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Roles of the Heme Proximal Amino Acids in the Active Site Structure and the NO Formation Activity in Neuronal Nitric Oxide Synthase

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Summary: Nitric oxide synthase is a fusion enzyme composed of an oxygenase domain and a reductase domain. The oxygenase domain has a cytochrome P450-type heme active site, whereas the reductase domain has FAD, FMN and NADPH binding sites similar to NADPH-cytochrome P450 reductase. For two stepwise monooxidation reactions from L-Arg to NO and L-citrulline to occur at the heme active site, electrons must be supplied from NADPH via the reductase domam perhaps in a similar manner as microsomal cytochrome P450s. To elucidate the role of the heme proximal amino acids in the catalytic electron transfer reaction, we mutated several conserved Lys and Arg located near the proximal site and examined the NO formation actrvities. It was found that the amino acids mutated are important to keep the heme bound in the active srte properly and also to help catalyse NO formation activity perhaps by facilitating electron transfer to the heme active site from the reductase domain.

Introduction

The nitric oxide synthases (NOS) are a family of heme enzymes that catalyze the generation of a physiologically important molecule, nitric oxide (NO) (1-7 and references therein). NOS consists of an oxygenase domain with a thiol-coordinated heme active site similar to that of cyytochrome P450 (P450), and an electron-transfer domain related to NADPH-cytochrome P450 reductase which binds FMN and FAD. It has been suggested that NOS catalysis is composed of two successive monooxygenation reactions; one from L-Arg to NG-hydroxy-L-Arg (NHA) and one from NHA to NO and L-citrulline. Based on the previous dlscussions and the obvious analogy with the P450, it is clear that the likely role of the iron-heme In NOS is to bind, reduce and activate molecular oxygen to generate the ultimate oxidant which is thought to be a high-valent, metal-oxo species. If the proposed mechanism is true, Introduction of electrons to the oxygenase domain is prerequisite for the activation of molecular oxygen during catalysis. It is possible that electron transfer to the heme from the reductase domain proceeds in an intramolecular fashion. This would be expected to cause an increase

in the efficiency of electron transfer and therefore catalysis in this enzyme because the Intermolecular electron transfer from the NADPH-P450 reductase to P450 is a rate-determining step (8-11). Ca2+/calmodulin regulates the electron transfer from the reductase domain to the oxygenase domain in NOS (12). A recent report proposed the existence of mtermolecular/intersubunit electron transfer from the adjacent reductase domain to the oxygenase domain in the homodimer of the inducible NOS (iNOS) (13) (Fig.1). Previous work in this laboratory suggested that basic amino acids such as Lys and Arg on the proximal surface of microsomal P450s are important for the interaction between the reductase and P450 for efficient electron transfer to occur (14, 15) (Fig. 2). It is possible that a similar intermolecular/intersubunit electron transfer occurs in the homodimer of other isomers of NOS, if the proposed electron transfer model is correct.

Cyt415 of rat neuronal NOS (nNOS) serves as the internal ligand of the heme iron at the proximal slte. Several charged amino acids are highly conserved around the heme binding site as shown in Fig. 3. Since highly conserved amino acids are also known to exist around the heme-bound cysteine at the P450 proximal site (Figs. 2 and 3), it is expected that highly conserved charged amino acids at the NOS proximal site may also have important roles in catalysis associated with intermolecular/intersubunit electron transfer.

In the present study, we mutated conserved basic amino acids, Arg410, Arg414, Arg418, Lys423 and Arg430 of nNOS to several amino acids and obtained difference spectra and catalytic activities for these enzymes. Some of the mutants obtained had high expression efficiency in yeast, while other mutants had absorption bands at around 420 nm for the CO-reduced complex, suggesting that the latter mutants were denatured by the mutations. Most of the mutants had very low NO formation activities compared with that of the wild type. From these results together with the three-dimensional structure of the nNOS heme active site deduced from the crystal structure of iNOS oxygenase domain, we discuss the role of these basic amino acids in NO formation catalysis with respect to the structure of the heme active site and the interdomain/intersubunit electron transfer reaction.

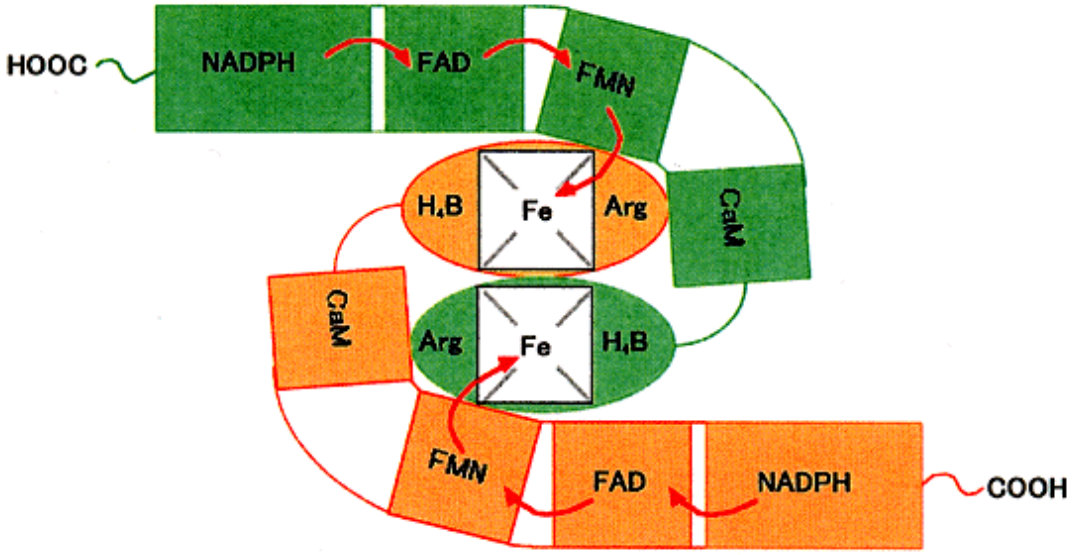

Fig.1: Proposed model for an iNOS dimer indicating domain swapping and electron transfer pathway. In this model, electrons form the adjacent reductase domain transfer to the heme active site of the oxygenase domain(13).

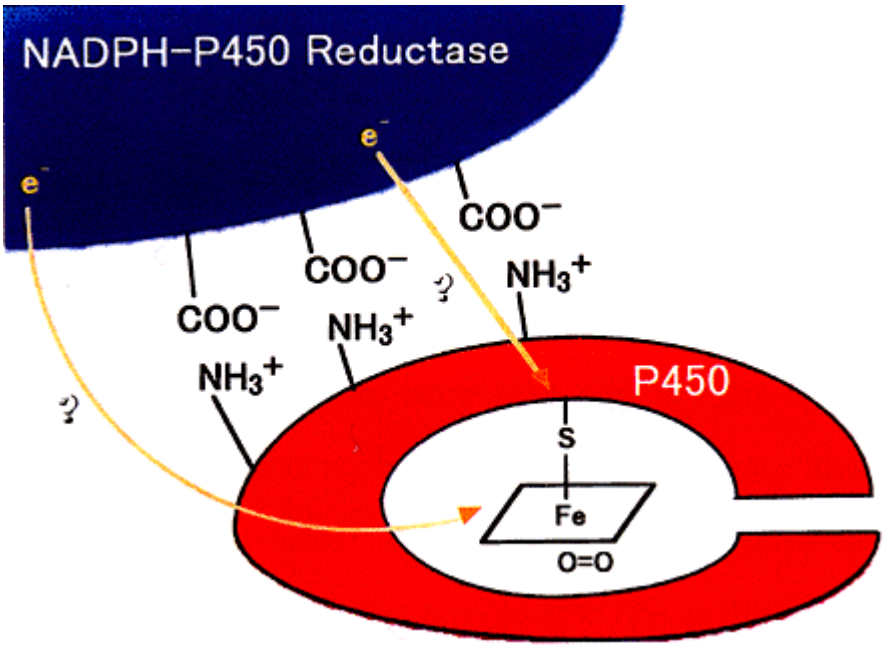

Fig.2: Hypothetical interaction between microsomal P450 and NADPH-cytochrome P450 reductase. Basic amino acids located on the proximal surface of P450 have ionic interactions with acidic amino acids of the reductase protein surface. Forming ionic bridges between the two proteins and/or orienting to the appropriate geometry may facilitate electron transfer across the interfacial surface between the two proteins.

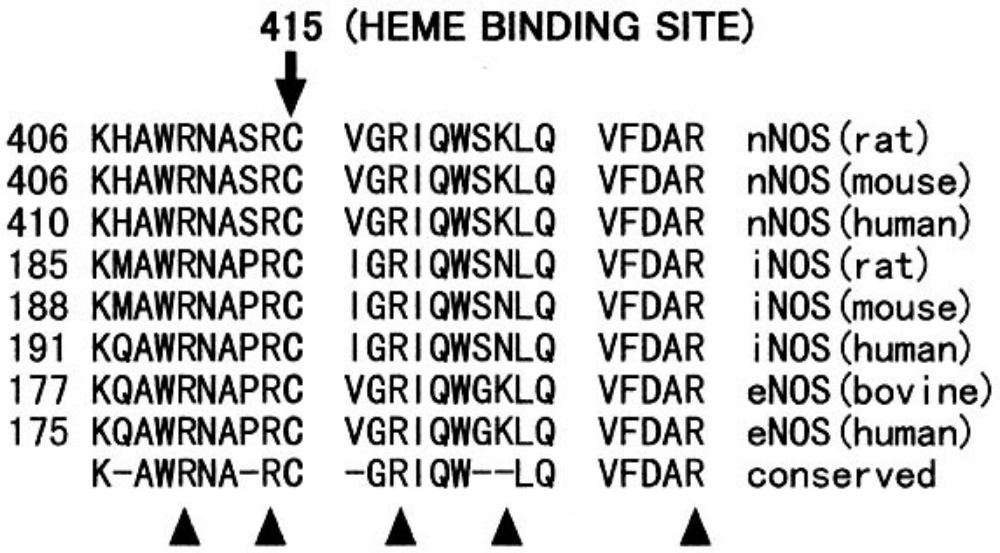

Fig. 3: Amino acid sequences at the proximal site of NOSs. Conserved basic amino acids (σ) of nNOS were mutated.

Experimental Procedures

Materials: Tetrahydrobiopterin (H4B) was purchased from Schirks Laboratories (Jona, Switzerland). Other reagents, that were obtained from Wako Pure Chemicals (Osaka, Japan), were of the highest guaranteed grade and were used without further purification.

Expression and punfication of nNOS: Wild type nNOS and proximal mutants were expressed in Saccharomyces cerevisiae using an acid phosphatase promoter as previously used for the expression of cytochrome P450 1A2 (16, 17). After the expression, the yeast cells were harvested by centrifuge at 5,000 rpm. The cell pellet was resuspended in buffer A (100 mM Tris HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 4 μM H_4B, 0.1 mM DTT, l mM PMSF, 2 mg/ml aprotinin, 2 mglml leupeptine and 2 mg/ml pepstatin). The yeast was crushed with an MSK cell homogenizer (B. Braun, FRG). After centrifugation at 100,000 g for 90 min at 4 °C, the resulting supernatant was loaded with 0.8 ml/min onto a 2', 5'-ADP-Sepharose column (Pharmacia, Uppsala, Sweden), previously reequilibrated with buffer A. After the yeast supernatant was applied to the column, the resin was washed with buffer B-1 (50 mM Tris HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 4 uM H4B, 0.1 mM DTT, 1 mM PMSF, 2 mg/ml aprotinin, 2 mg/ml leupeptine and 2 mg/ml pepstatin) containing 0. 1 M NaCl (50 - 100 ml). The column was then washed with 50 mL of buffer B-1 containing 0.4 M NaCl (0.3 M NaCl for mutants) to remove nonspecifically bound protein. The column was next washed sequentially with

50 ml of buffer B-1 containing 0.7 mM NAD+ (this was omitted for mutants), and with 50 ml of buffer B-2 (50 mM Tris HCl, pH 7.5, 0. 1 mM EDTA, 0.1 mM EGTA, 10% glycerol, 4 μM H4B and 0.1 mM DTT). To obtain larger quantity of mutant nNOSs, several washing steps were omitted due to the lower affinity of the mutants to the column m contrast to the wild type. The bound nNOS was eluted with 20 ml of buffer B-2 containing 10 mM NADPH. The fractions containing nNOS were pooled and concentrated into about 4 - 5 ml with centrifugation at 2,000 rpm using Centricut U50. To remove NADPH and further purify the protein, the sample was further purified by a calmodulin-Sepharose column (Pharmacla, Uppsala, Sweden) (2 - 3 ml) preequilibrated with buffer C (50 mM Tris HCl, pH 7.5, 0.1 mM EDTA, 10% glycerol, 10 μM H4B, and 1 mM DTT) containing 2 mM $CaCl_2$ The column was washed with 40 ml of buffer C containing 2 mM $CaCl_2$・ nNOS was eluted with 20 ml of buffer C containing 0.2 M NaCl and 5 mM EGTA. The fractions were monitored with UV-vis spectrometer at the range of 250 - 700 nm. The fractions containing nNOS were pooled and concentrated into about 2 - 3 ml with centrifugation at 2.000 rpm using Centricut U50. The purified and concentrated enzyme was dialyzed against 50 mM Tris HCl (pH 7.5) buffer containing 5 μM H4B/DTT, 0.1 mM EDTA and 10% glycerol. The enzyme was quickly frozen in liquid nitrogen and stored at -80 °C. The concentration of rat nNOS was determined optically from the [CO-reduced] - [reduced] difference spectrum using $\triangle \varepsilon_{444\text{-}467nm}$ = 55 $mM^{-1}cm^{-1}$. This Δε values was calculated by the pyridine hemochromogen method (17) assuming that one heme is bound to one subunit of this enzyme Expression efficiency was also estimated from the CO-reduced difference spectra. The specific activity of the purified wild type enzyme was approx. 70 μmole/min/μmole NOS heme (440 nmol/min/mg NOS) in terms of NO production as determined by the oxyhemoglobin method at 25 °C(1). In order to avoid denaturation of the mutants, most of experiments were conducted at 25 °C.

Assays of enzyme activity: The rate of NO formation was determined from the NO-mediated conversion of oxyhemoglobin to methemoglobin, monitored at 401 nm using a methemoglobin minus oxyhemoglobin extinction coefficient ($\triangle \varepsilon$ 401nm(MetHb-0_2Hb)) of 49 $mM^{-1}cm^{-1}$ (1). Unless otherwise indicated, assays were carried out in the reaction mixture which was incubated with enzyme at 25°C for 5 min containing 50 mM Tris HCl (pH 7.5), 10 μg/ml CaM 1 mM $CaCl_2$, 5 μM FAD/FMN, 5 μM H4B/DTT, 100 units/ml catalase, 10 units/ml SOD 100 μM NADPH and 10 μM HbO_2 in the presence or absence of 500 μML-Arg or NHA.

Optical absorption spectra: Spectral experiments under aerobic conditions were

carried out on a Shimadzu UV-2500 spectrophotometer maintained at 25 °C by a temperature controller. Anaerobic spectral experiments were conducted on a Shimadzu uv-160A spectrophotometer maintained at 14 °C in a glove box under an argon atmosphere with O_2 concentration less than 50 ppm. To ensure that the temperature of the solution was appropriate, the cell was incubated for 10 min prior to spectrometric measurements. The concentration of H4B was adjusted by dialysis against buffer containing an appropriate concentration of H4B . Titration experiments were repeated at least three times for each complex. Regression analyses were performed and lines giving an optimum correlation coefficient were selected. Linear least-squares fitting was carried out on a Power Macintosh 6100/60AV personal computer using DeltaGraph™ software as described previously (16, 18). Experimental errors were less than 20%.

Crystal Structure: The crystal structure cordinates of murine iNOS heme domain (19) was obtained through WEB from the Protein Data Bank. RasMac 2.6-ucbl.0 software was used to determine the distance between Asn202 and Trpl88 in iNOS

Results

In nNOS expression system with acid phosphatase promoter, the yeast cells were fist grown in high phosphate Berkholder minimum medium, and the medium was changed to low-phosphate minimal medium to induce the protein expression. This two step growth system of yeast is useful for the expression of NOS, because NOS and/or NO would be cytotoxic for the host. Actually the wild type protein of nNOS could be successfully overexpressed in yeast and purified with sufficient amounts for characterization of various spectral and kinetic parameters. However, some nNOS proximal mutant proteins are not properly folded and thus we often faced difficulties to obtain sufficient enzyme.

Fig. 4 shows CO-reduced difference spectrum of the wild type (———), Lys423Leu (……) and Arg430Leu (—•—•—•) mutants. Table I summarizes the expression efficiencies of the mutants, that were estimated from the intensity of the CO-reduced difference spectrum and the position of the CO-reduced difference spectrum of the crude yeast extract. The CO-reduced difference spectrum of the wild type has a single large peak around 445 nm and thus indicates that the enzyme is in Its active form in terms of the spectral character of the heme active site. The CO-reduced difference spectrum of the Lys423Leu mutant had two peaks around 420 mm and 445 nm. The 420-nm peak increased concomitant with a decrease of the 445-nm peak In 10 - 20 min at room temperature such that eventually only the 420-nm peak remained. The Arg418Leu and

Arg430Leu mutants provided only a 420-nm peak in the CO-reduced difference spectrum (Fig. 4). The 420-nm peak indicates that the coordinated thiol is dissociated from the reduced heme iron and perhaps a CO-Fe2+_N- (imidazole of His) complex Is formed due to denaturation of the heme surrounding.

The Arg410Leu and Arg414Leu mutants provided a very small peak at around 445 nm, although significant intensity of the peak at around 420 nm was not observed.

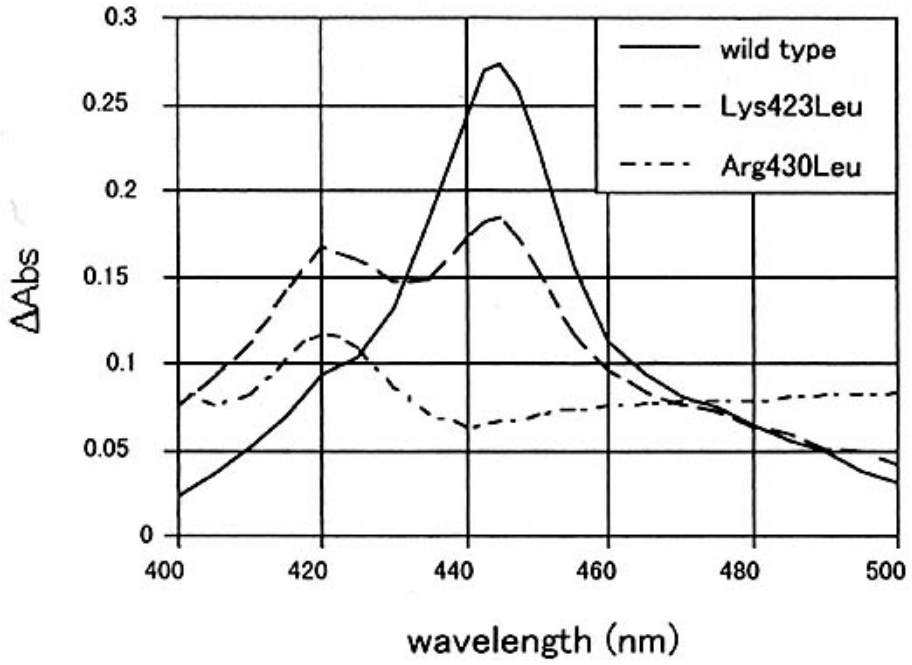

Fig. 4: The CO-reduced difference spectra of the wild type (———), Lys423Leu (----) and Lys430Leu (—・—・—・) mutants. Spectra were obtained for the supernatant of the crushed yeast per se harboring the expression vector of the wild type and the mutants.

This indicates that expression of the active enzyme is low because the intensity of the CO-reduced difference spectra tightly reflect the amount of heme containing enzyme in the solution. It is also possible that the protein of the mutant is sufficiently expressed in yeast, but only a part of the heme is bound to the active site. Without examining the mutant protein concentratron by Western blotting or other methods, it is difficult to deduce whether or not the protein expression efficiency is good enough. In any event, it is certain that the holoenzyme of the Arg410Leu and Arg414Leu mutants are not sufficiently expressed in yeast. A global conformational change and/or refolding might occur due to the mutations.

The Lys423Glu, Lys423Met and Lys423Asn mutants had absorption peaks at around 445 nm in the CO-reduced difference spectrum, and had enough expression efficiencies comparable to that of the wild type in terms of the intensity of the CO-reduced difference spectrum.

As expected, the mutants which showed only 420-nm peak had no NO formation activity (Table 1). The Arg414Leu and Lys423Glu mutants had no NO formation activity.

Table 1: Expression Efficiency, CO-reduced Difference Spectra and NO Formation Activity of the Proximal Mutants of nNOS

Enzymes	Expression [a] (nmol/36 L)	CO-reduced difference spectra (nm)	NO formation [b] (nmol/min/nmol heme)
Wild type	300	445	72
Arg410Leu	< 10	445	15
Arg414Leu	< 10	445	0
Arg418Leu	100 [c]	420	0
Arg423Leu	100	455 / 420	25
Lys423Glu	200	445	< 1
Lys423Met	200	445	18
Lys423Asn	250	445	78
Arg430Leu	< 10 [c]	420	0

a Expression efficiency was calculated from the intensity of the CO-reduced difference spectrum of yeast harboring the expression vector.

b Experiments were repeated at least twice. Experimental errors were less than 20%. Solutions consisted of 500 μM L-Arg, 50 mM Tris-HCI (pH 7.5), 10 μg/ml CaM, 1 mM CaCl2 5 uM FAD/FMN, 5 μM H4B/DTT, 100 units/ml catalase, 10 units/ml SOD, 100 μM NADPH, 10 μM Hb02 and 0.05 - 0. 1 μM nNOS.

c Calculated from a molar absorbance intensity assumed 10^5 M^{-1}.

The Arg410Leu, Lys423Leu and Lys423Met mutants had measurable NO formation activities, but these values were less than one third of that of the wild type. In terms of NO formation activity under the described conditions, the active site of the Lys423Asn

mutant does not appear to be greatly affected by the mutation.

Discussion

The X-ray crystal structure of the monomer of the iNOS oxygenase domain is now available (19). The crystal structure of the oxygenase domain of iNOS (Figs. 5 and 6) indicates that cysteine thiolate of the axial ligand, Cysl94, interacts with several arginines such as Argl89, Argl93, Argl97 as well as with Trpl88 via ionic or hydrogen bonds. By comparing the amino acid sequences at the proximal site of NOSs (Fig. 3), Arg410, Arg414, Lys423 and Arg430 of nNOS correspond to Argl89, Argl93, Argl97, Asn202 and Arg209 of iNOS, respectively. If the proximal structure of nNOS is similar to that of iNOS, it is conceivable that axial ligand, Cys415, interacts with Arg410, Arg414 and Arg418 in the proximal site of nNOS.

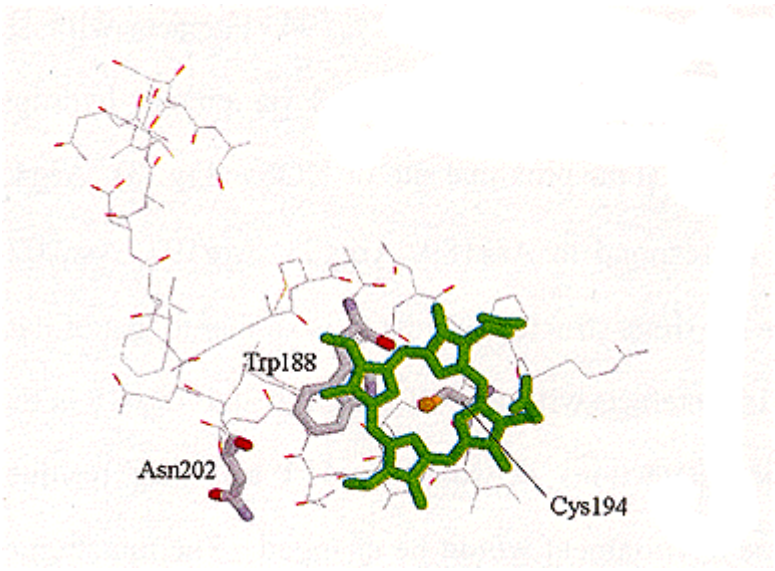

Fig. 5: The proximal structure of iNOS heme domain from the view of distal side. Trp188, Cys 194, and Asn202 of iNOS correspond with Trp409, Cyt4 15, and Lys423 of nNOS, respectively.

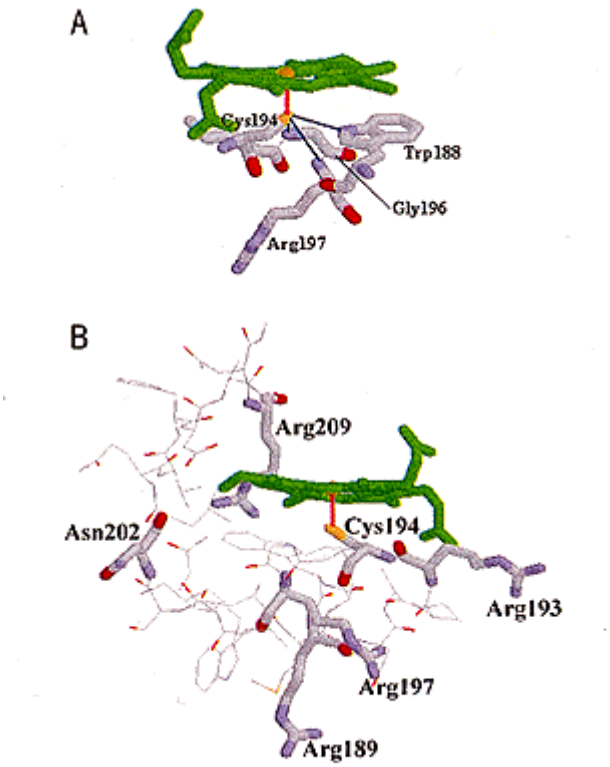

Fig. 6:　Structure in the heme active site of iNOS oxygenase domain. A: Amino acid residues directly interacting with heme-bound cysteine via hydrogen bonds are shown by blue lines. B: Corresponding amino acid residues of iNOS that were mutated for nNOS in this study are shown Trp188, Arg189, Arg193, Cys194, Gly196, Arg197. Asn202 and Arg209 correspond with Trp409, Arg410, Arg414, Cys415, Gly417, Arg418, Lys423 and Arg430 of nNOS, respectively.

If the ionic interactions between Cys415 and Arg residues are disrupted by the mutations, the heme environment would be changed. The mutation of Arg418Leu resulted in the denatured enzyme with a 420-nm peak in the CO-reduced difference spectrum, suggesting that the mutation largely affects the coordination structure of the Cys415. Perhaps, the thiol anion of Cys415 is inappropriately coordinated to the heme iron in the mutant. An ionic Interaction between Cys415 nitrogen and Arg nitrogen would support the axial coordination from back side maintaining the proper conformation or orientation of Cys415 for ideal ligation to iron. It is interesting to note that the mutation of Arg430 of nNOS, which corresponds with Arg209 of iNOS and is located more than 20 A from the heme plane, resulted in a 420-nm peak in the CO-reduced difference spectrum. Perhaps, a global conformational change was caused

by the Arg430Leu mutation.

The Arg410Leu and Arg414Leu mutations caused the very low expression effirciency in yeast. It is hard to explain whether the heme binding capability was decreased or the protein expression efficiency per se was decreased in these mutants. If the former is the case, then protein unfolding would take place and it would tend to be more degraded due to accessibility by peptidases. In this case, even Western plotting procedure may fail to detect the mutant protein In yeast extract. Perhaps, the Arg410Leu and Arg414Leu mutations profoundly change the heme active site structure especially at the proximal site and thus lowered the heme binding capability. However, the Arg410Leu and Arg414Leu mutations are not so drastic as to change the conformation or orientation of the coordinated Cys and to generate a denatured form with non-thiolate ligand on the heme iron.

The mutations at Lys423 neither caused to denaturation around the heme active site nor lower the expression efficiency in yeast. Nevertheless, NO formation activities of some Lys423 mutants were markedly lower than that of the wild type. The crystal structure of eNOS indicate that the corresponding Lys seems to stick out of the proximal protein surface (20). It Is possible that Lys423 is very important in catalysis perhaps in association with the electron transfer from the reductase domain to the oxygenase domain

In summary, it is suggested that (1) Arg418 and Arg430 are prerequisite for the coordination of the internal thiol anion to the ferrous heme perhaps by supporting the cysteinyl axial ligand from the backward position.; (2) Arg410 and Arg414 are important to keep the heme environment suitable to hold the heme plane in the active site.; and (3) Lys423 is Important in catalysis perhaps associated with the electron transfer from the reductase domam to the oxygenase domain.

The basic amino acids of the proximal site should be important in structuring the heme environment and/or heme proximal protein surface of nNOS appropriately for optimal catalytic activity. The crystal structures of bovine and human iNOS and endothelial NOS oxygenase domains (20, 21) showed that the proximal structures of the isomers are similar to that of rat iNOS. In regard to the electron transfer of NOS, the important role of Lys423 of nNOS in the catalysis associated with the interdomain/intersubunit electron transfer was studied more in detail elsewhere (22). Also extra 42-amino acids loop located in the FMN-binding site of nNOS appears to be important in controlling the interdomain/intersubunit electron transfer of nNOS under specific conditions (23).

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神経型－酸化窒素合成酵素内のヘム近位アミノ酸の活性部位構造と一酸化窒素合成活性における役割

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要旨－酸化窒素合成酵素は酸化反応ドメインと還元酵素ドメインとから構成される融合酵素である。酸化反応ドメインはチトクロム P450 型のヘム活性部位を保持しているが、還元酵素ドメインは NADPH チトクロム P450 還元酵素と同様に FAD,FMN、及び NADPH 結合部位を保持している。ヘム活性部位でアルギニンから一酸化窒素とシトルリンが合成される 2 段階の一酸素添加反応が進行するためには、電子はミクロゾーム型チトクロム P450 と同様に、NADPH から還元酵素ドメインを経由してヘムへ移動すると推定される。ヘム近位側に存在するアミノ酸の電子移動反応における役割を理解するために、我々はヘム近位側に良く保存されたリジンやアルギニンを変異させて一酸化窒素合成反応活性を調べた。その結果、変異させたアミノ酸はヘムを活性部位に都合良く結合させるのに重要であり、又、それらは、電子が還元酵素ドメインからヘム活性部位へ効率良く移動させるのに重要な働きをしているなど、一酸化窒素合成活性に重要な役割を果たしていることがわかった。