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Electron transfer in nitric-oxide synthase

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Contents

Abstract

Nitric oxide (NO) has many diverse biological functions as an important signaling and cytotoxic molecule in the cardiovascular, nervous, and immune systems. NO is generated from L-Arg via formation of N^G-hydroxyl-L-Arg as an intermediate by a family of enzymes termed nitric-oxide synthases (NOSs). NOS consists of two functional domains: one is an amino-terminal oxygenase domain that has a cytochrome-P450-like heme active site, and the other is a carboxy-terminal reductase domain that is similar to NADPH-cytochrome P450 reductase. In contrast to the well-known P450/NADPH-P450-reductase fusion protein, cytochrome P450BM3, however, all NOS isoforms are only active as homodimers and require the presence of both Ca^{2+}/calmodulin and (6*R*)-5,6,7,8-tetrahydrobiopterin for electron transfer and catalysis. We summarize recent results focusing on electron transfer reaction within this novel enzyme and demonstrate differences between the NOS and P450 systems.

Keywords: Nitric-oxide synthase; L-Arginine; Electron transfer; Site-directed mutagenesis; NADPH oxidation; Heme; Flavins; Reductase; Pterin

Abbreviations: NO, nitric oxide; NHA, N^G-hydroxyl-L-Arg; NOS, nitric oxide synthase; P450, cytochrome P450; CaM, calmodulin; H4B, (6*R*)-5,6,7,8-tetrahydrobiopterin.

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1. Introduction

Nitric oxide (NO) is one of the smallest and simplest biologically active molecules in nature. NO plays multiple important physiological roles in neuronal, skeletal muscle, macrophage, and endothelial cells as a neurotransmitter, mediator, cytoprotective molecule, vasodilator, and other numerous functions ([1–6] and references therein). NO also produces cytotoxic reactive oxygen species responsible for the destruction of microorganisms and tumor cells by phagocytic cells. NO is synthesized by NO synthase (NOS). NOS catalyzes monoxidation of the first substrate, L-Arg, and forms N^G-hydroxyl-L-Arg (NHA). The enzyme then catalyzes monooxidation of the second substrate NHA, and generates NO and L-citrulline as final products (Fig. 1). NOS has three isoforms, neuronal NOS (nNOS) (NOS I), endothelial NOS (eNOS) (NOS III) and inducible NOS (iNOS) (NOS II). All isoforms are composed of two domains: the oxygenase domain contains a cytochrome P450-like heme–thiol complex, substrate and cofactor (6*R*)-5,6,7,8-tetrahydrobiopterin (H4B) binding sites, the reductase domain, which is similar to NADPH-cytochrome P450 reductase, contains NADPH, FAD and FMN binding sites (Fig. 2). A calmodulin (CaM) binding site is located between these two domains. The CO–Fe(II) complex of NOS gives a Soret absorption band at around 450 nm, similar to that of the cytochrome P450. It is likely that monooxidation of the two substrates is conducted on the heme–thiol complex in the way similar to the P450 system. However, there are intriguing differences between the NOS and P450 systems in respect to function and structure. Recent crystal structures of NOS enzymes showed there are differences in the structures of NOS and P450 enzymes with respect to the heme distal sites, α-helix/β-sheet content, whole protein shape and many other aspects [7–9].

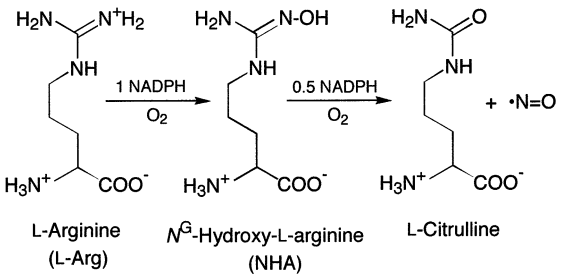

Fig. 1. NO formation from L-Arg catalyzed by NOS.

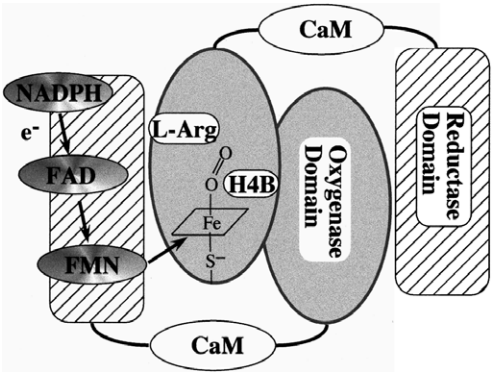

Fig. 2. Hypothetical structure of NOS.

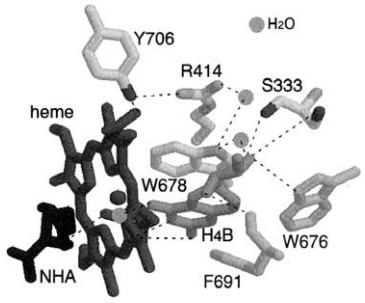

Fig. 3. H4B binding site is surrounded by aromatic amino acids.

In this article, we briefly summarize the current knowledge on regulation of NOS activity and on the structure–function relationships of the enzyme. We also describe our recent results on the nNOS enzyme focusing on the electron transfer reactions associated with catalysis.

2. Role of H4B in electron transfer

H4B is an absolute requirement for the NOS catalytic function. This is one of the important differences between the NOS and P450 systems, since no P450 enzymes require H4B in catalysis. H4B helps to stabilize the NOS enzyme, to enhance the substrate binding affinity, to increase the high-spin content of the heme, and to promote subunit dimerization which is prerequisite for NO formation activity ([1–6] and references therein). Recent studies suggest that the most important role of H4B in catalysis is likely to participate in the reaction as a redox-active cofactor ([1–11] and references therein). Bec et al. proposed that H4B donates the second electron to the Fe(III)–O–O^- complex generated during catalysis in order to cleave the O–O bond [12]. In fact, a cation radical of H4B has been detected by EPR under specific conditions [13].

2.1. Aromatic amino acids in the H4B binding site are important in catalysis and perhaps associated with electron transfer

The crystal structures of eNOS and iNOS indicate that the H4B is surrounded by several aromatic amino acids (Fig. 3) which are well conserved through all NOS isoforms [1–6]. In addition, it was suggested that the cation radical generated during catalysis may be stabi-

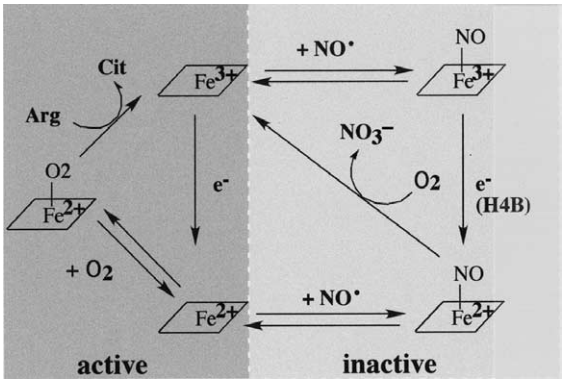

Fig. 4. Hypothetical scheme of feedback inhibition with NO.

lized by those aromatic amino acids [4]. Thus, we mutated conserved aromatic amino acids near the H4B binding site of nNOS and examined the catalytic parameters of the resultant mutants [10]. Most of the aromatic mutants became partly monomeric as observed for mutations at the dimer interface of nNOS. However, mutants at Trp676 and Phe691 had very low NO formation activities and heme reduction rates even if the proportion of enzyme in the dimeric form was taken into account. Therefore, we suggested that aromatic amino acids near the H4B binding site, including Trp678 and Phe691, are important in catalysis and electron transfer. However, the excellent work by Stuehr's group reported that these aromatic residues are not essential for the H4B binding or NO formation activity with the iNOS oxygenase domain [11]. It is not clear why their results are different from us, but may be due to differences between the two NOS isomers or/and between the heterodimer (consisting of the oxygenase domain subunit and the full-length subunit in their systems) and the full-length homodimer (in our systems). Although a well conserved Tyr706 interacts with the heme propionate and is located distant from H4B (Fig. 3), mutants at this position lost a significant amount of their H4B binding affinity. The hydrogen bonding network consisting of these aromatic and ionic amino acids appears to be crucial for catalytic activity and electron transfer in nNOS and perhaps the other isoforms.

2.2. H4B is important in the reduction of the nNOS–NO complex

It has been suggested that NO, the product of NOS, binds to the heme iron and inhibits the catalytic activity (Fig. 4) [2,5,14–16]. Stuehr's group first reported that a majority of nNOS is present as a stable ferrous heme–NO complex during the steady state and the heme–NO complex is an important regulator of NOS catalysis [14,15]. The extensive studies of the kinetics of heme–NO complex formation and heme reduction were also done by the same group. They proposed a kinetic model of NOS–NO complex formation in the catalytic turnover which can also be applied to the iNOS or eNOS systems [16]. However, it is not clear how the presence of substrates and cofactors influence NO complex formation and how the redox state (ferric or ferrous) of the heme iron–NO complex is controlled under various conditions. We examined the NO complex of nNOS under various conditions [17]. We found that the nNOS–NO complex is easily reduced by NADPH in the presence of both L-Arg and H4B even in the absence of Ca^{2+}/CaM. It appears that the NO complex reduction is strictly dependent on the presence of H4B. It is surprising to note that NO–heme reduction occurs even in the absence of Ca^{2+}/CaM. Ca^{2+}/CaM is known to be an important activator of electron transfer during catalysis as mentioned next, but may not be very important in the reduction of the heme iron under specific conditions. Automatic reduction of the NO–heme complex was reported for heme distal mutants of cytochrome P450 1A2 where the heme iron was reduced without adding NADPH [18].

3. Regulation of catalytic activity by Ca^{2+}/CaM

nNOS and eNOS isoforms are constitutive and their NO formation activities are switched on by adding Ca^{2+}/CaM ([1–6] and references therein). In the absence of Ca^{2+}, CaM loses the capability to bind to these isoforms, and is therefore able to switch catalytic turnover on/off by regulation of the free Ca^{2+} concentration in the cytosol. However, the iNOS isoform always binds CaM tightly, even in the absence of Ca^{2+}. Therefore, NO formation by iNOS is not controlled by the Ca^{2+} concentration, but by external factors such as liposaccharides, interleukin, lipopolysaccharides, and cytokines, which induce the enzyme, resulting in burst NO formation.

There is electron transfer from NADPH via the reductase domain (NADPH → FAD → FMN) to the heme active site in the oxygenase domain in all NOS isoforms (Fig. 2). The electron transfer is absolutely necessary in activating molecular oxygen during NO formation by this enzyme. It is important to note that Ca^{2+}/CaM markedly facilitates the intradomain electron transfer from FAD to FMN and the interdomain electron transfer from FMN to the heme in nNOS and eNOS. Without Ca^{2+}/CaM, no NO formation activity and very slow heme reduction rates were obtained for the constitutive NOSs. The requirement of Ca^{2+}/CaM for the activation of NOS is an unusual characteristic for a redox enzyme and can be contrasted with the similar fusion P450 enzyme, P450BM3. P450BM3 is also composed of an oxygenase domain and a similar reductase domain, but it is functionally independent of Ca^{2+}/CaM and other cofactors.

3.1. Deletion of an extra loop in the FMN-binding domain of nNOS results in a decrease in Ca^{2+}/CaM dependency

Although the amino acid sequence of the reductase domain of NOS is similar to NADPH-cytochrome P450 reductase, close inspection of the constitutive NOSs (nNOS or eNOS) shows that there is an extra loop located in the FMN binding domains of these enzymes (Fig. 5). Master's group excellently demonstrated that peptides based on the sequence of the eNOS insert inhibit CaM binding to eNOS and nNOS [19] and suggested that the extra loop acts as an autoinhibitory domain in the constitutive NOSs [3,19]. To investigate the role of the extra-loop of nNOS, we generated a nNOS deletion mutants lacking the extra loop (Pro831-Ser870) and examined the catalytic properties of the mutant [20]. The deletion mutant had significant NO formation activities and heme reduction rates even in the absence of Ca^{2+}/CaM, which is in contrast to the wild type enzyme. The Ca^{2+}/CaM-independency of the deletion mutant suggested that this extra loop regulate activity by inhibiting electron transfer in the absence of Ca^{2+}/CaM and by destabilizing CaM binding at low Ca^{2+} concentrations. Therefore, our results support the proposal that the extra loop is an autoinhibitory domain in the constitutive NOSs [3]. In contrast to our results, Gillemette and coworkers [21] reported that a deletion mutant of nNOS (Met828-Ser870) still has required CaM for NO formation activity. It is possible that a difference in the deleted-amino acids might affect the CaM dependency of each deletion mutant. Nishida and Ortiz de Montellano analyzed a series of chimera obtained by swapping the reductase domains of the three NOS isoforms [22,23]. Their results also supported the idea that the eNOS and nNOS inserts have autoinhibitory function. Note that iNOS has no extra insert in the FMN binding domain (Fig. 5) and as a result, CaM appears always to bind tightly this isoform.

3.2. C-terminal tail plays a role as a negative control for electron transfer

The reductase domains of NOSs are structurally similar to the NADPH-cytochrome P450 reductase (CPR) with the exception of the C-terminus, in which region all NOS isoforms are 20–40 amino acids longer than CPR. The truncated mutant of the C-terminal tail from iNOS exhibited 7–10-fold higher cytochrome *c* reductase activity and 20% faster NO formation rate than the wild-type enzyme [24]. Truncation of C termini from nNOS and eNOS resulted in a 7- and 21-fold increase of cytochrome *c* reductase activities without CaM, respectively [25]. Without CaM, the truncated nNOS and eNOS synthesized NO at rates 14 and 20%, respectively, more than those with CaM of wild-type enzymes. Thus, the C terminus of iNOS attenuate electron flow through the flavin domain, whereas the C termini of nNOS and eNOS regulate electron transfer through the flavin and heme domains and affect modulation by CaM. They propose a mechanism in that this C-terminal tail curls back to interact with the flavin domain so as to have a negative modulation of the FAD–FMN interaction. When CaM is bound and the autoinhibitory loop swings away, the flavins and the NADPH-binding site appear to be rearranged by a conformation change to have efficient electron transfer for catalytic activities [24,25].

3.3. Electron transfer from the isolated reductase domain to the isolated oxygenase domain is differently regulated by CaM

As mentioned in the introduction of this review, Ca^{2+}/CaM facilitates intradomain and interdomain electron transfer and results in the enhancement of NO formation activity ([1–6] and references therein). Catalytic properties of the oxygenase domains of iNOS [26] and eNOS [27] in reconstituted systems with the isolated recombinant reductase domain have been reported. The catalytic activities of iNOS or eNOS for

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                                           ↓                       _________
rat nNOS    AFDAKAMSME EYDIVHLEHE ALVLVVTSTF GNGDPPENGE KFGCALMEMR HPNSVQ---E 836
human eNOS  AFDPRVLCMD EYDVVSLEHE TLVLVVTSTF GNGDPPENGE SFAAALMEMS GPYNSSPRPE 603
human iNOS  AFNPKVVCMD KYRLSCLEEE RLLLVVTSTF GNGDCPGNGE KLKKSLFMLK ELNNKF---- 619
rat CPR     SADPEEYDLA DLSSLPEIDK SLVVFCMATY GEGDPTDNAQ DFYDWLQETD VDLTGV---- 165
                                   *      *  * **   *        *

                     Δ40 deletion                Δ2                  ↓
            ___________________________________ __
rat nNOS    ERKSYKVRFN SVSSYSDSRK SSGDGPDLRD NFESTGPLAN VRFSVFGLGS RAYPHFCAFG 896
human eNOS  QHKSYKIRFN SISCSDPLVS SWRRKRKESS NTDSAGALGT LRFCVFGLGS RAYPHFCAFA 663
human iNOS  ---------- ---------- ---------  ---------- -RYAVFGLGS SMYPRFCAFA 638
rat CPR     ---------- ---------- ---------  ---------- -KFAVFGLGN KTYEHFNAMG 184
                                                            *****     *  * *
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Fig. 5. Amino acid sequences of the FMN subdomains in NOSs and rat cytochrome P450 reductase (CPR). The 40 amino acids of rat nNOS region deleted in the Δ40 mutant and two additional residues (Δ2) in the Δ42 mutant are indicated. The two amino acids involved in FMN binding (Phe809 and Tyr889) are marked by arrows. Asterisks indicated conserved residues.

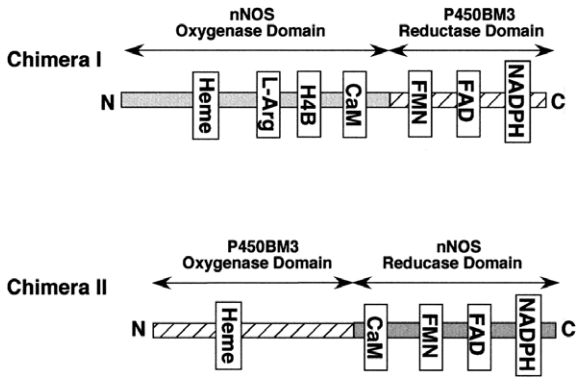

Fig. 6. Chimeric enzymes constructed by swapping the reductase domain of NOS and P450BM3.

NHA or L-Arg were obtained with the reconstituted enzyme composed of these two separate domains only when Ca^{2+}/CaM was added to the systems. Similarly, we found that the reconstituted system composed of the nNOS oxygenase domain and the isolated nNOS reductase domain showed significant NO formation activity with NHA. However, addition of Ca^{2+}/CaM to the reconstituted system markedly decreased the NO formation rate and the rate of heme reduction with NADPH [28]. A similar effect on catalytic activity and heme reduction were observed when Ca^{2+}/CaM was added to the reconstituted system composed of the nNOS oxygenase domain and NADPH-cytochrome P450 reductase [29]. Therefore, the role of CaM is manifest only in the full-length wild-type enzyme, while the role of CaM in the system reconstituted from separated domains is very different [18]. Redox potential studies indicate that Ca^{2+}/CaM does not alter the redox potentials of FAD and FMN, but appears to change the protein conformation so as to facilitate intramolecular electron transfer in the reductase domain [30].

3.4. Electron transfers in chimeric enzymes constructed by swapping the reductase domains of NOS and P450BM3

We generated a chimeric enzyme composed of the P450BM3 oxygenase domain and the NOS reductase domain in order to study interdomain electron transfer in the fusion enzymes (Chimera II in Fig. 6). This chimeric enzyme had a similar substrate binding affinity to wild-type P450BM3 enzyme [31,32]. Substrate binding appeared to facilitate electron transfer by increasing the redox potential of the heme in the chimeric enzyme as in P450BM3. The addition of Ca^{2+}/CaM also enhanced the rate of interdomain electron transfer similar to that in the native NOS enzyme. Another chimeric enzyme (Chimera I in Fig. 6) composed of the NOS oxygenase domain and the P450BM3 reductase domain was very unstable, but was able to catalyze NO formation [31,32].

4. Ionic and aromatic surface amino acids of the oxygenase domain of nNOS

All isoforms of the NOS enzyme are homodimeric in their active form, although the monomeric forms are indistinguishable from the active form in terms of optical absorption spectra [2,5]. It is intriguing to note that electrons from the reductase domain of one subunit are likely to move to the oxygenase domain of the other subunit in iNOS as proposed by Stuehr's group (Fig. 2) [33,34]. This crossing in the interdomain electron transfer is therefore, an intersubunit or intermolecular process, and is a characteristic of the protein–protein interaction in the NOS enzyme. The interdomain electron transfer process may be similar to that of the microsomal P450 system in which at least one of the electrons from the reductase domain passes through the heme proximal surface of the oxygenase domain. In the microsomal P450 system, it has been suggested that heme proximal surface cationic patch(es) composed of Arg and Lys form a docking site with surface anionic patch(es) of the NADPH-cytochrome P450 reductase [35]. Appropriate ionic interaction between the two proteins appears to facilitate the intermolecular electron transfer reaction required for microsomal P450 catalysis. Since the reductase domain structure of NOS is similar to the NADPH-cytochrome P450 reductase and the intermolecular or intersubunit electron transfer reaction is similar in NOS, it is conceivable that a similar mode of recognition is operative across the NOS interdomain boundary. We mutated the well-conserved Lys423 residue, which lies near the axial ligand, Cys415, in nNOS to other amino acids and examined the catalytic properties of the Lys423 mutants [36]. The NO formation activity, the heme reduction rate with NADPH and the NADPH oxidation rate of the Lys423Glu mutant were markedly lower than those of the wild type enzyme. Lys423 appeared to be located on the heme proximal surface and exposed to the solvent according to the crystal structure of eNOS (Fig. 7) [4]. Therefore, we suggest that the intersubunit and/or intermolecular electron transfer reaction in nNOS is similar to the microsomal P450 system in that, at least the first electron from the reductase domain is transferred to the heme via the proximal ionic surface of the oxygenase domain.

5. Aromatic amino acids at the heme proximal site

The internal axial ligand to the heme of NOSs is cysteine, similar to that found in P450s. However, there

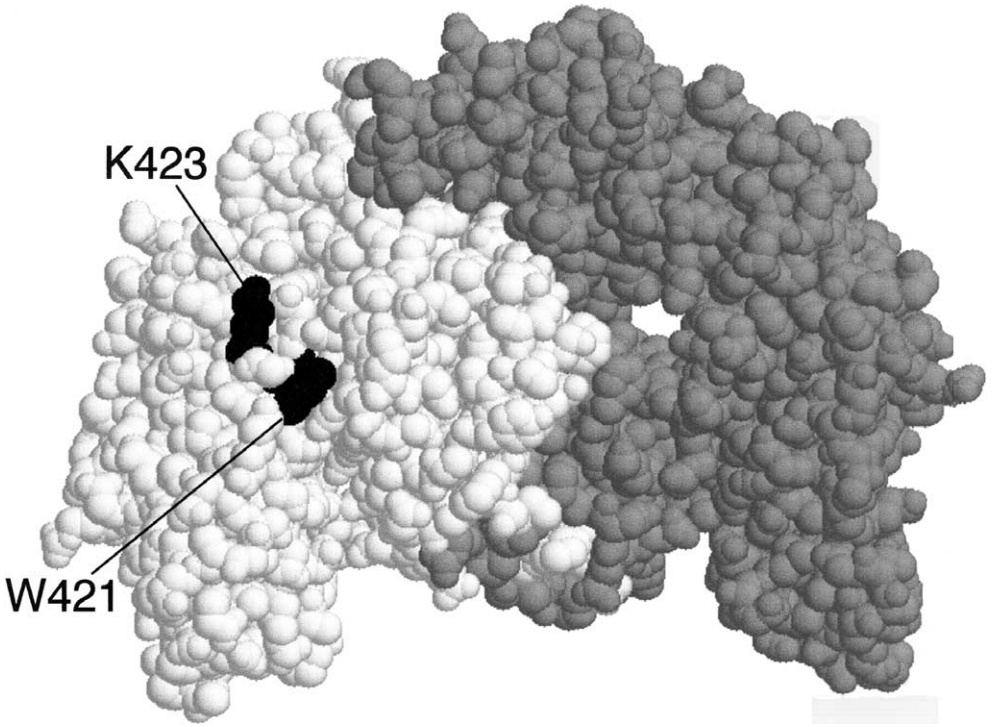

Fig. 7. Proximal surface of the oxygenase domain in NOS dimer which must interact with the reductase domain.

are differences between NOS and P450 in regard to the heme proximal structure. For instance, there is noticeable β-sheet content at the proximal site of NOS, whereas in the P450s, the α-helix content is in the majority [7–9]. Another important difference is that NOS has several conserved aromatic amino acids in the heme proximal site similar to peroxidases while P450s do not. In the nNOS structure, Trp409 hydrogen bonds to the heme coordinated Cys415 ligand (Fig. 8) and Trp421 is solvent exposed on the heme proximal surface (Fig. 7). We mutated the conserved Trp409 and Trp421 residues to other amino acids and examined the catalytic properties of the generated mutants [37]. The Trp409His, Trp421His and Trp421Leu mutants had significant NO formation activity with L-Arg and NHA. However, we found that the Trp421 mutants had NADPH oxidation rates and cytochrome *c* reduction rates much larger than those of the wild type and the Trp409His mutant. It is possible that Trp421 faces into the solvent and towards the reductase domain, playing an important role in electron transfer and the interdomain protein–protein interaction. It was puzzling that the heme reduction rates with the Trp409His and Trp421His mutants are much lower than that of the wild type although the NO formation rate of the Trp421His mutant was comparable to that of the wild type. Stuehr's group reported that Trp409Phe and Trp409Tyr mutants of nNOS showed hyperactivity of NO formation [14,16,38]. They suggested that the Trp409 mutations destabilized the ferrous–NO complex which was observed as a major component during catalytic turnover in their system as mentioned above. However, it is not in the case of Trp409His which had only 25% of catalytic activity compared with the wild type. It should be noted that mutations of hydrophobic amino acids at the proximal site of NOS often induce denaturation and/or heme dissociation, probably because the heme-binding involves some hydrophobic interactions [37,39].

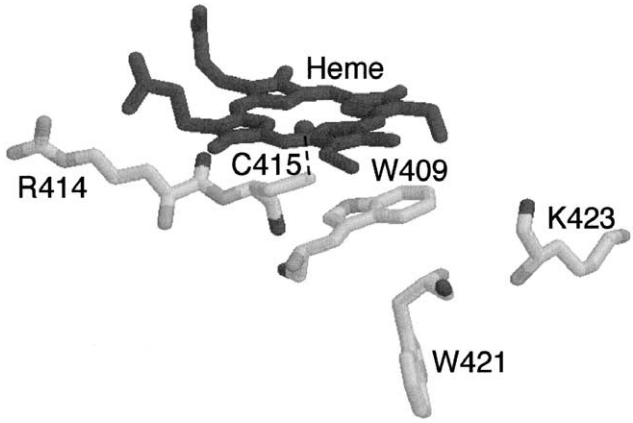

Fig. 8. The heme proximal structure of nNOS.

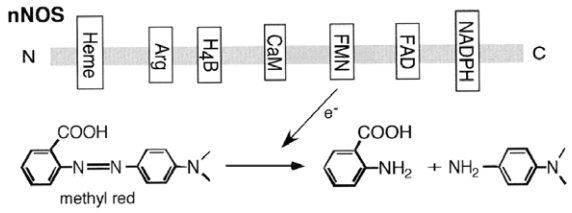

Fig. 9. Methyl red reduction by nNOS.

6. Substrate binding site and the heme distal site

We also studied the structure–function relationships at the substrate binding site and the heme distal site of nNOS using mutant and wild type enzymes [40–45]. Those sites should be distant from the electron transfer route according the concept of the P450 electron transfer system. However, it is interesting to note that mutations at these sites often increased the NADPH oxidation rates and cytochrome *c* reduction rates, suggesting that these sites are somehow involved in electron transfer and/or the redox reactions which accompany NO formation.

7. The reduction of the methyl red azo bond catalyzed by nNOS

NOS reduces dissolved molecular oxygen and generates superoxide anion ([1–6] and references therein). These reactions are not normal functions for NOS, however, it is physiologically very important for cells under some conditions. It also has the ability to reduce and/or metabolize several chemicals [3,4]. Some azo compounds are environmental contaminants and very toxic and carcinogenic due to their strong DNA modifying capability after radical formation in the cytosol. We examined whether or not nNOS degrades the azo compound, methyl red (Fig. 9). The azo bond of methyl red was efficiently cleaved with a turnover rate of 130 min^{-1} by nNOS in the presence of Ca^{2+}/CaM and NADPH under anaerobic conditions. This bond cleavage rate was strongly decreased by eliminating Ca^{2+}/CaM from the enzyme solution, suggesting that electron transfer to the azo compound was regulated by Ca^{2+}/CaM [46]. The rate of azo reduction with a FMN-deficient mutant was 5% of the wild type. The azo reduction rate of the autoinhibitory-loop deletion mutant [20] whose activity is not dependent on Ca^{2+}/CaM was observed even in the absence of Ca^{2+}/CaM. Thus, electrons delivered from the FMN subdomain of the nNOS enzyme are responsible for reduction of the azo bond of methyl red and that the reduction is regulated by Ca^{2+}/CaM [46].

8. Summary

We generated various mutant enzymes of nNOS and examined NO formation activities and the electron transfer reactions of these mutant enzymes. We found that the structure–function relationships of the NOS enzyme are fairly different from the corresponding P450 enzymes. We are further studying the electron transfer mechanism of this enzyme in focusing on the dimer–monomer equilibrium and the intersubunit nature of interdomain electron transfer [47,48].

Acknowledgements

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