Nitric oxide reductase (Nor) cytochrome P450nor (P450nor) is unique because it is catalytically self-sufficient, receiving electrons directly from NADH or NADPH. However, little is known about the direct binding of NADH to cytochrome. Here, we report that oxidized pyridine nucleotides (NAD⁺ and NADP⁺) and an analogue induce a spectral perturbation in bound heme when mixed with P450nor. The P450nor isoforms are classified according to electron donor specificity for NADH or NADPH. One type (Fnor, a P450nor of Fusarium oxysporum) utilizes only NADH. We found that NAD⁺ induced a type I spectral change in Fnor, whereas NADP⁺ induced a reverse type I spectral change, although the Kₐ values for both were comparable. In contrast, NADP⁺ as well as NAD⁺ caused a type I spectral change in Tnor, a P450nor isozyme from Trichosporon cutaneum that utilizes both NADH and NADPH as electron donors. The B’ helix region of Tnor (7SAGGKAA⁸⁶⁰) contains some Ala and Gly residues, whereas the sequence is replaced at a few sites with more bulky amino acid residues in Fnor (7SASGKQAA⁸⁶⁰). A single mutation (S75G) significantly improved the NADPH-dependent Nor activity of Fnor, and the overall activity was accelerated via the NADPH-enhanced reduction step. These results showed that pyridine nucleotide cofactors can bind P450nor and that only a few residues in the B’ helix region determine cofactor specificity. We further showed that a poor electron donor (NADPH) could also bind Fnor, but an appropriate configuration for electron transfer is blocked by steric hindrance mainly by Ser⁷⁵ against the 2’-phosphate moiety. The present results along with previous observations together revealed a novel motif for cofactor binding.

Cytochrome P450 (P450) consists of a group of hemoprotein enzymes that are involved in the metabolism of a variety of substrates (1). P450s are widespread in eukaryotic and prokaryotic organisms, having attained unparalleled functional and molecular diversity during evolution (2, 3). The P450 superfamily consists of about 200 known families that have been classified according to amino acid sequence data (2, 4). P450 enzymes usually function as monooxygenases, but many catalyze other reactions such as reduction, isomerization, and dehydration (5). Nitric oxide reductase (Nor) cytochrome P450nor (P450nor) is involved in fungal denitrification (6). This enzyme is the most functionally diverse among known P450 enzymes as it catalyzes the reaction shown in Reaction 1 without a redox partner such as P450 reductase and thus receives electrons directly from NADH or NADPH (NAD(P)H). The catalytic turnover is over 30,000 min⁻¹ at 30 °C (7). The overall reaction can be divided into three partial reactions (Reactions 2–4) (7).

All of these partial reactions except for the last step (Reaction 4) can be observed as isolated events (7, 8). The chemical nature of the specific intermediate (I) with a Soret absorption peak at 444 nm that is formed upon reduction of the ferric-NO complex (Fe³⁺-NO) with NADH (Reaction 3) is an iron-hydroxyl amine radical complex (9).

We have isolated and cloned the genes for several fungal P450nor isoforms that belong to the CYP55A subfamily (10–13). These are P450norA and P450norB from F. oxysporum (Fnor) (14), P450nor1 and P450nor2 from Cylindrocarpon tonkinense (Cnor1 and Cnor2) (12, 15), and P450nor from T. cutaneum (Tnor) (13). Recent progress in genome analyses has further revealed that P450nor and thus denitrification is generally distributed among fungi. The Fnor species has been extensively analyzed both physicochemically and kinetically, and the structure has been determined by x-ray crystallography (16). These P450nor isoforms are specific for the electron donors, NADH and NADPH. Fnor and Cnor1 are specific to NADH (6, 12, 15), whereas Cnor2 and Tnor can utilize both (13) or prefer NADPH (15).

The P450 superfamily has attained vast diversity by rapidly changing the substrate recognition sites (SRSs) of its many
forms (3). Each P450 species has adapted as the result of molecular evolution to a specific substrate. In contrast to other P450s that catalyze the monooxygenase reaction against an organic substrate, the substrate of P450nor is NO (an inorganic gas). Since P450nor interacts directly with NAD(P)H, the enzyme seems to have evolved to fit this electron donor instead of the organic substrate of the monooxygenase reaction. The crystal structure (16) revealed a unique configuration of P450nor despite its overall structural similarity to other P450s, such as P450cam (17) and P450BM-3 (18). The B’ helix and the loop between F- and G-helices (F, G-loop) form an entrance to the heme-distal pocket. The F, G-loop rises toward the outside to form a wide entrance and a large cavity inside the distal pocket. In addition, positive charges due to Arg and Lys residues are abnormally concentrated outside and inside the heme-distal pocket. We supposed that these structural features are associated with the direct NAD(P)H interaction and showed that the positive charge cluster functions in attracting negatively charged NAD(P)H (19).

SRS-I that contains a B’ helix is the most structurally variable among P450 species (20, 21). Thus, the B’ helix of P450nor may also play an important role in direct interaction with NAD(P)H. However, direct evidence has yet to show that NAD(P)H forms a reversible complex with P450nor prior to transferring electrons to the bound heme. Here, we constructed various mutant proteins of P450nor based on the B’ helix sequence and found evidence of a key role for the B’ helix in determining specificity for NAD(P)H along with the direct binding of NAD(P) analogues to wild type and mutant P450nor proteins.

**EXPERIMENTAL PROCEDURES**

Site-directed Mutagenesis—Plasmids were constructed, enzymatically modified, and electrophoretically resolved as described (22). Recombinant proteins of Fnor (11) and Tnor (13) were produced using the expression vector for P450nor (pT7Nor) (16, 19). Site-directed mutagenesis of the Fnor gene was achieved by PCR (23) using template pfp (450)-20 (11) that consisted of the Fnor cDNA and pUC18 vector. Primers M13-47 and M13-RV (Takara, Otsu, Japan) were specific for pUC18. The following lists the mutants and primers used in this study (mutated sites are underlined): 3A, GAGCTTAGCGCCGCTGCAGCC; S73A, TTCCCTGAGCTTAACGCCAGTGGAAAG; NS, TTCCCTGAGCTTAACGCCGGTGGAAAG; GG, TTCCCTGAGCTTAACGCCGGTGGAAAG; GS, TTCCCTGAGCTTGGCGCGCTGAGCC; SG, CTTAGCGCCGGTGGAAAGCAAGCA; NG, TTCCCTGAGCTTAACGCCGGTGGAAAG; GS, TTCCCTGAGCTTGGCGCGCTGAGCC; SG, CTTAGCGCCGGTGGAAAGCAAGCA; NG, TTCCCTGAGCTTAACGCCGGTGGAAAG. The resulting supernatant was dialyzed overnight against Tris buffer.

**FIG. 1.** Alignment of the amino acid sequences of P450nor isozymes around B’ helix and mutagenesis strategy. A double arrow indicates the extent of the B’ helix. Bold characters show amino acid residues 73 and 75 (upper) or residues at mutation sites in each mutant protein.

![Alignment of the amino acid sequences of P450nor isozymes around B’ helix and mutagenesis strategy](image)

**FIG. 2.** NADH- or NADPH-dependent overall Nor activity of Fnor alanine mutants. Column 1, Wild type; column 2, 3A; column 3, 73A; column 4, 75/6A; column 5, 77A; column 6, R77A; column 7, Q78A.

![NADH- or NADPH-dependent overall Nor activity of Fnor alanine mutants](image)
presence of 1.0 mM NADH, and the amount of N₂O produced was determined by gas chromatography. The content of high spin heme at the resting state of the enzyme was estimated from the ratio of the absorbance of the high spin form (390 nm) to that of the low spin form (414 nm) as described (24). The P450 nor concentration was spectrophotometrically determined from the CO-difference spectrum as reported (25) using an extinction coefficient of 86.3 mM⁻¹cm⁻¹ for the difference in absorbance between 448 and 490 nm (6). NADH was determined from the absorbance at 340 nm using a molar absorption coefficient of 6.22 mM⁻¹cm⁻¹.

RESULTS

Heme-dependent Absorption Spectra of the Mutant Proteins—We constructed mutant proteins of Fnor in which the amino acid residues of the B helix (74ASGKQA79) and its neighboring residue (Ser 73) were replaced or deleted, respectively (Fig. 1, bottom). The absorption spectra of all mutant proteins produced in heterologous E. coli cells were the same as those of the native protein in the ferric (Fe³⁺) or the CO-bound (Fe²⁺-CO) form (data not shown). The bound heme in the native purified protein is in a Fe³⁺ form that is in equilibrium between the high and low spin states. The ratio of the high spin state of wild type P450 nor is 58% in 50 mM TES buffer (pH 7.2). The ratio of the spin states of mutant proteins was similar (54–59% high spin). These results indicate that the mutant proteins were adequately folded and that the fine structure at the active site was maintained.

Effects of Mutations in the B’ Helix on Catalytic Turnover—The B helix should be destroyed in mutant 3A in which the sequence 75SGK77 was deleted and Gln 78 was replaced with Ala. This mutation resulted in a decline of NADH-dependent Nor activity to the same level as the NADPH-dependent activity of wild type Fnor (Fig. 2). Other single mutations in which individual residues were replaced with Ala (S73A, S75A, G76A, K77A, and Q78A) decreased the NADH-dependent activity to various degrees (Fig. 2). Among them, the activity of G76A mostly decreased to 40%, confirming the importance of the smallest residue (Gly) at this position (26). These mutagenesis results showed that the B helix plays an important role in conferring full Nor activity upon P450 nor.

Tnor and Cnor 2, which can utilize both NADH and NADPH or prefer NADPH to NADH, contain many Ala and Gly residues in the region 73SAGGKAAA80 (Fig. 1, top). The only bulky residue (Lys77) is oriented toward the opposite side so that the entrance is clear (16, 19). The A/G-rich sequence is replaced at sites 73, 75, and/or 78 with bulky residues in Fnor and Cnor 1 that can utilize only NADH. These facts suggest that the substitution of a few residues in this region is responsible for determining electron donor specificity. The NADPH-dependent activity of the mutant Q78A was slightly improved, although...
the NADH-dependent activity decreased to some extent (to 75% of the wild type) (Fig. 2). This suggests that position 78 is important for NADH interaction but not for determining specificity. We therefore focused upon positions 73 and 75 in subsequent investigations (Fig. 1).

Among several mutants of Fnor, the results from SG (S75G) and GG (S73G/S75G) are notable. Fig. 3 shows that these mutations significantly improved NADPH-dependent Nor activity without affecting, or even slightly increasing, the NADH-dependent activity. These results indicate that position 75 must be Gly for NADPH to be an effective electron donor of Nor activity as observed in wild type Cnor2 or Tnor. Cnor1 also conserves Gly75, but NADPH-dependent Nor activity is very low. However, Asn with a bulky residue replaces the Ser 73 in Fnor. The double mutation (NG) that confers the same sequence as Cnor1 did not modulate either the NADH- or the NADPH-dependent activities (Fig. 3), consistent with the specificity of wild type Cnor1. By contrast, the single mutation that replaced Ser73 with Gly (GS mutant) or with a bigger residue (NS mutant) decreased NADH-dependent activity without improving that dependent on NADPH. Thus, the size of the residues at positions 73 and 75 cooperatively determines specificity for NAD(P)H.

**Effects of Mutations at Ser73 and Ser75 on the Reducing Half-reaction**—The mutations at Ser73 and/or Ser75 in Fnor modulate the interaction with NAD(P)H as well as the overall Nor activity. This process (Reaction 3) can be observed as an isolated reaction by rapid scan analysis (7). Accumulation of the intermediate (I) upon reduction of the ferric enzyme-NO complex (Fe3+-NO) with NADH (7) is detectable under controlled conditions (Fig. 4C). This intermediate cannot be detected when NADPH is used instead of NADH as the electron donor as in wild type Fnor (Fig. 4D). This is possibly because the reduction (I formation) is slower than the decomposition of I (7, 19). However, I accumulated upon reduction with NADPH as well as with NADH in the SG (Fig. 4, A and B) or GG (data not shown) mutants, showing that the rate of reduction with NADPH was enhanced by the mutations. These results are consistent with the finding that overall activity was enhanced by the mutations (Fig. 3). The apparent rate constant for the reduction at a fixed NADH (or NADPH) concentration (kobs) was obtained with each mutant from the time-dependent spectral change during I formation (7) (Table I). The results demonstrated that the NADH- or NADPH-dependent kobs value for each enzyme species parallels the overall activity (Fig. 3). In other words, the overall Nor activity was modulated by each mutation via action against the reduction step.

**Binding of NAD(P)H Analogues**—Various ligands cause spectral perturbation in bound heme upon binding to P450 (24). However, we have not detected spectral perturbation upon mixing P450nor and NADH. This may be because the powerful absorbance due to NADH overwhelms the minor spectral perturbation that would occur in the bound heme of P450nor. Here, we tested the specificity of various NADH analogues for

### Table I

| Substrate | WT | 3A | SG | NG | GS | NS | GG |
|-----------|----|----|----|----|----|----|----|
| NADH      | 35.1 ± 1.6 | ND | 56.2 ± 3.9 | 37.3 ± 1.0 | 17.1 ± 0.5 | 12.7 ± 0.5 | 35.6 ± 1.3 |
| NADPH     | ND | ND | ND | ND | ND | ND | ND |

a Calculated from three to five replicates.
b WT, wild type.
P450nor binding as described under “Experimental Procedures.” Among these analogues, PAAD and NAD+ caused a type I spectral change in wild type Fnor (Fig. 5A). A spectral change is defined as an increase in absorbance around 390 nm due to high spin state heme accompanying a concomitant decrease in low spin heme (413 nm). On the contrary, NADP+ caused a reverse type I spectral change. The SG mutant also underwent a type I spectral perturbation by PAAD or NAD+ but to a greater extent than those caused in the wild type (Fig. 5B). However, NADP+ conferred little spectral change on the SG mutant. Mutant 3A was inert against PAAD and NAD+ but perturbed by NADP+ similarly to the wild type (Fig. 5C). NADP+ as well as PAAD and NAD+ induced a type I spectral change in Tnor that can utilize both NADH and NADPH as electron donors (Fig. 5D). Thus, these NADH analogues can bind to P450nor, whereas in the bound form, there would be two alternative states that cause either a type I or a reverse type I spectral change. The effects of these mutations in the B’ helix region also indicate that these analogues bind P450nor from the distal side.

Arg64 and Arg174 are located beneath the B’ helix and the F,G-loop, respectively, and play a central role in the positive charge cluster to interact with NADH (19). Our R64E and R174E mutants completely lose the ability to interact with NADH. We concluded from the results that Arg64 and Arg174 must play the central role in binding NADH by fixing the pyrophosphate moiety of NADH from both sides (19). In fact, neither PAAD, NAD+, nor NADP+ caused any spectral perturbation in these mutants (R64E and R174E; data not shown). These and previous results support the notion that P450nor evolves during the interaction with NADPH. The tertiary structure of Fnor shows the Ser73 and Ser75 residues are serine residues both of which have the 2-phosphate moiety (B’ helix and the附近 residues). The Ser73 residue is located in the helix and the neighboring residue 73 play a key role in the interaction of P450nor with NADPH. The Ser75 residue is also oriented toward the entrance of the distal heme pocket displaying the side chains of Ser73 and Ser75 is shown in Fig. 6. These side chains are oriented toward the entrance. It is thus highly likely that this side chain of Ser75 prevents NADPH from interacting properly with Fnor through steric hindrance of the 2-phosphate moiety. Asn73 in Cnor1 substitutes for Gly75 in causing steric hindrance. Position 75 must be Gly for NADPH to be an effective electron donor. Only a single mutation at this site (SG mutant) in Fnor remarkably increased the overall activity of NADPH-dependent reduction of the Fe3+/NO complex (Reaction 3).

That NADP+ induced a reverse type I spectral change in wild type Fnor in contrast to the type I change caused by NAD+ is notable (Fig. 5), although the $K_a$ values for NAD+ and NADP+ were comparable (Table II). The results indicate that Fnor can bind NADPH with affinity comparable with that for NADH, but their configurational state in the bound form differs. In other words, the configurational state of NADPH bound to Fnor is not adequate for subsequent electron transfer. This notion is supported by the results that Fnor and its SG mutant, a finding that was in striking contrast to the results obtained using halogen ions (Br− and Cl−). Halogen ions are reverse type I ligands of P450nor that also inhibit the reduction process (19). The $K_a$ and $I_{50}$ values of halogen ions were comparable (Table II) (19).

**DISCUSSION**

The present results indicate that the B’ helix and the neighboring residue 73 play a key role in the interaction of P450nor with NADPH. The A/G-rich sequence of Tnor/Cnor2 type P450nor evolves during the interaction with NADPH. The sequence was modulated at positions 73, 75, and/or 78 to restrict interaction with NADPH. The tertiary structure of Fnor around the entrance of the distal heme pocket displaying the side chains of Ser73 and Ser75 is shown in Fig. 6. These side chains are oriented toward the entrance. It is thus highly likely that this side chain of Ser75 prevents NADPH from interacting properly with Fnor through steric hindrance of the 2-phosphate moiety. Asn73 in Cnor1 substitutes for Gly75 in causing steric hindrance. Position 75 must be Gly for NADPH to be an effective electron donor. Only a single mutation at this site (SG mutant) in Fnor remarkably increased the overall activity of NADPH-dependent reduction of the Fe3+/NO complex (Reaction 3).

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**FIG. 6. Structure of the heme-distal side of P450nor showing B’ helix and F,G-loop from top (left) and side (right).** Ser73 and Ser75 residues are shown.

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**TABLE II**

| Ligand  | Type of spectral change | $K_a$ | $I_{50}$ of $k_{obs}$ | $k_{red}$ | $k_{red}$ |
|---------|------------------------|------|---------------------|----------|----------|
|         |                        | WT   | SG                  | WT       | SG       |
|         |                        |       | NADH                | NADH     | NADPH    |
| Analogue|                        |      |                     |          |          |
| PAAD    | I                      | 4.0  | 1.4                 | 0.9      | 0.2      |
| NAD+    | I                      | 10.4 | 7.6                 | 1.4      | 0.5      |
| NADP+   | Reverse I              | 9.1  | 3.2                 | 1.6      | 1.7      |
| Anion   | Br−                    | Reverse I | 100   | 50     |          |
|         | Cl−                    | Reverse I | 100   | 120   |          |

$^a$ WT, wild type.

$^b$ Ref. 19.

$^2$ $k_{red}$ indicates the first order rate constant for the reduction.
further supported by the finding that Tnor can utilize both NADH and NADPH and that NADP⁺ induced a type I spectral change like NAD⁺ (Fig. 5). Thus, the reverse type I spectral change appears to be caused by binding the analogue (NADP⁺ for Fnor) of the unfavorable electron donor. We propose that the SG mutation altered the configuration of NADPH bound to Fnor to improve the fit for subsequent electron transfer. The cofactor specificity of P450nor might be mainly determined only by the presence or absence of a small side chain of Ser75. This notion could be confirmed if the $K_d$ and $K_m$ values for NADP⁺/NADPH for the reduction step can be determined and compared between the wild type and SG mutant. However, the reduction is too rapid to determine the kinetic constants.

That the $I_{50}$ value was smaller than the $K_d$ value for each analogue with both the wild type and SG mutant of Fnor is also notable. The $I_{50}$ value should usually be comparable with or larger than the $K_d$ value (i.e., $K_d$) for an inhibitor, as seen with halogen ions (Table II). The results suggested two kinds of ligand (pyridine nucleotide) binding with different affinity. This notion assumes that high binding affinity does not cause spectral perturbation, but it can inhibit the reduction step, and the binding affinity corresponds to the $I_{50}$ value. Another mode of binding with a lower affinity causes spectral perturbation in the bound hem by which the $K_d$ values were obtained. This assumption agrees with the small spectral changes caused by pyridine nucleotides (Fig. 5) since the population of the protein-ligand complex with lower affinity should be smaller. Dinucleotides such as pyridine nucleotide are symmetrical molecules with a 2-fold axis at the pyrophosphate moiety. Considering the importance of the Arg⁶⁴ and Arg¹⁷⁴ for cofactor binding along with the big gap in the distal heme pocket, cofactor binding in a reverse orientation would be possible. This means that the pyridine nucleotide molecule can penetrate the pocket with the adenine moiety as a head and the nicotinamide moiety as a tail. The nicotinamide moiety of oxidized pyridine nucleotide (NAD⁻ and NADP⁻) is positively charged, so the positive charge cluster inside the heme pocket would repel this molecule. Thus, the P450nor-ligand complex should become more stable when NAD⁻ or NADP⁻ binds in the reverse, instead of the normal direction. The big cavity as well as the hydrophilic environment of the distal heme pocket would allow bound pyridine nucleotide cofactors to assume several modes or configurations.

We concluded that P450nor forms reversible complexes with the electron donor (NAD(P)H) before receiving electrons from it. Therefore, the mode of P450nor interaction should be compared with those of other enzymes that require pyridine nucleotides. An ionic interaction or repulsion is important for many NAD(P)-binding proteins to discriminate NADP from NAD. For example, the M. G.conserved Asp at the C terminus of the $\beta\beta$ motif (Rossman fold) in NAD-specific dehydrogenases impairs interaction with NADP by both ionic repulsion and steric hindrance against the 2'-phosphate moiety (27, 28). By contrast, NADP-specific dehydrogenases or hydroxylases alter cofactor specificity by replacing the negatively charged residue with a smaller and neutral residue and by arranging a positively charged residue in its neighbor to improve interaction with the 2'-phosphate moiety of NADP (29, 30). P450nor has only two types of cofactor specificity. One is specific to only NADH, and the other can utilize both NADH and NADPH. No isofrom has yet been discovered that utilizes only NADPH as an electron donor. This seems reasonable when the manner in which P450nor discriminates NADH and NADPH is considered. A positive charge is not used to improve interaction with NADPH, but simply canceling steric hindrance caused by a few residues alters the specificity. Thus, the binding motif in P450nor for the cofactors, such as a large hydrophilic cavity (16, 31), a positive charge cluster (19), and steric hindrance by a few residues in the B’ helix, is unique among proteins that interact with NAD(P).

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The B’ Helix Determines Cytochrome P450nor Specificity for the Electron Donors NADH and NADPH
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