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Basic Studies on Fungal Unique P450s for Industrial Applications

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We isolated two unique cytochromes P450, P450nor and P450foxy, from the fungus *Fusarium oxysporum*. P450nor is involved in fungal denitrification and functions as nitric oxide (NO) reductase (Nor). P450foxy catalyzes subterminal (ω-1$\sim\omega$-3) hydroxylation of fatty acids. It is unique in that it is a fused protein consisting of P450 and its reductase domains. P450 is an attractive material not only for basic research but also in the aspect of industrial application of biological reactions. P450 usually catalyzes a monooxygenase reaction, as below.

$$S+O_2+NAD(P)H+H^+ \rightarrow SO+H_2O+NAD(P)^+$$

The reaction is usually supported by other protein(s) that transport two electrons of NAD(P)H to P450 (redox partner), since P450 cannot receive the electrons directly from NAD(P)H. Many of eukaryotic P450s are bound to endoplasmic reticulum membrane, and their reactions are supported by their redox partner, NADPH-cytochrome P450 reductase (P450 reductase). P450 reductase is comprised of, from its N-terminus, FMN-, FAD-, and NADPH-binding domains (Fig. 1). Electrons are transferred in the order of: NADPH$\rightarrow$FAD$\rightarrow$FMN$\rightarrow$P450. A single eukaryotic organism usually contains many P450 species while it contains only one P450 reductase species (two species are found in plants). Therefore, a single P450 reductase supports many P450 species, several tenth and in some cases, several hundreds of P450 species. P450 and P450 reductase is usually separate proteins, while in rare case, they are fused as a single polypeptide chain. P450foxy is the first instance of such a fused protein among eukaryotes (Fig. 1). On the other hand, P450nor is not a monooxygenase but catalyzes the following reaction as Nor.

$$2NO+NAD(P)H+H^+ \rightarrow N_2O+H_2O+NAD(P)^+$$

Surprisingly, P450nor does not need a redox partner, and thus is the only P450 species that can receive electrons directly from NAD(P)H. P450-catalyzed reactions are extremely diversified, and therefore, are very attractive in the aspect of industrial application. However, P450 reactions are generally difficult to be reconstituted because of their complex redox system, and their catalytic turnover is usually low. Such properties of P450 enzyme system make a high barrier for industrial application. On the other hand, fused P450 proteins such as P450foxy and P450BM3 realize a high catalytic turnover possibly because the intramolecular electron transfer is faster than the intermolecular electron transfer. Further, P450nor exhibits exceptionally high catalytic turnover as P450 (in the order of 100,000 min^{-1}). Therefore, elucidation of the reaction mechanisms of P450nor and P450foxy that exhibit exceptionally high speed catalyses, are important also for industrial application.

Determination of crystal structure of P450nor in complex with an NAD analogue (NAAD)

P450nor is the only hemeprotein (or even the only metalloprotein) that can receive electrons directly from the physiological electron donor NADH or NADPH. Such an electron-transferring mechanism is unprecedented, because two

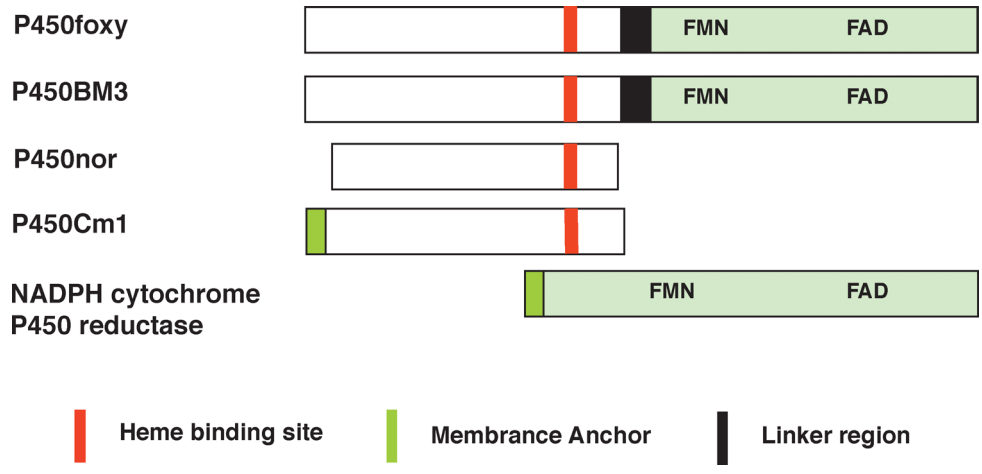

Fig. 1. Structures of several types of P450 and its redox partner (P450 reductase).

electron-transfer between NAD and its redox couple is conducted by transporting two electrons as a hydride ion (H^-) and thus two electrons are released simultaneously whereas a hemeprotein that contains only one electron-redox center (heme) can never receive H^- directly. Crystal structure of P450nor in several forms, that is, resting ferric (Fe^{3+}), CO-bound (Fe^{2+}–CO), and NO-bound (Fe^{3+}–NO) forms, have been so far determined. Here we succeeded in obtaining a crystal of P450nor in complex with NAAD and determining its structure, as shown in Fig. 2, which provided conclusive evidence for the direct H^- transfer from NADH to P450nor.

(1) Whole structure: A global conformational change of P450nor protein was observed upon binding of NAAD, which was accompanied by various intriguing local movements. Entrance of the heme pocket was closed, while the I-helix near heme moved a little away from heme. These changes are typical of the "induced fit".

(2) NADH-binding site: The heme-distal pocket is comprised of three sides, as shown in Fig. 3. One is extended beneath the B′-helix, in which many hydrophilic amino acid residues are concentrated (hydrophilic side). On the other hand, the area beneath the F,G-loop is hydrophobic. These two sides form the NADH-access channel. The third side forms a proton channel.

Arg174 is the only charged amino acid residue on the hydrophobic side. By contrast, the hydrophilic side contains many charged groups; Glu71, Arg64, Asp88, Lys291,

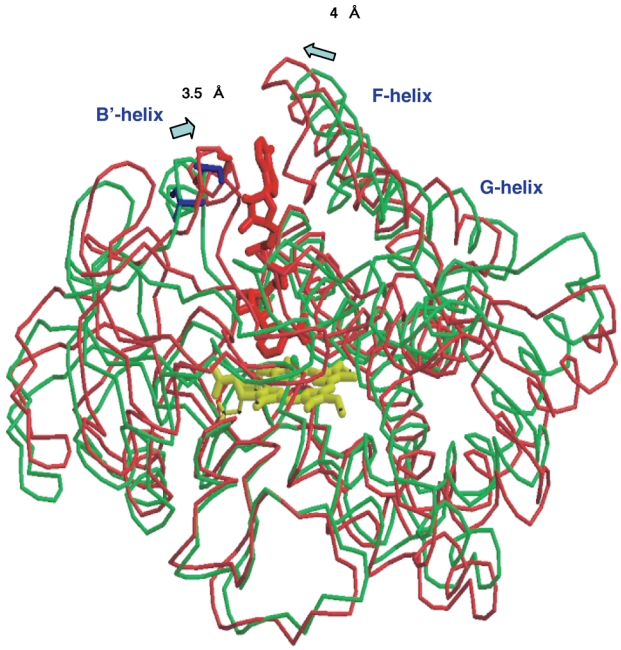

Fig. 2. Crystal structure of P450nor.
Two structures of P450nor in complex with NO (green) and with NAAD (nicotinic acid adenine dinucleotide) (red) superimposed centering heme (yellow). The B′-helix and F,G-loop moved by 3.5 and 4.0 Å, respectively, upon binding NAAD so that the entrance of the heme-distal pocket is closed. NAAD is shown by red, bold line.

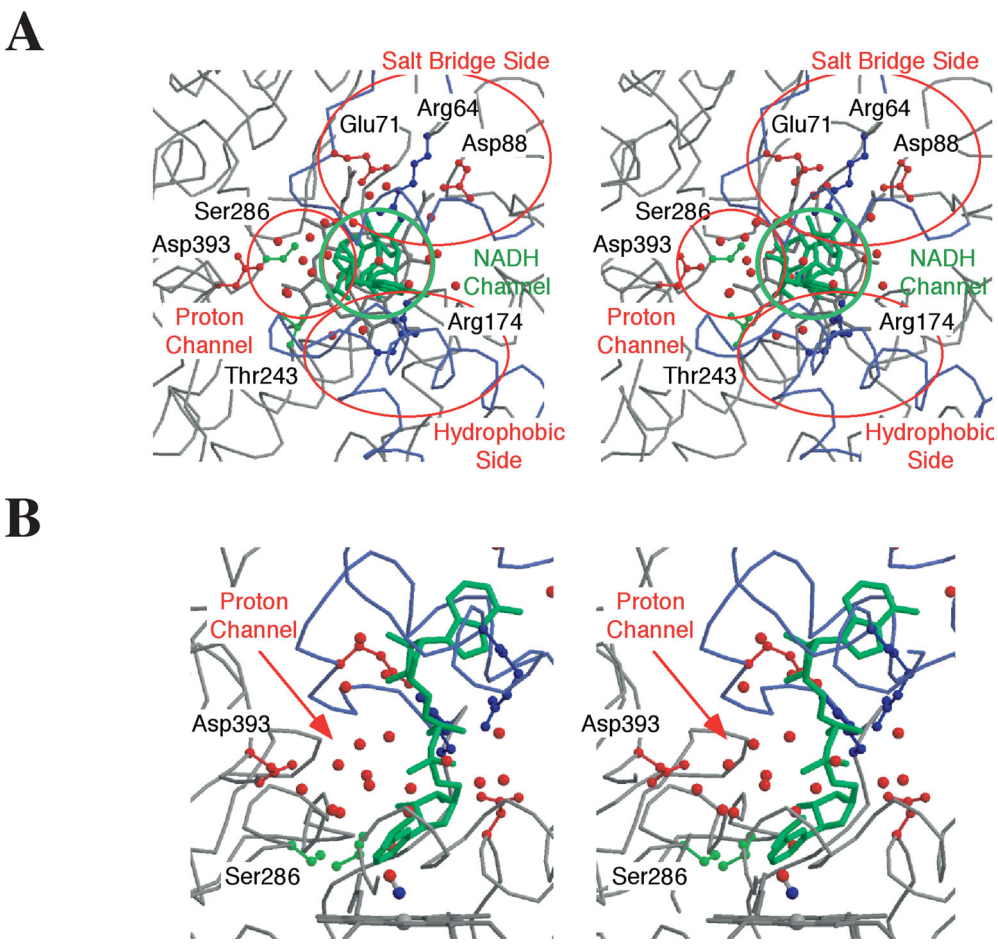

Fig. 3. NADH channel (bold, green circle) and proton channel (small, red circle).
A, A view from the heme-distal side perpendicular to the heme plate. B, Parallel to heme (bottom). Red, small spheres represent water. Side chains of Arg64 and Arg174, Glu71 and Asp88, Ser286 and Thr243, are depicted in blue, red, and green. NAAD is depicted by bold, green line.

Lys62, and Arg292. As expected, Arg174 and Arg64 bind the pyrophosphate moiety of NAAD between them. Thr243 in the I-helix binds the side chain of nicotinic acid ring and thus is crucial for stereo-selective hydrogen transfer from NADH. Also as expected, Ser73 and Ser75 are located near the 2'-hydroxyl group of adenine ring, and thus play the crucial role in determining the electron donor specificity, NADH or/and NADPH. Arg292 binds one of the propionate side chains of heme. Arg292 accompanies the mass movement of the charge cluster in the hydrophilic side that is caused upon binding of NAAD. The marked movement of Arg292 is accompanied by upward shift of the propionate side chain of heme, which play crucial role in fixing nicotinic acid ring in a stereo-selective manner.

(3) Active site: C4 carbon atom on nicotinic acid ring is located in the close vicinity of the hemebound NO and faces its proR side to heme (NO). This stereo-selective binding is mainly supported by Thr243 and the propionate side chain, as noted above. This structure of NAAD-binding is consistent with our previous results on such as kinetic isotope effect of hydrogen atoms on C4 of NADH, strongly supporting the direct H^- transfer from NADH to the Fe^{3+}–NO complex. In parallel with the NADH channel is formed a proton channel that is comprised of Asp393, Ser286, and many water molecules. The simultaneous formation of the proton channel upon binding NAAD suggests occurrence of a proton transfer subsequent to the H^- transfer to form the intermediate (equivalent to Fe^{3+}-hydroxylamine complex).

(4) Salt bridge network Glu71-Arg64-Asp88: In the NAAD-free structures of P450nor three charged groups, Glu71, Arg64, and Asp88 form a salt bridge network. Upon binding of NAAD, Glu71 and Arg64 accompany the mass movement of the charge cluster beneath the B′-helix, whereas Asp88 does not move so much. This means that the salt bridge between Arg64 and Asp88 is cut off upon the NAAD binding. It would appear that formation of the salt bridge network stabilizes the protein structure and breakage of the network is energetically unfavorable. This results in the low affinity of P450nor against NADH/NAD^+ by increasing the rate of release of NAD^+ (*k*off), but in realizing the high catalytic turnover. That is, the salt bridge network is essential for the elegant reaction mechanism to realize high catalytic turnover at the sacrifice of the affinity against NADH.

P450nor as multi-functional enzyme: P450nor exhibits NADH-peroxidase activity, as below.

$$H_2O_2 + NADH + H^+ \rightarrow 2H_2O + NAD^+$$

Here, we found a new function of P450nor, codenitrifying activity (below).

$$NO + N_3^- \rightarrow N_2O + N_2 \quad \text{or} \quad NO + NH_3 \rightarrow N_2O + H_2O$$

This means that P450nor forms N_2O when another nitrogen donor (azide, ammonium, and so on) is supplied instead of NADH, by combining two nitrogen atoms of NO and the other nitrogen donor. This reaction can elucidate the phenomenon of codenitrification observed with intact fungal cells.

Protein engineering of P450foxy: P450foxy is the fungal counterpart of bacterial P450BM3. They resemble each other, while a noticeable difference is observed. According to the crystal structure of P450BM3, Phe42, Arg47, and Tyr51 located at the entrance of the active site pocket, play crucial role in binding the substrate fatty acid. Interestingly, these amino acid residues are respectively replaced by Leu43, Lys48, and Phe52 in P450foxy. We constructed many mutant proteins of P450foxy in which these amino acid residues are replaced with the corresponding amino acids at each site. The results showed that these amino acids are important not only in binding fatty acid substrate but also in determining the substrate specificity.

References

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