Site-Directed Mutagenesis of the Conserved Threonine (Thr243) of the Distal Helix of Fungal Cytochrome P450nor†

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ABSTRACT: Cytochrome P450nor (P450nor) is a heme enzyme which catalyzes NO reduction in denitrifying fungi. Threonine 243 (Thr243) of P450nor, which corresponds to the conserved threonine of monooxygenase cytochrome P450s, was replaced by 18 different amino acids via site-directed mutagenesis. The mutation did not seriously affect the optical absorption and the CD spectral properties of the enzyme in several oxidation, ligation, or spin states or the association rate constant for association of NO with the ferric iron, suggesting subtle and local structural changes in the heme environment on Thr243 mutation. However, the NO reduction activity was dramatically altered by Thr243 mutation, depending on the properties of the replaced amino acids. The catalytic activity, as measured by N_2O formation and NADH consumption, was considerably retained on substitution of Asn, Ser, and Gly for Thr243, while it was profoundly decreased or lost on substitution with other amino acids. Kinetic analysis of the reaction of the enzymes with NO and NADH indicated that the decrease in the enzymatic activity upon Thr243 mutation mainly results from a decrease in the rate of reduction of the ferric−NO complex with NADH. On the basis of these enzymatic, kinetic, and spectroscopic results, as well as on the basis of the crystal data for native P450nor [Park, S.-Y., et al. (1997) *Nat. Struct. Biol. 4*, 827−832], the role of the conserved threonine at the 243 position in the NO reduction reaction by P450nor is discussed. We also discuss structural similarities or differences in the vicinity of the conserved threonine between P450nor and other monooxygenase P450s.

The fungal nitric oxide reductase (NOR) is involved in denitrification by fungi, in which nitrate (NO_3^-) and nitrite (NO_2^-) are converted into nitrous oxide (N_2O) via nitric oxide (NO) (*4*). This enzyme catalyzes the NO reduction reaction, in which NO is reduced to N_2O using NAD(P)H as the electron donor; $2NO + NAD(P)H + H^+ \rightarrow N_2O + H_2O + NAD(P)^+$ (*2*). The enzyme is a monomeric protein with a molecular mass of 46 kDa and contains a protoheme as a prosthetic group in its active site. The spectroscopic and some biochemical properties of the enzyme are similar to those of heme monooxygenases, the so-called cytochrome P450s. Indeed, the primary structure is up to 40 and 25%, on average, identical to those of the monooxygenase P450s (*5*). Therefore, the fungal NOR can be classified as a member of the P450 superfamily and is given a systematic name P450 55A1, although the common name, P450nor,[1] which is specifically representative of its functional characteristics, is normally used.

We recently determined the crystal structure of P450nor isolated from the denitrifying fungus *Fusarium oxysporum* (*6*) and showed that its molecular structure is basically similar to those of the monooxygenase P450s (*7*−*11*), e.g., a triangular molecular shape, α- and β-domain structures, a proximal Cys loop, and a long distal I helix. In this structure, it is noteworthy that the threonine residue (Thr243) in the distal I helix is located in the immediate vicinity of the heme active site (Figure 1A) and that its hydroxyl group is hydrogen-bonded with the main chain carbonyl group of Ala239 through two water molecules, making a groove in the helix.

The threonine residue at the distal I helix is conserved in almost all P450s (*12*), and they are thought to play a crucial role in the monooxygenation reaction as a key residue. For example, it has been reported that the catalytic property of *Pseudomonas putida* P450cam was dramatically altered when its conserved threonine (Thr252) was replaced with other amino acid residues via site-directed mutagenesis; e.g., on substitution of alanine for the conserved threonine (Thr252) in P450cam, the hydroxylation reactivity of *d*-camphor was

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[1] Abbreviations: P450nor, product of the CYP55 gene (*2*); P450cam, product of the CYP101 gene (*3*). The nomenclature of cytochrome P450 is adopted from ref *1*.

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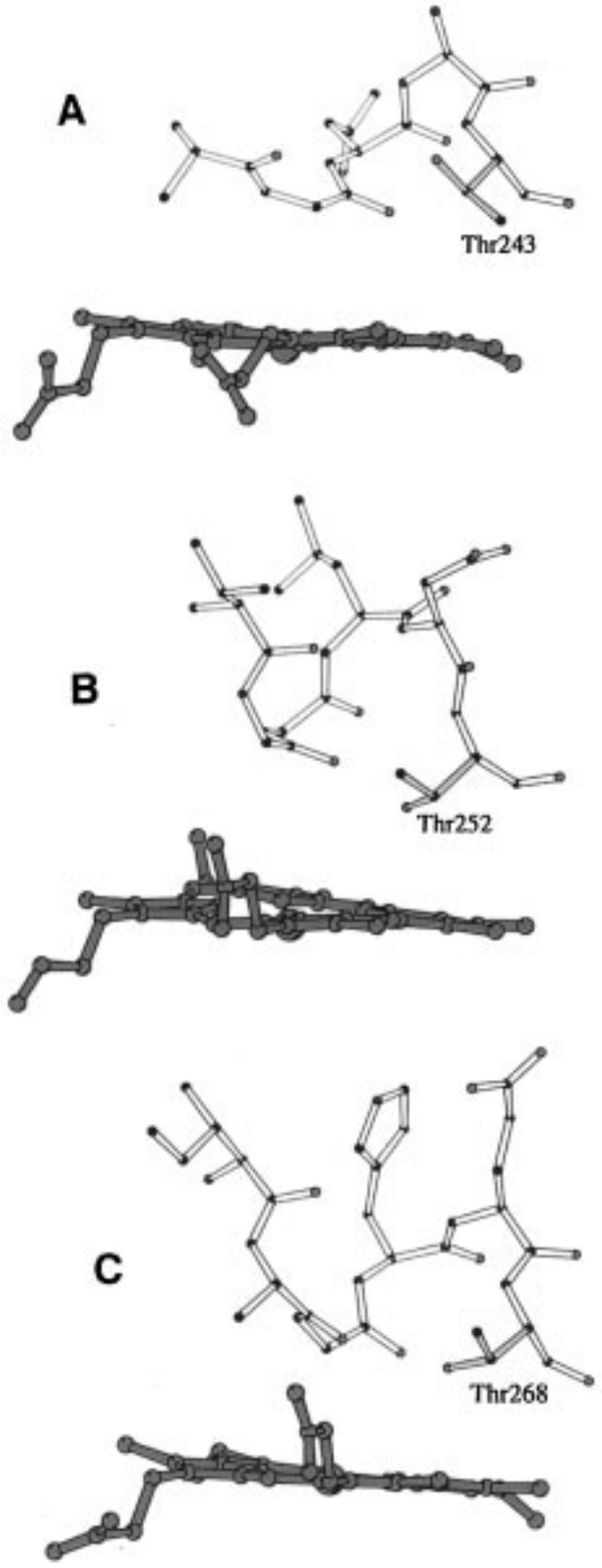

FIGURE 1: Structural comparison around the conserved threonine in the distal I helix for (A) P450nor, (B) P450cam, and (C) P450BM3.

lost, but the mutated enzyme (T252A) generated H_2O_2 as a product of the uncoupling reaction (*13*, *14*). On the basis of this observation, it is generally believed that the conserved threonine is involved in the proton transfer in the monooxygenation reaction (*15*). We also reported that the spectral and catalytic properties of P450 2C2 are altered when a variety of amino acid residues are substituted for its conserved Thr (Thr301) and suggested that the conserved Thr301 of P450 2C2 is probably located very close to the heme surface (*16*−*18*). The suggestion was entirely consistent with the relatively narrow substrate specificity of P450 2C2 as a drug-metabolizing enzyme. Shimizu et al. (*19*) have also pointed out the importance of the conserved threonine (Thr319) in P450 1A2 in its catalytic reaction through their mutagenesis studies.

In addition, we have also found that, when lysine was substituted for the conserved threonines of P450 2C2, P450 2E1, and P450 2C14, the optical spectra were changed from those of the wild-type enzymes in a different manner, among these three P450s (*20*, *21*). These observations were likely interpreted to be due to the different bindings of the ϵ-amino group of the substituted lysine to the iron among the three P450s, possibly because of certain differences in the topology of the conserved threonine relative to the heme iron. Indeed, in the crystal structures of some P450s (*8*−*11*), some differences in the location and configuration of the conserved threonine relative to the heme plane can be observed, as in Figure 1. In addition, it was also found that the Ser mutant of the conserved threonine exhibited different mutation effects on the stereoselectivity of fatty acid monooxygenation between P450 2C2 and P450 2E1 (*22*). These differences appear to be related to the individuality of P450 as an enzyme which is a member of the P450 superfamily having versatile functions.

As stated above, the fungal P450nor also contains a conserved threonine (Thr243) in the distal I helix, although this enzyme is not a monooxygenase but a NO reductase. Most recently, we observed that the lysine mutant of Thr243 in P450nor does not exhibit an absorption spectrum characteristic of the nitrogenous ligand-bound ferrous iron (*23*), which is in sharp contrast to the mutation results in P450 2C2, P450 2E1, and P450 2C14. These observations have led us to question the nature of the differences in structure around Thr243 between P450nor and other P450s. In addition, the exact function of Thr243 in the NO reduction reaction with P450nor is not known. In this study, we performed a mutagenesis of P450nor, by which the Thr243 was replaced by every amino acid which normally occurs in protein sequences, and examined the spectroscopic, kinetic, and enzymatic properties of the Thr243-mutated enzymes to characterize the structure in the vicinity of Thr243 in detail and to evaluate the role of Thr243 in the catalytic reaction of P450nor.

MATERIALS AND METHODS

Materials. The following chemicals and biochemicals were from the sources indicated in parentheses: Klenow fragment of *Escherichia coli* DNA polymerase I and DNA ligation kit (Takara Shuzo), restriction enzymes (Nippon Gene), Sequenase DNA sequencing kit (United States Biochemical Corp.), in vitro mutagenesis kit (Amersham Japan), [α-^{35}S]dATP-αS (37 TBq/mmol) (Radiochemical Center, Amersham), Bacto Tryptone, Bacto Yeast Extract, and Bacto Agar (Difco Laboratories), butyl isocyanide (Aldrich Chemical Co.), and mutagenic oligonucleotide primers and the cDNA sequencing primer for screening the mutants, 5′-AATATCGACAAGTCCGA-3′ (Cruachem). Plas-

Table 1: Spectral and Kinetic Properties of the Thr243 Mutants of P450nor

residue 243	spin state of ferric form (high spin[a] %)	rate constant of NO binding ($\times 10^7$ M^{-1} s^{-1})	ferrous−butyl isocyanide complex λ_{max} (nm)
Thr (wild-type)	44	4.0	427
Ser	55	ne[c]	427
Cys	53	ne	423
Asn	42	3.8	427
Gln	37	1.8	425
Gly	41	ne	426
Ala	52	ne	ne
Val	37	3.9	427
Ile	31	2.8	427
Leu	46	3.2	427
Met	35	ne	ne
Pro	37	ne	ne
Tyr	47	1.3	427
Phe	43	ne	427
Trp	87	1.1	426
Asp	23	ne	ne
His	37	ne	426
Lys	N[b]	1.6	425
Arg	60	ne	426

[a] The content of the high-spin form, as an approximate value for comparing the mutants with one another, was estimated from the ratio of the absorbance at γ_{max} of the low-spin form (414 nm) to that of the high-spin form (390 nm) (*23*). [b] Spectrum containing the nitrogenous ligand-bound form. [c] ne, not examined.

mid pCW (*24*) was kindly provided by A. Roth of R. Dahlquist's laboratory (University of Oregon, Eugene, OR). Other chemicals and biochemicals used were from the same sources as previously described or of the highest quality commercially available.

Mutagenesis and Expression Plasmids. The *Bss*HII−*Xba*I fragment of pGFP450-1 was inserted between the *Bss*HII−*Xba*I site of M13mp18/P450nor(Nmod) (*23*) and subjected to site-directed mutagenesis by the method of Nakamaye and Eckstein (*25*) according to the instructions provided by the kit supplier. The desired mutants were selected by sequencing the single-stranded DNA inserts (*26*) prepared from progeny phages. In the case of some mutants, preliminary tests were performed by digestion of the inserts (RF forms) with restriction enzymes. The *Bss*HII−*Xba*I fragment of pCW/P450nor was replaced by the corresponding fragment of the mutants to yield the plasmids for expression of the mutated P450s.

Bacterial Expression. A single ampicillin-resistant colony of *E. coli* JM109 cells, transformed with the mutated P450nor expression plasmid DNA, was grown overnight at 37 °C while it was shaken in terrific broth (TB) containing 200 μg of ampicillin per milliliter. The preparation was diluted 1/1000 in TB medium containing 200 μg of ampicillin per milliliter, and the cells were cultured at 37 °C to an optical density of 0.7−0.9 at 600 nm. Expression of the P450 was then induced with 1 mM isopropyl β-D-thiogalactoside, and the culture was cooled to 30 °C. After 24 h, the cells were harvested by centrifugation at 5000*g* for 10 min.

Purification of Mutated P450nors. The cells were treated with lysozyme, and the supernatant fraction obtained by centrifugation was subjected successively to DEAE-cellulose and hydroxyapatite column chromatographies by essentially the same procedures as those described for the wild-type P450nor (*23*).

Analytical and Assay Methods. Protein concentrations were determined by the method of Lowry et al. (*27*). The concentration of the mutated P450nor was determined from the CO-difference spectrum (*28*) using molar absorption increments for the wild-type P450nor of 86.9 mM^{-1} cm^{-1} between 448 and 490 nm (*2*). The concentration of NADH was determined from the absorbance at 340 nm using a molar absorption coefficient of 6.22 mM^{-1} cm^{-1}. Catalytic activity was assessed by two methods. (a) The reduction of NO to N_2O was determined by gas chromatography as described (*2*), and (b) the NADH consumption was monitored by the absorbance change at 340 nm (*29*). Flash photolysis and stopped-flow rapid scan measurements were carried out using equipment constructed by Unisoku (Osaka, Japan) as described previously (*29*, *30*). Absorption spectra were measured using JASCO Ubest 50 spectrophotometer. CD spectra were measured using a JASCO J720 automatic recording spectropolarimeter.

RESULTS

Expression of Thr243-Mutated P450nor. We undertook the preparation of the Thr243 mutants of P450nor, in which Thr243 in the distal I helix was replaced by every amino acid. We have already constructed the expression system of the recombinant enzyme in *E. coli* cells and have prepared its Arg, His, and Lys mutants (T243R, T243H, and T243K, respectively) (*23*). In this study, the cDNAs of the other mutants were constructed by the oligonucleotide-directed mutagenesis of the P450nor cDNA. Overall yields of the mutants varied, depending on the amino acid introduced at position 243. For example, the expression level of the recombinant enzyme, as detected spectrophotometrically, was 60 nmol/L of culture for the Asp mutant (T243D) and 1000 nmol/L of culture for the Trp mutant (T243W). We were unable to obtain the Glu243 mutant of P450nor because the preparation of the cDNA for the Glu243 mutant has not yet been successful.

Optical and CD Spectral Properties of Thr243-Mutated P450nor. We measured the optical absorption spectra of all Thr243 mutants of P450nor in the ferric resting, ferric NO-bound, ferrous, ferrous CO-bound, and ferrous butyl isocyanide-bound forms. In Figure 2, the spectra of the Asn mutant (T243N), the Val mutant (T243V), the Gln mutant (T243Q), and the Trp mutant (T243W) are shown. When these spectra were compared with the corresponding spectra of wild-type (WT) P450nor, it was found that the spectra of the Thr243 mutants in all states, except for the ferric resting form, basically exhibited features similar to those of WT P450nor.

The ferric−NO complexes of the Thr243 mutants exhibited a Soret absorption at 432 nm. As shown in Table 1, the bimolecular rate constant, k_1, in the NO association to the ferric resting enzyme is on the order of 10^7 M^{-1} s^{-1} for the Thr243 mutants examined, showing that the Thr243 mutation does not practically affect NO binding. The affinity of CO for the ferrous enzyme was not altered by these Thr243 mutants, and the spectral features of the resultant ferrous CO complex of the Thr243 mutants were also indistinguishable between the WT and the mutants, where an intense absorption was observed at 448 nm (data not shown). The ferrous isocyanide complex of P450nor gave

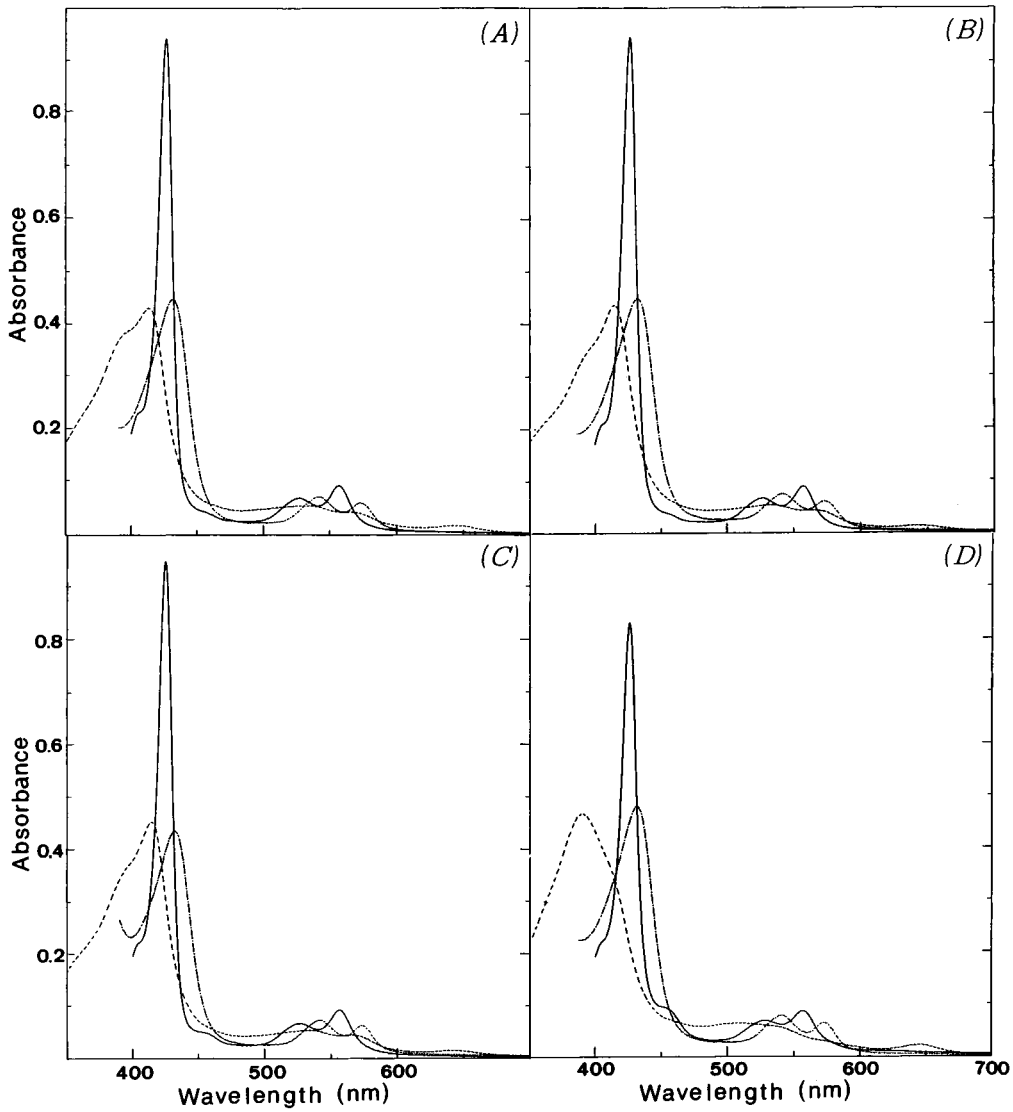

FIGURE 2: Optical absorption spectra of (A) T243N, (B) T243V, (C) T243Q, and (D) T243W mutants of P450nor in the ferric resting (- - -), ferric NO-bound (− · −), and ferrous isocyanide-bound (—) forms.

a single absorption around 427 nm (*31*), and this spectral feature was basically unchanged for the Thr243 mutation (see Figure 2 and Table 1). However, the affinity of butyl isocyanide for the ferrous enzyme varied among the mutants; the dissociation constants are 0.1 mM (WT), 0.25 mM (T243N), 0.2 mM (T243V), 0.5 mM (T243Q), 0.25 mM (T243I), 0.45 mM (T243L), 2.5 mM (T243W), and 2.5 mM (T243Y).

In the ferric resting state, the Thr243 mutants, as well as the WT enzyme, gave spectra typical of the low- and high-spin mixed type in P450s, but the high spin/low spin ratio varies slightly among the mutants in the range of 30−60% for the high-spin content, as shown in Table 1. In the tryptophan mutant (T252W), the ferric high-spin state is exceptionally predominant.

To evaluate the structural difference in more detail, especially in the protein secondary structure, induced by the Thr243 mutation, we measured the CD spectra of the WT and the Thr243 mutants of P450nor in the ferric resting state. Figure 3A shows the CD spectra in the far-ultraviolet (UV) region for WT, T243N, T243V, and T243Q. It is noteworthy that these spectra are completely superimposable. The helical content, as estimated from the $[\theta]_R$ value at 222 nm, is 50% for these enzymes, which is consistent with the value obtained from the crystal structure of the WT enzyme. The other Thr243 mutants showed the same feature, exhibiting no change in the gross secondary structure of the enzyme on Thr243 mutation.

We also measured the CD spectra of P450nor in the near-UV region. In Figure 3B, the absolute CD spectrum of WT and the difference spectrum between T243N and T243V are illustrated. It was found that the CD spectrum of P450nor in the near-UV region was essentially unchanged upon substituting any amino acid residue for Thr243. Since the CD spectrum in the near-UV region reflects fine structures around aromatic amino acid residues in proteins, these data suggest that environmental structures of the aromatic amino acid residues contained in P450nor, i.e., two tryptophan, eight tyrosine, and nineteen phenylalanine residues, are not influenced by the Thr243 mutation. The CD spectral results allowed us to conclude that the Thr243 mutation does not induce a large change in the protein secondary structure of P450nor.

Catalytic Activity of Thr243-Mutated P450nor. The enzymatic activities of the Thr243 mutants of P450nor were measured by two methods, i.e., N_2O production and NADH

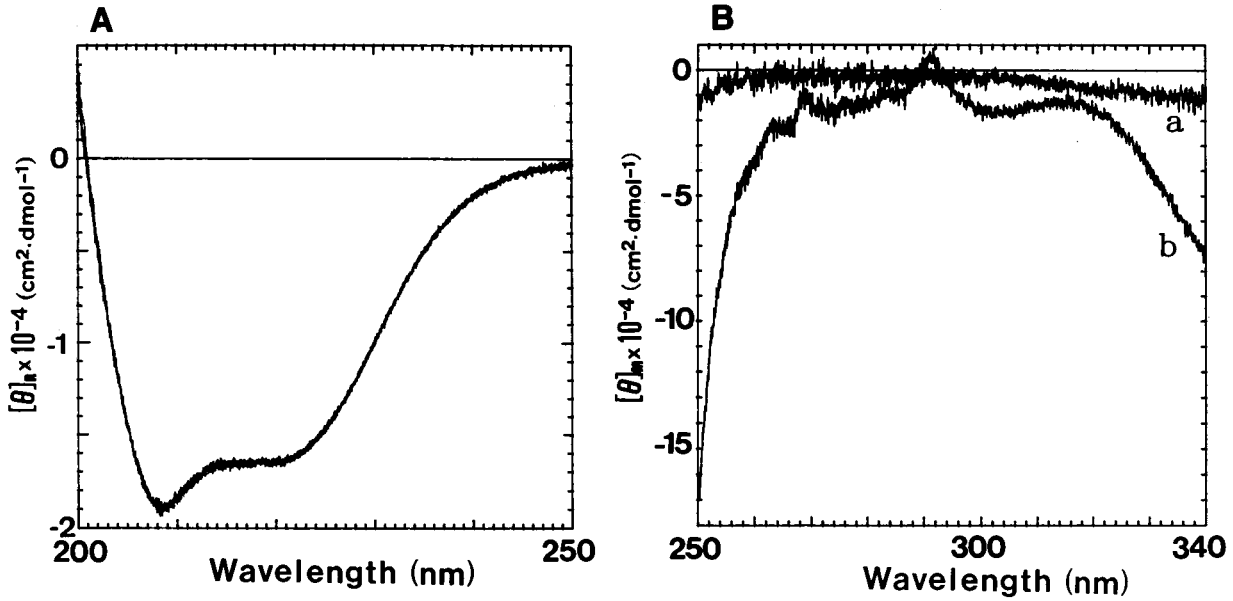

FIGURE 3: (A) CD spectra in the far-UV regions for WT and Thr243 mutants of P450nor: (—) WT, (- - -) T243N, (···) T243V, and (— · —) T243Q. Mean residue ellipticities, $[\theta]_R$, are expressed on the basis of the numbers of amino acid per molecule of P450nor. (B) CD spectra in the near-UV region: (a) a difference spectrum between T243V and T243N and (b) an absolute spectrum of the WT enzyme. Molar ellipticities, $[\theta]_M$, are expressed on the basis of the molar concentration of P450nor.

Table 2: Catalytic and Kinetic Properties of the Thr243 Mutants of P450nor

	catalytic activities [relative values[a] (%)]		rate constant	
residue 243	NADH consumption	N_2O production	intermediate formation ($\times 10^5$ M^{-1} s^{-1})	intermediate decomposition (s^{-1})
Thr (wild-type)	100	100	9.0	0.027
Asn	67	83	3.2	0.005
Gly	37	47	2.9	0.006
Ser	33	18	2.3	0.004
Ala	5.4	9.3	1.0	0.014
Gln	4.4	2.1	0.06	0.004
Cys	1.3	6.1	ne[c]	ne
Val	0.8	2.2	0.08	0.004
Met	0.8	1.5	ne	ne
Arg	0.8	3.2	ne	ne
Lys	0.7	1.3	0.16	0.053
Pro	0.7	2.0	ne	ne
Asp	0.4	1.1	ne	ne
His	0.3	0.9	ne	ne
Ile	0.2	0.8	0.008	0.002
Leu	nd[b]	0.3	0.029	0.49
Phe	nd	0.1	ne	ne
Tyr	nd	0.3	0.006	0.41
Trp	nd	nd	0.001	0.002

[a] Absolute values for the wild-type P450 were 5.21 μmol of NADH consumed per minute per nanomole of P450 and 2.57 μmole of N_2O produced per minute per nanomole of P450. [b] nd, not detected. [c] ne, not examined.

consumption, and the values obtained by these methods were consistent with each other within experimental accuracy, as is seen in Table 2. Inspection of Table 2 shows that the enzymatic activity of P450nor was significantly altered by the single mutation of Thr243, depending on the properties of the amino acid which replaced it. On the basis of the activity levels of the NO reduction, the Thr243 mutants can be classified into three groups. (1) Mutants having amino acids with a small and polar but nonionic side chains (Asn or Ser) or no side chain (Gly) instead of Thr243, i.e., the T243N, T243S, and T243G mutants, have significant activities, comparable to that of the WT enzyme. (2) Mutants having Ala, Gln, and Cys at the 243 position, i.e., T243A, T243Q, and T243C, have significantly lowered activities. (3) Other mutants, which have a large aliphatic or aromatic or an ionic side chain at the 243 position, exhibit little enzymatic activity. Groups 2 and 3 are practically inactive.

Formation Rate of the Reaction Intermediate for Thr243-Mutated P450nor. In our previous study, we showed that the ferric−NO complex of P450nor is reduced with NADH to yield a characteristic intermediate (λ_{max} = 444 nm), which is spectrophotometrically distinguishable from both the ferrous− and ferric−NO complexes of the enzyme (*29*). We have thought of the intermediate as a two-electron-reduced product of the ferric−NO complex, formally $[Fe^{3+}NO]^{2-}$. On the basis of this observation, we proposed that this step is important in the molecular mechanism of the NO reduction catalyzed by P450nor. Therefore, to discuss the effect of mutation of Thr243 on the catalytic reaction of P450nor in more detail, we kinetically and spectrophotometrically followed the NADH-dependent reduction steps of the ferric−NO complex for some Thr243 mutants.

Figure 4 shows the optical absorption spectral changes in the reaction of the ferric−NO complex with NADH for T243N and T243V. T243N exhibits about 80% of the activity of the WT enzyme, while T243V is practically inactive with respect to the NO reduction, although the spectral characters and the NO binding properties of both mutants are the same as those of the WT enzyme. In the case of the T243N mutant, the absorption at 432 nm for the ferric−NO complex was decreased in intensity, with the concomitant appearance and increase in intensity of the 444 nm absorption for the intermediate in the millisecond time range. The rate constant, k_2, for the intermediate formation was estimated to be 3.2×10^5 M^{-1} s^{-1} from the time course of the spectral change. The intermediate was spontaneously decomposed into its ferric form with a rate constant k_3 of 0.005 s^{-1}. The spectral changes in the NADH reduction of the T243N mutant were basically similar to those of the WT P450nor, and its k_2 value is about 40% of that of the WT

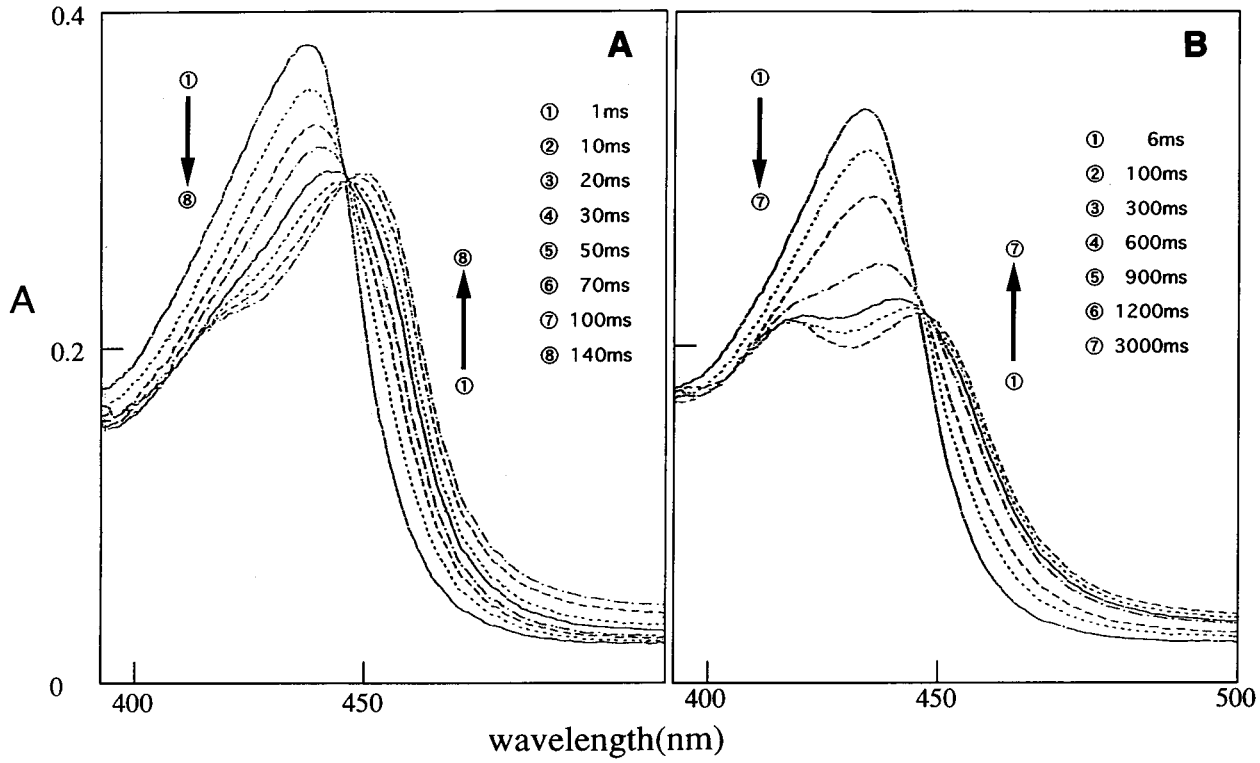

FIGURE 4: Spectral changes of the ferric−NO complexes of (A) T243N and (B) T243V mutants of P450nor in the reaction with NADH. The final enzyme concentration was 10 μM. The final NADH concentrations were 100 and 250 μM for T243N and T243V, respectively. The other conditions are basically the same as reported previously (*29*).

enzyme. In contrast, T243V gave a rate constant for intermediate formation (8.4×10^3 M^{-1} s^{-1}) which was decreased by about 2 orders of magnitude upon substituting isosteric valine for Thr243. Due to the extremely low k_2 value for T243V, its intermediate was not completely isolated in the optical absorption spectrum and was present in the mixture along with the ferric−NO and resting states. However, k_3 (0.004 s^{-1}) was not so seriously affected by the mutation.

An inspection of Table 2 shows that the effect of the Thr243 mutation on k_2 is nearly comparable to that of the enzymatic activity of P450nor; i.e., T243N, T243G, T243S, and T243A have significant k_2 values, while the others have extremely lowered k_2 values. The results suggest that the Thr243 mutation substantially affects enzymatic activity through a decrease in the NADH-dependent reduction rate (the intermediate formation rate) of the enzyme.

DISCUSSION

We have studied the mutation effect of Thr243 of P450nor on its enzymatic, spectroscopic, and kinetic properties. On the basis of the data obtained in this study, it is possible to discuss three points concerning the structure−function relationship of P450nor, namely, the structural change of P450nor on the Thr243 mutation, the role of Thr243 in the P450nor catalytic reaction, and structural characteristics of the conserved threonine (Thr243) of P450nor in comparison with those of other monooxygenase P450s.

Structural Change on Thr243 Mutation. The mutation of Thr243 in P450nor changes the high spin/low spin ratio in the ferric resting state. The crystal structures of the *d*-camphor-bound and -free forms of P450cam have shown that the high-spin form of P450 is a five-coordinate iron, while the low-spin form is a six-coordinate iron with a water molecule as the sixth ligand (*9*). On the basis of this structural information, the Thr243 mutation influences the water coordination in the sixth site of P450nor in the ferric resting state. In addition, the CD spectral results show no change in the gross secondary structure of the enzyme on Thr243 mutation. Therefore, it is likely that the change in the water coordination in the ferric resting state is possibly caused by a local structural change around Thr243 on its mutation.

In the crystal structure of P450nor in the ferric resting state (*6*), we found that the hydroxyl group of Thr243 is hydrogen-bonded with two water molecules (Wat63 and Wat72) which also interact with the main chain carbonyl group of Ala239 (see Figure 5A). The Wat72 is also hydrogen-bonded with another water molecule (Wat113, not shown). Temperature factors of these three water molecules are 24, 26, and 31 $Å^2$, showing that they are immobile. In addition, the water molecule which presumably coordinated with the ferric heme iron in the low-spin state was crystallographically invisible, probably because it rapidly exchanges with the bulk solvent water. Therefore, the introduction of other amino acid residues at the 243 position on the mutation might slightly disturb the hydrogen bond network in the heme pocket, since the residue has a side chain with a polarity and size different from those of threonine, resulting in changes in the accessibility or stability of the water molecule at the sixth position.

The structural change in the heme environment induced by the Thr243 mutation appears to be local and subtle, because the optical absorption spectra of the mutants in the ferric NO-bound, ferrous, ferrous CO-bound, and ferrous

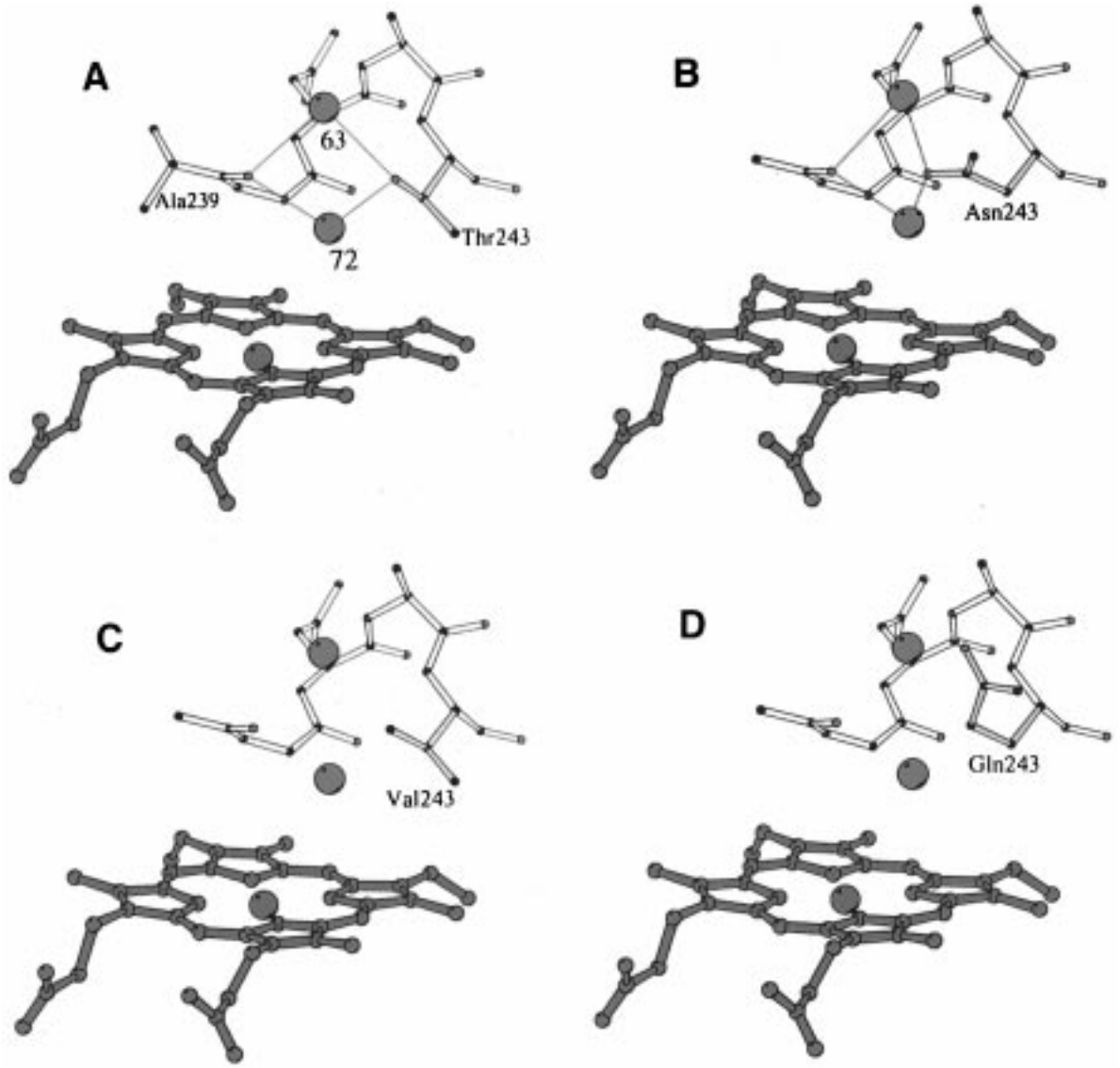

FIGURE 5: (A) Hydrogen bond network involving Thr243 in the distal I helix of P450nor. The images of the heme environmental structures of (B) T243N, (C) T243Q, and (D) T243V mutants of P450nor, as described by computer graphics on the basis of the crystal structure of the native P450nor in the ferric resting state. Side chains of the amino acid residues are shown at the allowable position, where there is no contact with surrounding residues. The amide group of Asn243 is shown in the position at which the hydrogen bonds with Wat63 and Wat72 are allowed without any steric constraint.

isocyanide-bound forms are basically indistinguishable from the corresponding spectrum of the WT enzyme. Support for this suggestion is provided by the kinetic data on NO binding to the ferric resting enzyme and equilibrium data on CO binding to the ferrous enzyme, in which the rate constant and the dissociation constant, respectively, were not significantly different between the WT and the mutant enzymes. The Thr243 mutation slightly but not seriously alters the heme environmental structures of P450nor, which is responsible for its NO and CO binding properties.

Role of Conserved Thr243 in NO Reduction by P450nor. The most interesting finding in this study is that, despite a subtle and local change in the heme environmental structure, the NO reduction activity of P450nor was dramatically affected by Thr243 mutation, depending on properties of the side chain of the amino acid residue introduced at the 243 position; i.e., T243N, T243S, and T243G are active, while other mutants have a largely decreased or no reactivity. The finding clearly indicates that the structural change in the vicinity of heme induced by the Thr243 mutation is subtle but very significant in terms of the NO reduction reactivity of P450nor. Furthermore, we also found that the change of the entire enzymatic activity, as a result of Thr243 mutation, can be predominantly attributed to a change in the reduction process of the ferric NO-bound form with NADH. Thus, the structural difference between active and inactive enzymes appears to be closely related to the intermediate formation.

In this context, the most interesting comparison is between the WT, T243N, and T243V enzymes. The T243N mutant is active with respect to the NO reduction like the WT enzyme, but the T243V is practically inactive. The side chain of valine is the same size as that of threonine (isosteric) but does not possess a polar group, while asparagine has a side chain which is longer by one methylene group than threonine and contains a polar (amide) group. When an asparagine is substituted for Thr243 in the P450nor crystal structure on computer graphics, it was found that its amide group can interact reasonably well with the Ala239 main chain carbonyl in the same manner (see Figure 5B); i.e., the water molecules (Wat63, Wat72, and Wat113) are possibly

retained in their original positions. This would also be the case for the T243S mutant, because a serine residue also contains a hydroxyl group and a less bulky side chain. However, a glutamine, because of its long side chain, is not able to maintain the original hydrogen-bonding network in the distal I helix of P450nor (see Figure 5C), yielding an inactive enzyme, although it also contains an amide group as asparagine. In T243V, the isopropyl group of the valine residue is able to adopt the same configuration as the threonine side chain (see Figure 5D), but due to a lack of hydrogen-bonding ability, the location of the water molecules (Wat63, Wat72, and Wat113) might be changed and/or the hydrogen bond network in the heme pocket would be disturbed in the T243V mutant, compared with that in the WT and the T243N enzymes. We can conclude that Thr243 and its hydrogen-bonding network, Ala239···(Wat63, Wat72, Wat113)···Thr243, could directly or indirectly play a crucial role in the reduction of the ferric−NO complex with NADH (the intermediate formation).

The roles of the conserved threonine residue in the monooxygenase reaction catalyzed by P450s have been examined with crystallographic, spectroscopic, and mutagenesis techniques (*32*). Since the electron transfer from NAD(P)H to the heme iron of the monooxygenase P450s takes place at the heme proximal side with the aid of the proteinous mediators (its reductase), it has generally been thought that the conserved threonine, which is located in the heme distal helix, is possibly responsible for the proton delivery utilized in the monooxygenation reaction (*33*). The hydroxyl group of Thr252 in P450cam makes a hydrogen-bonding network for the proton supply from the bulk water to the heme moiety (*15*). Indeed, the network was altered on substituting alanine for Thr252, resulting in the inactive T252A mutant (*13*, *14*). In other P450s, the hydrogen-bonding networks in the heme distal side have also been identified (*8*, *11*, *34*).

In contrast, a crystallographic study has predicted that a NADH binding site of P450nor is presumably present in its distal pocket (*6*), because electrons required for the reaction are directly transferred from NADH to the heme without the aid of any proteinous mediators (*2*). This prediction leads to a suggestion that, in the NO reduction by P450nor, both proton and electron transfers might take place at the heme distal side and that, if so, the hydrogen bond network containing Thr243 is possibly related to either proton or electron transfer.

From this point of view, we make an interesting observation in the crystal structure of the ferrous−CO complex of P450nor (*6*). Upon CO binding, the hydrogen bond network in the heme distal pocket is rearranged to give a new hydrogen bond network to the solvent water through Ser286 and Asp393, i.e., Wat22···Ser286···Wat8···Asp393···solvent water. The Wat22 hydrogen-bonded with the hydroxyl group of Ser286 is 3.0 Å from the iron-coordinated ligand, and its temperature factor is relatively high (42 Å^2), indicating a high degree of mobility. In addition, we also found that the replacement of Ser286 with valine dramatically reduced the NO reduction reactivity of P450nor. On the basis of these observations, we previously assigned the hydrogen-bonding network, Wat22···Ser286···Wat8···Asp393···solvent water, as the proton delivery pathway in the NO reduction reaction with P450nor.

In the previous discussion (*6*), we excluded the possibility of Thr243 and its hydrogen bond network being the proton delivery pathway, primarily because the Thr243 hydrogen bond network is isolated from the solvent water. However, these results, based on the Thr243 mutation, indicate that Thr243 is responsible for either the proton or electron transfer in the NO reduction reaction of P450nor. If the Thr243 hydrogen-bonding network is related to the proton transfer, another water molecule which would be crystallographically invisible at room temperature should participate in this network for connecting Thr243 with the bulk water through the Ser286 network. Alternatively, if this network is related to the electron transfer step, the electron transfer pathway should be identified from the bound NADH to the heme through Thr243 in the NADH-bound form of the enzyme.

In this respect, it is noteworthy that the affinity of butyl isocyanide for the ferrous enzyme was altered on the Thr243 mutation, in sharp contrast to showing no effect on CO and NO binding. In particular, the T243W and T243Y enzymes, both of which are completely inactive mutants, have relatively high dissociation constants, possibly due to steric repulsion of the long carbon chain of the butyl group of the iron-bound isocyanide against the large side chain of Trp243 or Tyr243. This observation suggests that the residue at position 243 modulates the accessibility of the large ligand molecule to the heme site. Furthermore, this result allows us to suggest that the Thr243 mutation, especially the T243W and T243Y mutations, might inhibit the access of NADH to the heme distal pocket for the electron and/or proton transfer to the heme site, making completely inactive mutants. In any event, to directly assess this problem, we are now preparing single crystals of the NADH-bound enzyme, the Thr243 mutants, and the Ser286 mutants of P450nor for crystallographic studies, and a cryocrystallographic study of the ferric−NO complex is in progress.

Structural Characteristics of the Heme Distal Pocket of P450nor. When we compare the mutation effect of Thr243 in P450nor with those of the conserved threonine in other monooxygenase P450s, it is possible to discuss the structural characteristics in the vicinity of Thr243. Here, it is noteworthy that the T243V of P450nor is only 0.8% as active as the WT enzyme, while the Val mutants for the conserved threonine of P450 2C2, P450 2C14, and P450 2E1 exhibited about 20% the activity of the corresponding WT enzymes (*17*, *35*). These results clearly indicate that Thr243 in P450nor is strictly essential to its enzymatic activity, compared with those of other monooxygenase P450s. In the case of P450nor, the correct hydrogen bond donor−acceptor relationship is critical for the NO reduction reaction, and Thr243, as well as Ser286, are critical in this reaction.

In addition, the Thr243 mutation influenced the water coordination in the ferric resting form slightly, as judged from the iron high spin/low spin ratio. This is in sharp contrast to results dealing with the mutation of the conserved threonine in other monooxygenase P450s; e.g., upon substitution of Leu or Ser for Thr301 in P450 2C2, the high-spin content (five-coordinate iron) was increased from 20% (wild-type) to 83% (T301L) or decreased to 12% (T301S), respectively (*18*). Such a large effect on the water coordination at the sixth site has been explained in terms of the close location of Thr301 to the heme. In P450cam, the Thr252 mutation largely affects water coordination to the sixth site,

even in the *d*-camphor-bound form (*36*).

In addition, it is noteworthy that the ferrous−isocyanide complexes of P450nor characteristically exhibited a single Soret band around 427 nm and that this spectral characteristic is not essentially altered for the Thr243 mutants. In the case of other monooxygenase P450s, double Soret bands are observed at 427 and 455 nm, and the absorption at 453 nm is predominant in P450cam (*37*). The absorption ratio of these two bands was sensitively altered on the mutation of the conserved threonine (*17*). The mutation effect suggests that the conserved threonine in monooxygenase P450s is located sufficiently close to the iron-bound isocyanide to alter its coordination character, since double Soret bands have been considered to be representative of the presence of two different coordinations in the binding of isocyanide to the ferrous iron of P450s (*21*). In relation to this discussion, we could expect that the side chain of Thr243 of P450nor is sufficiently far from the heme surface to have an effect on the coordination structure of the isocyanide or is not oriented toward the heme sixth site, and/or that the heme distal pocket might be opened widely. The suggestion, drawn from this mutagenesis work, is consistent with the crystal structure of P450nor, which shows that the hydroxyl group of Thr243 is 6.5 Å from the heme iron and extends in a different direction from those of the conserved threonine in P450cam and P450BM3, as shown in Figure 1.

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