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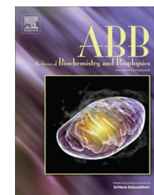

Intra- and inter-molecular effects of a conserved arginine residue of neuronal and inducible nitric oxide synthases on FMN and calmodulin binding

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ABSTRACT

Nitric oxide synthases (NOSs) synthesize nitric oxide (NO), a signaling molecule, from L-arginine, utilizing electrons from NADPH. NOSs are flavo-hemo proteins, with two flavin molecules (FAD and FMN) and one heme per monomer, which require the binding of calcium/calmodulin (Ca^{2+}/CaM) to produce NO. It is therefore important to understand the molecular factors influencing CaM binding from a structure/function perspective. A crystal structure of the CaM-bound iNOS FMN-binding domain predicted a salt bridge between R536 of human iNOS and E47 of CaM. To characterize the interaction between the homologous Arg of rat nNOS (R753) and murine iNOS (R530) with CaM, the Arg was mutated to Ala and, in iNOS, to Glu. The mutation weakens the interaction between nNOS and CaM, decreasing affinity by ~3-fold. The rate of electron transfer from FMN is greatly attenuated; however, little effect on electron transfer from FAD is observed. The mutated proteins showed reduced FMN binding, from 20% to 60%, suggesting an influence of this residue on FMN incorporation. The weakened FMN binding may be due to conformational changes caused by the arginine mutation. Our data show that this Arg residue plays an important role in CaM binding and influences FMN binding.

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Introduction

Nitric oxide synthases (NOSs)[3] catalyze the conversion of L-arginine to L-citrulline and the signaling molecule nitric oxide (NO) using NADPH as the electron donor [1]. There are three known isoforms of NOS, neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) transcribed from three different genes. NOSs have two domains, the N-terminal oxygenase or heme domain, where catalysis occurs, and a C-terminal reductase or flavin domain with two flavin co-factors, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), which transfer electrons from NAPDH to the heme domain. NO plays critical roles in both physiological and pathological situations, necessitating that its production be highly regulated. Inter- and intra-molecular electron transfer in nitric oxide synthases (NOSs) is regulated by both intrinsic and extrinsic factors. Regulatory subdomains present in the enzyme, such as the autoregulatory insert (AR), the C-terminal tail (CT) and the beta loop, control the flow of electrons within the NOS protein [2]. Interactions of NOS with other proteins also influence electron flow, the most notable of which is its interaction with calmodulin (CaM), an obligatory step for the production of NO [3,4]. CaM interaction with nNOS and eNOS is dependent on intracellular Ca^{2+} levels, whereas it is bound to iNOS at very low Ca^{2+} levels, independent of Ca^{2+} influx.

The interaction of NOS with CaM triggers a series of structural rearrangements in NOS, positioning various co-factors in an optimum state for efficient electron transfer from the reductase domain to the heme domain for NO production [1,2,5]. The CaM binding site of NOSs interacts with the EF hands of CaM (the helix-loop-helix structures) in an antiparallel orientation [6,7]. The binding of Ca^{2+} to CaM, which allows CaM to interact with NOSs, is cooperative [8]. As the affinities of different lobes of CaM to calcium are different, calcium concentration dictates which lobe(s) interact with NOS. For example, the C-terminal lobe interacts with nNOS first, but binding of the N-terminal lobe is necessary for the enzyme activation [9,10]. Direct interaction between CaM and the small insertion (SI) in the reductase hinge region and autoregulatory regions of NOSs has been proposed, based on various NOS con-

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[3] *Abbreviations used:* NOSs, Nitric oxide synthases; NO, nitric oxide; nNOS, neuronal NOS; eNOS, endothelial NOS; iNOS, inducible NOS; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; CaM, calmodulin; HPLC, high-performance liquid chromatography; AUC, analytical ultracentrifugation; 2DSA, 2-dimensional spectrum analysis; SV, sedimentation velocity; AR, autoregulatory insert; CT, C-terminal tail; SI, small insertion.

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structs and catalytic measurements. However, this interaction appears to be quite limited and direct interaction between the carbon backbones, at least in the closed conformation, seems to be restricted to a single arginine residue, which is conserved throughout the NOSs [11]. It has been proposed that the AR element in the FMN domain of the constitutive NOSs acts as a conformation-stabilizing element, stabilizing the closed conformation in the absence of NADPH oxidation by binding to the CT or a nearby region [3]. When NADPH is oxidized, the CT shifts [12], breaking the contact of the AR on the FAD domain and freeing it to stabilize the open conformation potentially by interacting directly with CaM [3]. However, as no structure has been solved of the complete CaM-bound reductase domain of either of the constitutive NOSs, direct proof of such interactions is still elusive.

The structure of the human iNOS-FMN domain bound to CaM reported by Xia et al. [11] sheds light for the first time on the interacting residues between NOS and CaM [11]. Most notable is the salt bridge between iNOS Arg536, which is conserved across isoforms and species, and Glu47 of CaM. Arg536 also makes hydrogen bonds with the main chain carbonyl oxygens of Ser562, Cys563, Ala564, and Phe565 of the iNOS FMN domain, as well as with Asn42 of CaM. It was proposed that Arg536 in the iNOS FMN domain, along with its salt bridge with Glu47 of CaM, acts as a hinge around which the two domains (FMN- and CaM-binding) of iNOS swivel, at least partially enabling the structural movement of the FMN domain necessary for catalysis to occur. A mutational study involving the homologous Arg752 residue of nNOS and Glu47 of CaM endorsed the pivoting motion of CaM and NOS around the conserved arginine of NOSs to orient FAD, FMN and heme for efficient electron transfer [13]. Furthermore, the apparent K_d of the NOS and CaM interaction was unaffected in these studies by the mutation of Arg752 of nNOS or Glu47 of CaM, as measured by rates of NO produced at different CaM concentrations [13].

Herein, we report that mutation of the conserved arginine residue in reductase domain (red) constructs of rat nNOS (R753A) and murine iNOS (R530A/E; mouse R530 is analogous to human R536) leads to destabilization of FMN binding. The reductase domain was used to elucidate the role of this conserved arginine residue as it allowed us to draw conclusions specific to the domain and its interacting partner, CaM, without interference from the heme domain. The structural alteration triggered by the mutation does not allow for reconstitution of the enzyme with FMN either permanently or transiently, as judged by catalytic measurements. This FMN depletion is much more severe in the case of nNOSred than in iNOSred, which is co-expressed with CaM, perhaps indicating a role for CaM in stabilization or incorporation of FMN in nNOS. We also report the direct binding constant for the nNOSred/CaM interaction derived from analytical ultracentrifugation. Thus, the conserved arginine plays a role in FMN binding and its mutation weakens, but does not obliterate, the nNOSred interaction with CaM, suggesting that other interactions remain intact.

Experimental procedures

Clone generation and protein purification

Plasmids. The plasmids nNOSpCW and iNOSpCW were constructed by Roman et al. (1995) [14] and Roman et al. (2000) [15] using pCWori$^+$ [16]. The plasmid encoding calmodulin, CaM pACMIP, was kindly provided by Anthony Persechini of the University of Missouri – Kansas City.

Recombinant DNA manipulations

iNOSred R530A, R530E, and nNOSred R753A mutations were created using a nested PCR technique. Primers were designed to amplify the regions of recombinant iNOS and nNOS from the start of the CaM binding site, incorporating a 6-His sequence just after the initiating ATG, to the mutation (PCR #1), and from the mutation to the STOP codon (PCR #2). Products from this amplification were then used as template for a third PCR reaction (PCR #3), spanning the entire reductase domain sequence.

The primers used were:

iNOSred R530A PCR#1: Upstream – 5′ GGA GGT CAT ATG GCT CAC CAC CAC CAC CAC CAC AAG CTG AGG CCC AGG AGG; Downstream – 5′ GAG GAC TGT GGC TCT GAC CGC TGA AGC CAT GAC CTT TCG.

iNOSred R530A PCR#2: Upstream – 5′ CGA AAG GTC ATG GCT TCA GCG GTC AGA GC CACA GTC CTC; Downstream – 5′ TCA TCG ATA AGC TTA GAG ACG CGT.

iNOSred R530A PCR#3: Upstream – 5′ GGA GGT CAT ATG GCT CAC CAC CAC CAC CAC CAC AAG CTG AGG CCC AGG AGG; Downstream – 5′ TCA TCG ATA AGC TTA GAG ACG CGT.

iNOSred R530E PCR#1: Upstream – 5′ GGA GGT CAT ATG GCT CAC CAC CAC CAC CAC CAC AAG CTG AGG CCC AGG AGG; Downstream – 5′ GAG GAC TGT GGC TCT GAC TTC TGA AGC CAT GAC CTT TCG.

iNOSred R530E PCR#2: Upstream – 5′ CGA AAG GTC ATG GCT TCA GAA GTC AGA GC CACA GTC CTC; Downstream – 5′ TCA TCG ATA AGC TTA GAG ACG CGT.

iNOSred R530E PCR#3: Upstream – 5′ GGA GGT CAT ATG GCT CAC CAC CAC CAC CAC CAC AAG CTG AGG CCC AGG AGG; Downstream – 5′ TCA TCG ATA AGC TTA GAG ACG CGT.

nNOSred R753A PCR#1: Upstream – 5′ TAC GTA CAT ATG CAC CAC CAC CAC CAC CAC AAA CGG CGA GCT ATC GGC; Downstream – 5′ GAG AAT GGT CGC CTT GAC AGC CTT GGC CAT GGC CTG CCC.

nNOSred R753A PCR#2: Upstream – 5′ GGG CAG GCC ATG GCC AAG GCT GTC AAG GCG ACC ATT CTC; Downstream – 5′ GTC GAC TCT AGA TTA GGA GCT GAA.

nNOSred R753A PCR#3: Upstream – 5′ TAC GTA CAT ATG CAC CAC CAC CAC CAC CAC AAA CGG CGA GCT ATC GGC; Downstream – 5′ GTC GAC TCT AGA TTA GGA GCT GAA.

The resultant product is a NOS reductase domain coding sequence with an N-terminal 6-His tag and the indicated mutation. The PCR products from PCR #3 were restriction digested with NdeI/XbaI (nNOS) or NdeI/HindIII (iNOS) and ligated with NdeI/XbaI- or NdeI/HindIII-digested pCW vector, respectively. This ligation was used to transform XL10-gold cells (Stratagene) and colonies were screened by restriction digest. The correct construct was then used to transform *E. coli* BL21 cells.

Purification

Transformed BL21 cells with the plasmid were grown to an $O.D._{600nm}$ of 0.8–1 prior to induction of protein expression by an induction cocktail containing 0.25 mM IPTG and riboflavin. Cells were grown at room temperature (22 °C–24 °C) for 24 h in the dark. Harvested cells were suspended in buffer with lysozyme (100 μg/ml) and protease inhibitor cocktail prior to sonication. Sonicated cells were centrifuged at 32,000 rpm for 1 h and the supernatant was collected and loaded onto a 2′5′-ADP-Sepharose column. Protein was eluted with 5 mM 2′ AMP after extensive washing on the column. Eluted protein was loaded onto a Ni–NTA column for further purification and eluted with 100 mM imidazole. Protein eluted from the Ni-column was dialyzed extensively to eliminate residual imidazole prior to its use in experiments. Notably, the nNOSred R753A protein was less stable than the other reductase domain constructs as evidenced by its proclivity to precipitation.

Activity measurements

Steady state kinetic measurements of reduction of external electron acceptors such as cytochrome *c* and $K_3Fe(CN)_6$ as well as

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