67 ± 10 (82%)	6.8 ± 3.0 (18%)
	+ H ₄ B	NP ^b	22.6 ± 1.8
iNOSoxy N115L	-/-	1660 ± 220 (52%)	372 ± 68 (48%)
	+ Arg	104 ± 13 (44%)	33.0 ± 5.0 (56%)
	+ H ₄ B	NP	10.5 ± 0.8
wild type nNOSoxy	-/-	702 ± 85 (83 %)	25 ± 6 (17 %)
	+ Arg	NP	24.5 ± 0.8
	+ H ₄ B	NP	22.0 ± 1.0
nNOSoxy L337N	-/-	305 ± 50 (47%)	23.4 ± 1.1 (53%)
	+ Arg	NP	29.3 ± 1.2
	+ H ₄ B	87 ± 6 (17%)	21.0 ± 1.2 (83%)
nNOSoxy L337F	-/-	312 ± 30 (37%)	25.0 ± 2.0 (63%)
	+ Arg	NP	21.9 ± 0.2
	+ H ₄ B	NP	22.9 ± 0.9

^aThe fast (if present) and slow observed first order rate constants k_1 and k_2 are shown along with their relative amplitudes in parentheses (if applicable). “-/-” stands for H₄B and Arg free. The final concentration of H₄B or Arg (if added) is 20 μM and 2 mM, respectively.

^bNP: not present.