A Role for Highly Conserved Carboxylate, Aspartate-140, in Oxygen Activation and Heme Degradation by Heme Oxygenase-1

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Abstract: Heme oxygenase (HO) catalyzes the oxygen-dependent degradation of heme to biliverdinIXα, CO, and free iron ion via three sequential monooxygenase reactions. Although the distinct active-site structure of HO from cytochrome P450 families suggests unique distal protein machinery to activate molecular oxygen, the mechanism and the key amino acid for the oxygen activation have not been clear. To investigate the functionality of highly conserved polar amino acids in the distal helix of HO-1, we have prepared alanine mutants: T135A, R136A, D140A, and S142A, and found drastic changes in the heme degradation reactions of D140A. In this paper, we report the first evidence that D140 is involved in the oxygen activation mechanism in HO-1. The heme complexes of HO mutants examined in this study fold and bind heme normally. The pK_a values of the iron-bound water and autoxidation rates of the oxy-form are increased with R136A, D140A, and S142A mutations, but are not changed with T135A mutation. As the wild-type, T135A, R136A, and S142A degrade heme to verdohemeIXα with H_2O_2 and to biliverdinIXα with the NADPH reductase system. On the other hand, D140A heme complex forms compound II with H_2O_2, and no heme degradation occurs. For the NADPH reductase system, the oxy-form of D140A heme complex is accumulated in the reaction, and only 50% of heme is degraded. The stopped flow experiments suggest that D140A cannot activate iron-bound dioxygen and hydroperoxide properly. To investigate the carboxylate functionality of D140, we further replaced D140 with glutamic acid (D140E), phenylalanine (D140F), and asparagine (D140N). D140E degrades heme normally, but D140N shows reactivity similar to that of D140A. D140F loses heme degradation activity completely. All of these results indicate that the carboxylate at position 140 is essential to activate the iron-bound dioxygen and hydroperoxide. On the basis of the present findings, we propose an oxygen activation mechanism involving the hydrogen-bonding network through the bridging water and D140 side chain.

Introduction

Heme oxygenase (HO), an amphipathic microsomal protein, catalyzes the oxygen-dependent degradation of heme (iron protoporphyrinIX) to biliverdinIXα, CO, and free iron ion.[1−4] The enzyme exists in two isoforms with essentially identical activity, the inducible HO-1 being primarily involved in heme catabolism at liver and spleen and the constitutive HO-2 primarily serving as a source of CO, a putative messenger molecule in various physiological functions.[3,5] Despite the difference in physiological function, the two isoforms have homologous secondary structures and spectroscopic properties, suggesting a similar catalytic mechanism.

The HO reaction proceeds via a multistep mechanism that depends on reduction equivalents provided by NADPH and NADPH cytochrome P450 reductase, as shown in Figure 1. In HO catalysis, the enzyme initially binds 1 equiv of heme to form a ferric heme−HO complex.[2] The first step of the reaction is an O_2-dependent oxidation of heme to ferric α-meso-hydroxyheme. The ferric heme in HO is reduced to a ferrous complex by the reductase, and the ferrous complex binds O_2 to form an oxy-form.[6] The oxy-form is then reduced to a ferric hydroperoxide intermediate by the electron from the reductase.[7] The iron-bound hydroperoxide of the ferric hydroperoxide intermediate oxidizes the α-meso carbon to form ferric α-meso-hydroxyheme. In the second step of the reaction, ferric α-meso-hydroxyheme is converted to ferrous verdohemeIXα with the concomitant formation of CO from the hydroxylated α-meso carbon by molecular oxygen and the electron from the reductase. The reaction of synthetic ferric α-meso-hydroxyheme in HO-1 has revealed the requirement of molecular oxygen and the electron from the reductase in this step.[8,9] In the third step, ferrous verdoheme is cleaved in an NADPH- and O_2-dependent manner

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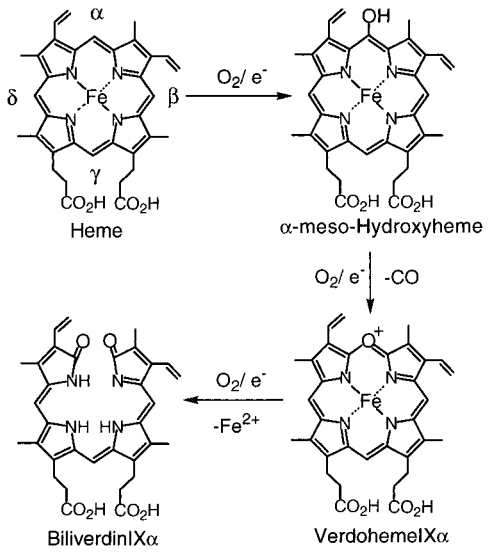

Figure 1. Reaction intermediates in the heme degradation by heme oxygenase.

to give ferric biliverdinIXα. From the reaction of synthetic ferrous verdohemeIXα in HO-1, this step also contains the activation process of molecular oxygen by verdoheme iron and the electron from the reductase, such as the first step.[10] Finally, biliverdinIXα and free iron are released from the enzyme upon the reduction of the ferric iron of ferric biliverdinIXα.

The activation of molecular oxygen is the key process in HO reaction. Although both HO and P450 activate the iron-bound dioxygen by electron donation from the reductase, the active-site structures and the proposed reaction intermediates of these enzymes are different (Figure 2). In cytochrome P450, cysteine binds to heme iron as a proximal ligand.[11] The strong push effect of the thiolate ligand assists to activate the iron-bound dioxygen. Furthermore, highly conserved threonine and aspartate in the distal side form a proton relay system, which provides protons to activate the iron-bound dioxygen. This protein machinery serves to produce a high-valent oxo-iron species such as compound I as an active oxidizing species. On the other hand, the proximal ligand in HO is histidine, which is a much weaker electron donor than cysteine.[12,13] In its distal heme pocket, a α-helix named the distal helix is placed within 4 Å from heme plane, and the closest amino acid residues to the heme are two glycine residues (G139 and G143),[13] which have no side chain. Thus, different from P450, the HO protein-machinery may not be designed to form the high-valent oxo-iron species but appears to be consistent with the ferric hydroperoxide intermediate to oxidize the heme.

The differences in the active-site structure and the active species between HO and cytochrome P450 imply that unique

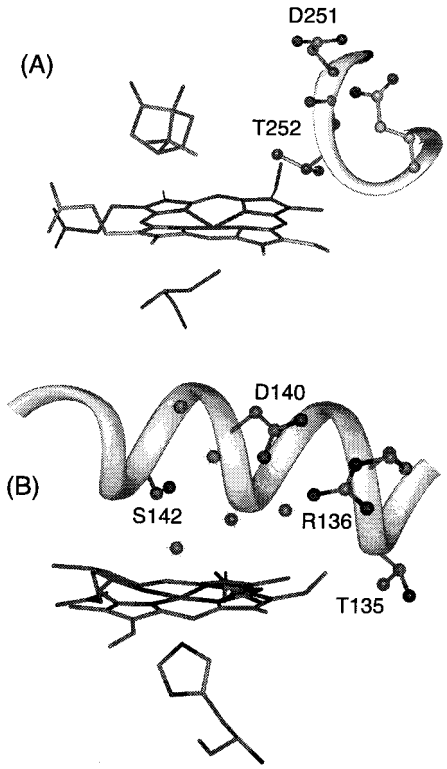

Figure 2. Conserved amino acid residues in the distal sides of ferric heme complex of cytochrome P450 and HO-1. (A) Cytochrome P450 (data from PDB accession code 1DZ9). (B) HO-1 (data from PDB accession code 1QQ8).

distal protein machinery is present in HO. To elucidate the HO oxygen activation mechanism, we have mutated highly conserved polar amino acid residues in the distal helix of HO isoform-1 (HO-1), threonine-135 (T135), arginine-136 (R136), aspartic acid-140 (D140), and serine-142 (S142), (Figure 2), and their enzymatic activities have been examined. We have found loss of or drastic decrease in the HO activity with the mutation of D140, which is located 7.5 Å away from the heme iron in the heme distal pocket.[13] In this paper, we report the first evidence that D140 is the key functional amino acid for the oxygen activation by HO. The mutations of D140 show that the carboxylate functionality at position 140 is essential to activate the iron-bound dioxygen and hydroperoxide. On the basis of the present results and the crystal structure,[13] we propose an oxygen activation mechanism by HO and discuss the difference in oxygen activation mechanism between HO and cytochrome P450.

Experimental Section

Materials. The wild-type and mutants of truncated water-soluble forms of recombinant rat HO-1 (30 kDa) and their heme complexes were prepared as described previously.[14] The mutations were confirmed by DNA sequencing. The homogeneity of each purified enzyme was monitored by SDS-PAGE. The heme complexes of HO-1 and its mutants were purified by Sephadex G-25 and DEAE cellulose column chromatography with 0.1 M phosphate buffer (pH = 7.0) after mixing 1.2 equiv of heme to the enzyme. NADPH cytochrome P450 reductase used in this work was a recombinant human enzyme using the gene obtained from Dr. F. Gonzalez of NIH.[15] All other chemicals were

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purchased from commercial sources and used without further purification.

Spectroscopy. UV−visible absorption spectra were recorded on a Shimadzu MultiSpec-1500 spectrometer. EPR spectra were obtained by a Bruker ESP-300 spectrometer operated at 9.45 GHz equipped with an Oxford liquid He flow cryostat. ^{1}H NMR spectra were obtained by a JEOL JNM-LA500 spectrometer at 25 °C. CD spectra were obtained on a JASCO J720W spectrometer. Resonance Raman spectra were measured on a Ritsu Oyo Kogaku, DG-1000 spectrometer equipped with a Princeton Instrument liquid-N_2 cooled CCD detector. Excitation sources consisted of a krypton laser (406.7 nm, Spectra Physics, BeamLok 2060) with 2 mW for ferric heme complexes and a helium−cadmium laser (441.6 nm, Kinmon Electronics, CD4805R) for ferrous deoxy complexes. Spectra were collected in a 90° scattering geometry on samples at room temperature with a collection time of 5 min. Frequencies were calibrated relative to indene.

Rate Constants of the HO Reactions. Single-turnover HO reactions of the wild-type and the mutants were monitored by stopped flow using a Hi-Tech SF-43 equipped with an MG 6000 diode array spectrometer. In general, syringe A of the stopped flow instrument contained 15 μM ferric heme complex of HO-1 or its mutants as well as 0.35 μM NADPH-cytochrome P450 reductase in 0.1 M phosphate buffer (pH 7.0). Syringe B of the stopped flow instrument contained 0.75 mM NADPH in the same buffer. The O_2 concentration in the reaction was taken to be 250 μM calculated for air-saturated solution. All of the measurements were performed at 25 °C, and spectra were recorded over the range of 380−740 nm at logarithmic time interval for 96 s. The kinetic time traces for individual wavelength between 380 and 740 nm were also recorded with the diode array detector. The data analyses were followed as in the previous report.[16] The absorption spectral changes from the ferric heme complex to verdoheme were analyzed as a sequential two-phase reaction, from the ferric heme complex to the ferrous oxy-form and from the ferrous oxy-form to verdoheme. The rate constant of each reaction was obtained by fitting the absorption change at 420 and 555 nm recorded with a logarithmic time interval with a double-exponential function.

The reduction of the ferric heme complexes of HO-1 and its mutants by sodium ascorbate under CO atmosphere was monitored using Shimadzu MultiSpec-1500 spectrometer at 25 °C. In a sealed quartz cuvette, 3 mL of 10 μM ferric heme HO-1 complex was degassed well by vacuum, and then CO gas was introduced in the cuvette. Sodium ascorbate (15 μL, 1.0 M) was added by syringe, and the spectral change was monitored at 10 s interval. The reduction rate constant was determined by fitting the absorption spectral change at the Soret band (405 nm) with single-exponential function.

Results

Mutations of Conserved Amino Acid Residues in the Distal Helix. The X-ray crystal structure of the heme−HO-1 complex has revealed that highly conserved glycine-139 (G139) and glycine-143 (G143) in the distal helix are the closest amino acids to the heme iron.[13] Although no polar amino acid side chains are in position to form a hydrogen bond to the heme iron-bound ligands, threonine-135 (T135), arginine-136 (R136), aspartic acid-140 (D140), and serine-142 (S142) are highly conserved polar amino acids placed near the heme in the distal helix (Figure 2). To investigate the function of these polar amino acids in the HO activity, we prepared alanine (A) mutants of T135, R136, D140, and S142: T135A, R136A, D140A, and S142A. The purified mutants bind heme with a 1:1 ratio to form ferric heme complexes. The absorption spectra of the heme complexes of the mutants are very similar to those of the wild-type complex. The far-UV CD spectra of the ferric heme complexes of these mutants are identical to those of the wild-type. These data indicate that ferric heme complexes of these HO mutants are folded properly and they are water-bound, six-coordinate high-spin species as is the wild-type heme complex. The high-spin absorption spectra of the heme complexes of these mutants exhibit pH-dependent reversible changes to the hydroxide-bound species at alkaline pH, as seen for the wild-type complex (see Supporting Information).[17] The estimated pK_a values of the iron-bound waters for these mutants are summarized in Table 1. While the pK_a value of the acid/base transition of the iron-bound water in T135A is similar to that (7.9) of the wild-type, those of R136A, D140A, and S142A are shifted to alkaline region with the mutations. The mutations of R136, D140, and S142 to alanine alter the polarities of the distal heme pockets and the hydrogen-bonding network with the iron-bound water in the heme pocket.

Table 1. Apparent Reaction Rate Constants, Auto Oxidation Rates, and pK_a values of Wild-Type HO-1 and Its Mutants in 0.1 M Phosphate Buffer, pH = 7.0 at 25 °C[a]

enzyme	k_1 ($s^{-1} \times 10^2$)	k_2 ($s^{-1} \times 10^2$)	k_1/k_2	k_1' ($s^{-1} \times 10^3$)	autooxidation ($s^{-1} \times 10^4$)	pK_a
wild-type	6.49 (1.00)	1.78 (1.00)	3.65	1.59 (1.00)	1.50	7.9
T135A	6.25 (0.96)	1.63 (0.92)	3.86	1.03 (0.65)	2.97	8.0
R136A	3.73 (0.57)	0.88 (0.49)	4.24	1.95 (1.23)	19.1	8.5
D140A	17.59 (2.71)	1.81 (1.02)	9.72	1.30 (0.82)	38.2	8.6
S142A	5.12 (0.79)	1.20 (0.67)	4.27	0.54 (0.34)	13.6	8.8

[a] The reaction conditions are described in Experimental Section. The numbers in parentheses indicate the ratio to the wild-type.

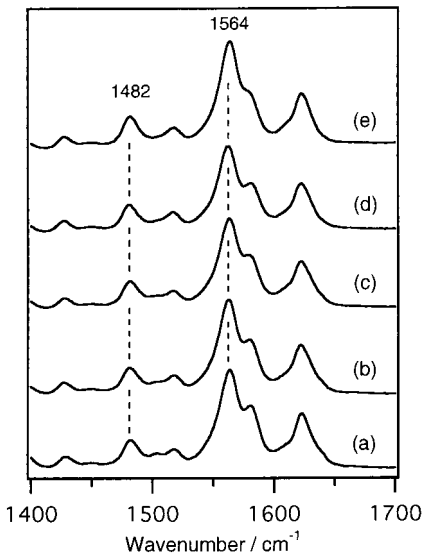

Figure 3. High-frequency region of the resonance Raman spectra of ferric heme complexes of wild-type and mutants in 0.1 M phosphate buffer, pH = 7.0. (a) Wild type. (b) T135A. (c) R136A. (d) D140A. (e) S142A.

The coordination of water to the ferric heme irons of these mutants are also supported by resonance Raman spectra. As shown in Figure 3, resonance Raman spectra of the ferric heme complexes of the mutants are similar to those of the wild-type. The ν_2 and ν_3 bands, which are distinct frequencies for five-(1570−1575 and 1490−1500 cm^{-1}, respectively) and six-coordinate high spin complexes (1560−1565 and 1475−1485 cm^{-1}, respectively),[18,19] are observed at 1562 and 1482 cm^{-1} for the wild-type and the mutants, respectively. These resonance Raman spectra are consistent with water coordination to the

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ferric heme iron to form six-coordinate state for the mutants, as established for the wild-type.[20]

Autoxidation Rates of the Mutants. To investigate the effect of the mutations on the stability of the ferrous oxy-form, we have measured the autoxidation rates of the oxy-forms of the wild-type and the mutant heme complexes. The results are summarized in Table 1. The autoxidation rate of the oxy-form of the wild-type heme complex is found to be $k_{obs} = 1.4 \times 10^{-4}$ s^{-1}, which is much higher than that of myoglobin ($\sim 10^{-5}$ s^{-1}) or hemoglobin.[21] The autoxidation rate of the oxy-form of T135A heme complex is the same as that of the wild-type heme complex. On the other hand, the autoxidation rates of the oxy-forms of R136A, D140A, and S142A heme complexes are drastically increased. The autoxidation rate of the oxy-form of D140A heme complex is about 25 times faster, and those of R136A and S142A heme complexes are about 10 times faster than that of the wild-type complex. Increase in the autoxidation rate has been observed for myoglobin mutants in which the distal histidine (His-64) is substituted with nonpolar amino acids and for a cytochrome P450 mutant in which threonine-252 is replaced by an alanine and have been due primarily to the changes in the hydrogen-bonding interaction of the bound dioxygen with distal protons.[21,22] R136, D140, and S142 residues appear to be involved in stabilizing the HO oxy-form.

Single Turnover HO Reactions by H_2O_2. Previous studies have shown that HO degrades heme in the wild-type to verdohemeIXα with H_2O_2.[23] The heme oxygenase reaction of the wild-type heme complex with H_2O_2 is shown in Figure 4a. With addition of 3 equiv of H_2O_2, heme in the wild-type is changed to verdohemeIXα, which has the Soret band with reduced intensity around 400 nm and a broad absorption around 650 nm, within 5 min. To examine the HO activities of T135A, R136A, D140A, and S142A HO-1 mutants, we have carried out the reactions of the heme complexes of these mutants with H_2O_2. Figure 5 shows absorption spectral changes of heme complexes of the mutants with addition of 3 equiv of H_2O_2. As shown in Figure 5a, 5b, and 5d, the reactivities of T135A, R136A, and S142A with H_2O_2 are similar to those of the wild-type. With addition of 3 equiv of H_2O_2, the Soret bands decrease their intensities and new absorptions appear around 650 nm. Within 5 min, the absorption spectra of the heme complexes are changed to those of the verdohemeIXα complexes. These spectral changes indicate that T135A, R136A, and S142A retain a normal HO activity toward H_2O_2.

On the other hand, the drastic effect of the mutation has been found in the reaction of D140A heme complex (Figure 5c). The absorption spectrum of the D140A heme complex quickly changes to a new one having the absorption peaks at 418, 528 and 553 nm and no further spectral change is observed. Presence of excess H_2O_2 results in bleaching of the heme, and no HO reaction takes place for D140A with addition of H_2O_2. The absorption spectrum of the new species is identical to that of an oxo ferryl species (compound II) of HO.[23] The formation of the oxo ferryl species by the reaction of D140A heme complex with H_2O_2 is further confirmed by the reproduction of the starting heme complex by reduction of the ferryl oxo species with sodium ascorbate. All of these findings indicate that the heme in D140A is not degraded to verdoheme by H_2O_2, but forms a ferryl oxo species (compound II).[24] The mutation of D140 to alanine completely loses HO activity to H_2O_2.

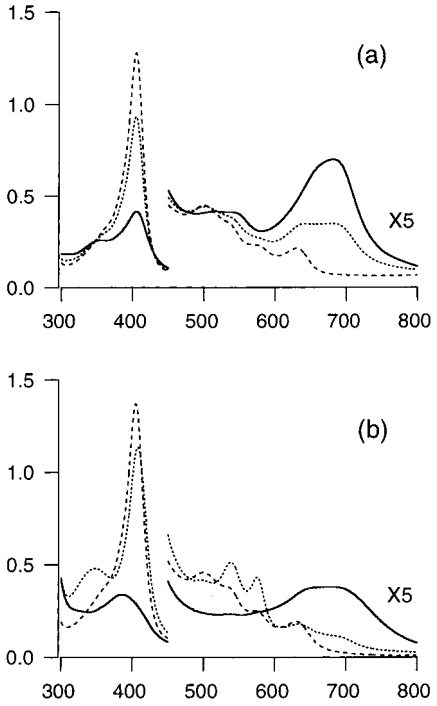

Figure 4. Absorption spectral changes in the HO reactions of the wild-type HO in 0.1 M phosphate buffer, pH = 7.0, at 23 °C. (a) With hydrogen peroxide. Broken line: ferric resting state, concentration: 10 μM, dotted line: immediate after addition of 3 equiv of hydrogen peroxide, solid line: after 5 min. (b) With cytochrome P450 reductase and NADPH. Broken line: ferric resting state, concentration: 10 μM, dotted line: immediate after addition of 3.5 equiv of NADPH and 0.08 μM of the reductase, solid line: after 20 min

Single Turnover HO Reactions by NADPH Reductase System. The mutations also cause drastic changes in the HO activity for the physiologically relevant NADPH-cytochrome P450 reductase system. Figure 4b shows the absorption spectral change of the wild-type heme complex after addition of NADPH-cytochrome P450 reductase and 3.5 equiv of NADPH under air. The wild-type heme complex immediately forms ferrous oxy-form upon addition of the reductase and NADPH, and then changes to biliverdinIXα with the decrease in the Soret band and the increase in an absorption around 650 nm. The heme in wild-type HO is completely degraded to biliverdinIX_α within 20 min. Figure 6 shows the absorption spectral changes of the heme complexes of the HO mutants with addition of NADPH-cytochrome P450 reductase and NADPH (3.5 equiv). As observed for the wild type, T135A and S142A degrade heme

(20) Recently, resonance Raman spectra of ferric heme complexes of human-D140 mutants have been reported by Lightning et al. (*J. Biol. Chem.* **2001**, *276*, 10612−10619). Although they report that the water ligand was lost from the heme iron to form five coordinated structure in human-D140A and -D140F mutants, our resonance Raman spectra of the rat-HO-1 mutants clearly indicate that the water ligand is present on the heme iron. To further investigate this inconsistency, we prepared heme complex of human-D140A. The heme complex of human-D140A prepared in our group shows the HO activity similar to theirs. However, the resonance Raman spectrum of our ferric heme complex of human-D140A indicates the presence of the water ligand on the heme iron, like in rat-D140A (See Supporting Information). Therefore, the reason for the discrepancy is not clear at present.

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(24) To investigate the change in the fashion of the O−O bond cleavage: homolysis versus heterolysis, the reactions of ferric heme complexes of the wild-type and D140A with cumene hydroperoxide were carried out. The HPLC analyses of the reaction products indicate that the ratio (3.3) of cumyl alcohol (heterolysis) over acetophenone (homolysis) for D140A is close to that (3.0) for the wild-type.

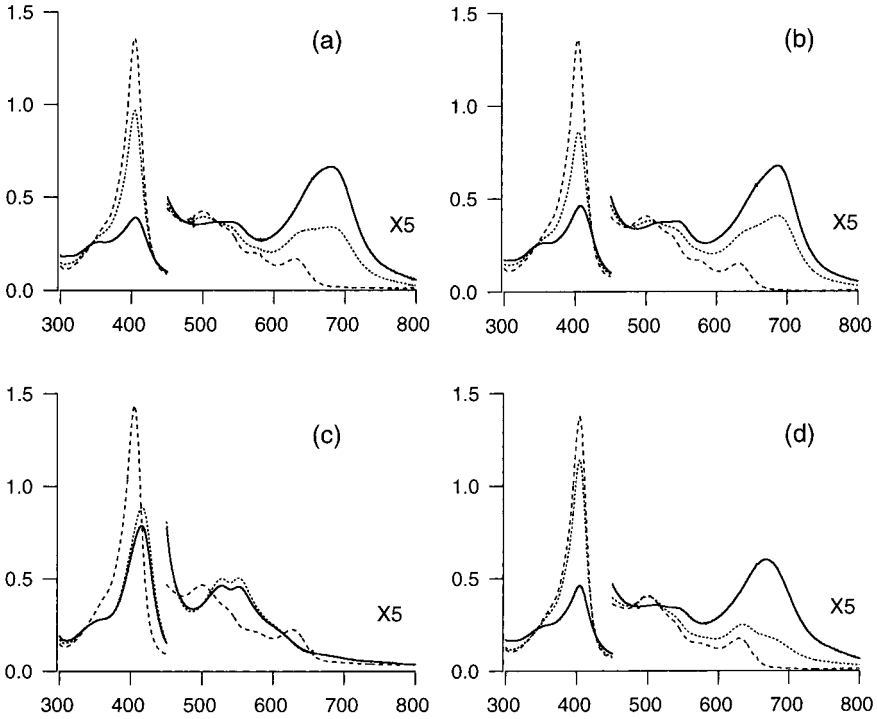

Figure 5. Absorption spectral changes in the HO reactions of the HO mutants with hydrogen peroxide in 0.1 M phosphate buffer, pH = 7.0, at 23 °C. Broken line: ferric resting state, concentration: 10 μM, dotted line: immediate after addition of 3 equiv of hydrogen peroxide, solid line: after 5 min. (a) T135A. (b) R136A. (c) D140A. (d) S142A.

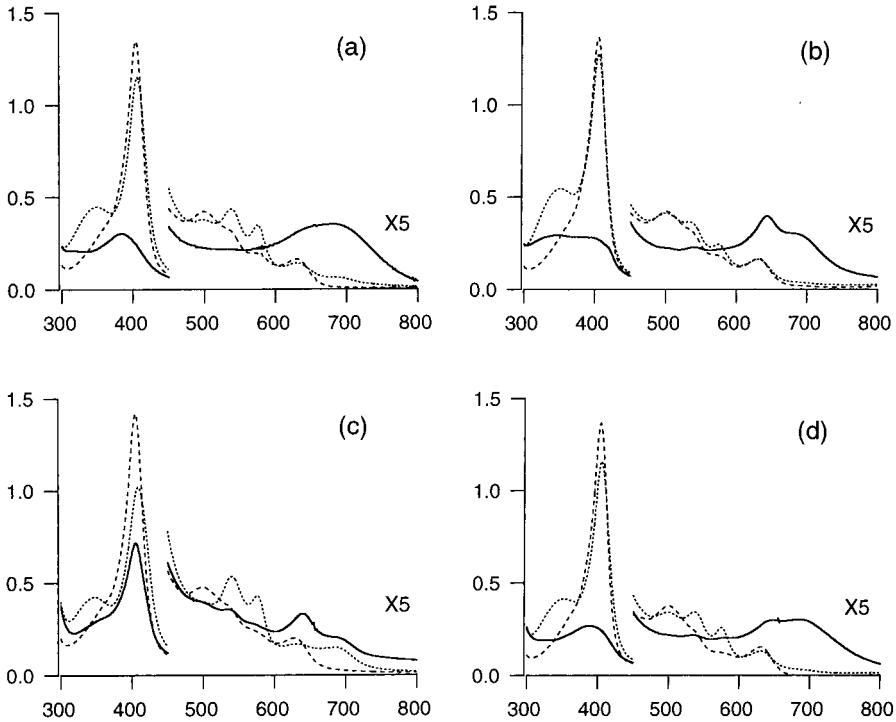

Figure 6. Absorption spectral change in the HO reactions of the HO mutants with NADPH and NADPH cytochrome P450 reductase in 0.1 M phosphate buffer, pH = 7.0, at 23 °C. Broken line: ferric resting state, concentration: 10 μM, dotted line: immediate after addition of 3.5 equiv of NADPH and 0.08 μM of the reductase, solid line: after 20 min. (a) T135A. (b) R136A. (c) D140A. (d) S142A.

to biliverdinIXα within 20 min, indicating normal HO activity. On the other hand, heme in R136A is degraded to biliverdinIXα, but the reaction is much slower than the wild-type, and more than 30 min is required to consume 3.5 equiv of NADPH. The absorption spectrum after consumption of 3.5 equiv of NADPH indicates that approximately 90% of heme is changed to biliverdinIXα, and more than 3.5 equiv of NADPH is required to degrade all of heme to biliverdinIXα.

More drastic change is found in the HO reaction by D140A (Figure 6c). As for the wild-type enzyme, the absorption spectrum of the D140A heme complex rapidly changes to its oxy-form with addition of 3.5 equiv of NADPH and the reductase. However, subsequent heme oxygenase reaction does not proceed effectively, and the oxy-form is accumulated in the reaction mixture. The decrease in the absorption at 340 nm indicates that NADPH is consumed normally, although the

reaction does not lead to the heme degradation. The oxy-form is oxidized to the initial ferric complex by air after all of NADPH is consumed. Finally, only 50% of heme is degraded by the reaction. The absorption at 639 nm resulting from verdohemeIXα CO complex in the spectrum suggests that the degradation of verdohemeIX$_\alpha$ is also slowed by the D140A mutation. The remaining heme could be degraded to biliverdinIXα by further addition of NADPH. All of these results indicate that the heme oxygenase activity is drastically lowered by the mutation of D140 to A.

Apparent Reaction Rates for Single-Turnover HO Reactions. To further investigate the effects of the mutations on each reaction step of the HO reaction, we measured apparent rates of the reaction steps from heme complex to verdoheme complex for the present HO mutants by a stopped-flow technique. The changes in the absorption spectrum between 380 and 740 nm were monitored by the stopped-flow spectrophotometry when the ferric heme complexes of the wild-type and the mutants was mixed with cytochrome P450 reductase and NADPH. As in the previous report, the reactions for the wild-type and the mutants appear to go through two phases and can be described by the two-step model as shown in eq 1.[16]

$$\text{ferric heme complex} \xrightarrow[k_1]{} \text{ferrous oxy heme complex} \xrightarrow[k_2]{} \text{verdohemeIX}\alpha \quad (1)$$

Table 1 shows the apparent reaction rates from the ferric heme complex to ferrous oxy heme complex (k_1) and from the ferrous oxy heme complex to verdoheme (k_2) by NADPH cytochrome P450 reductase and NADPH. We have also measured the apparent rates from the ferric heme complex to ferrous CO complex by sodium ascorbate (k_1') and the results are included in Table 1. Since O_2 and CO binding rates to the ferrous heme complex are much faster than the reduction rate of ferric heme complex to ferrous heme complex,[25] both k_1 and k_1' values reflect the reduction rate of the ferric heme complex by the reductase and sodium ascorbate, respectively.

As expected from the single-turnover HO reaction, the k_1 and k_2 values for T135A and S142A are similar to those of the wild-type. The reaction steps of the HO reactions for T135A and S142A are not changed by the mutations. However, the k_1 values of R136A and D140A are smaller and larger than that of the wild-type, respectively. The electron transfer from the reductase is not efficient for R136A, but efficient for D140A. Interestingly, the k_1' values for R136A and D140A are close to those of the wild-type, suggesting that the mutations of R136 and D140 hardly change the redox potentials of the ferric heme. As observed for k_1, the k_2 value for R136A is also smaller than that for the wild-type. Thus, the ratio of k_1 and k_2 for R136A is close to that of the wild-type (Table 1). On the other hand, while the k_1 for D140A is much larger than that of the wild-type, the k_2 value for D140A is similar to that of the wild-type. Therefore, the ratio of k_1 and k_2 for D140A is much smaller than that of the wild-type. Since the ratio of k_1 and k_2 indicates the oxygen activation rate relative to the iron reduction rate, these results suggest that the D140 side chain is involved in the oxygen activation process of HO reaction but the T135, R136, and S142 side chains are not.

Carboxylate Functionality of D140 residue on HO Activity. To investigate the carboxylate functionality at position 140, we further prepared mutants by replacing D140 with glutamic acid (D140E), phenylalanine (D140F), and asparagine (D140N). D140E, D140F, and D140N mutants bind heme with a 1:1 ratio and the absorption spectra of these heme complexes are very similar to that of the wild-type complex. The absorption spectra of the ferric heme complexes of D140E and D140N show pH-dependent reversible changes, as the wild-type heme complex. Ferric heme complexes of these D140 mutants exhibit far-UV CD spectra similar to those of the wild-type heme complex, suggesting that these mutants fold and bind heme properly. The resonance Raman spectra of the ferric heme complexes of D140E, D140F, and D140N are shown in Figure 7. As observed for D140A, these complexes exhibit the spectra typical to the six-coordinate high-spin ferric heme complex.[20] These spectral data indicate that a water molecule binds to the heme iron of these mutants.

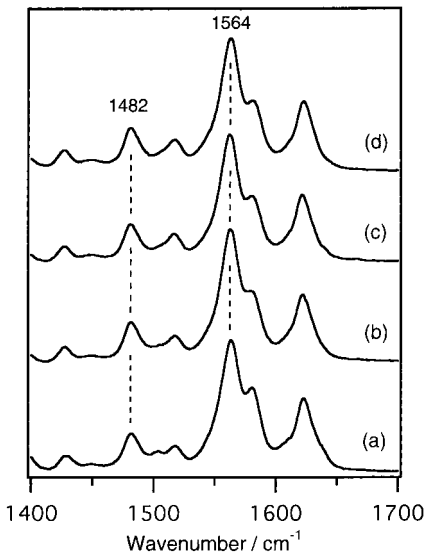

Figure 7. High-frequency region of the resonance Raman spectra of ferric heme complexes of wild-type and D140 mutants in 0.1 M phosphate buffer, pH = 7.0. (a) Wild type. (b) D140E. (c) D140N. (d) D140F.

The absorption spectral changes of the heme degradation reactions for D140E, D140F, and D140N are shown in Figures 8 and 9. As the wild-type, D140E degrades heme by H_2O_2 or NADPH and the reductase. The spectral change indicates that the HO activity of D140E is almost the same to that of the wild-type complex. The HO activity is retained even though the carboxylate side chain at position 140 is changed from aspartate to glutamate. On the other hand, D140N heme complex shows reactivity similar to that of D140A heme complex. The D140N heme complex quickly forms the ferryl oxo species (compound II) with addition of H_2O_2 and no heme degradation is observed. When NADPH and the reductase are added to the D140N heme complex, the oxy-form is accumulated, as observed for D140A. Although the oxy-form is accumulated, NADPH is consumed as same as the wild-type. The HO activity of D140N is not as low as that of D140A and approximately 70% of heme are finally degraded by 3.5 equiv of NADPH. Interestingly, D140F loses heme degradation activity both for H_2O_2 and NADPH reductase system. Like D140A and D140N, the D140F heme complex forms a ferryl oxo species with addition of H_2O_2, and no heme degradation occurs. Furthermore, the oxy-form is immediately produced when NADPH and the reductase are added to the D140F heme complex, but no further heme degradation is observed. About 90% of starting ferric

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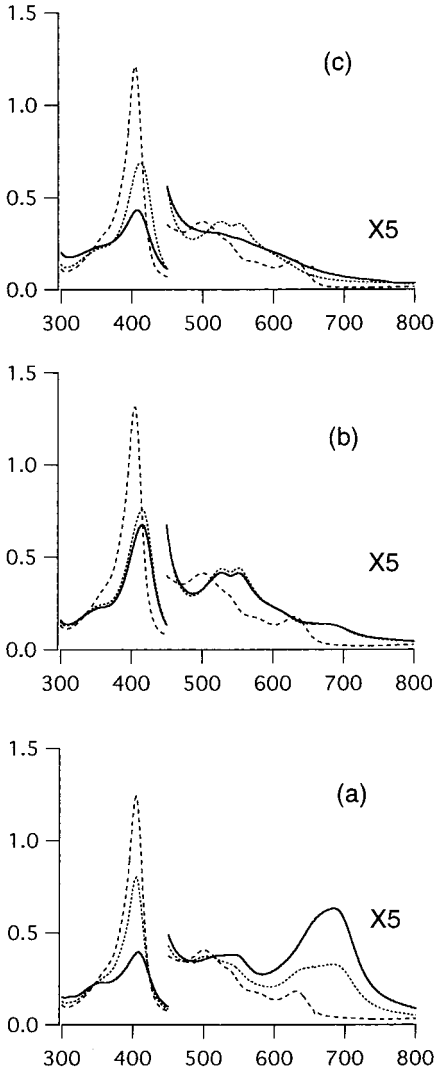

Figure 8. Absorption spectral changes in the HO reactions by D140 mutants with hydrogen peroxide in 0.1 M phosphate buffer, pH = 7.0, at 23 °C. Broken line: ferric resting state, concentration: 10 μM, dotted line: immediate after addition of 3 equiv of hydrogen peroxide for D140E and D140N and 10 equiv of hydrogen peroxide for D140F, solid line: after 5 min. (a) D140E. (b) D140N. (c) D140F.

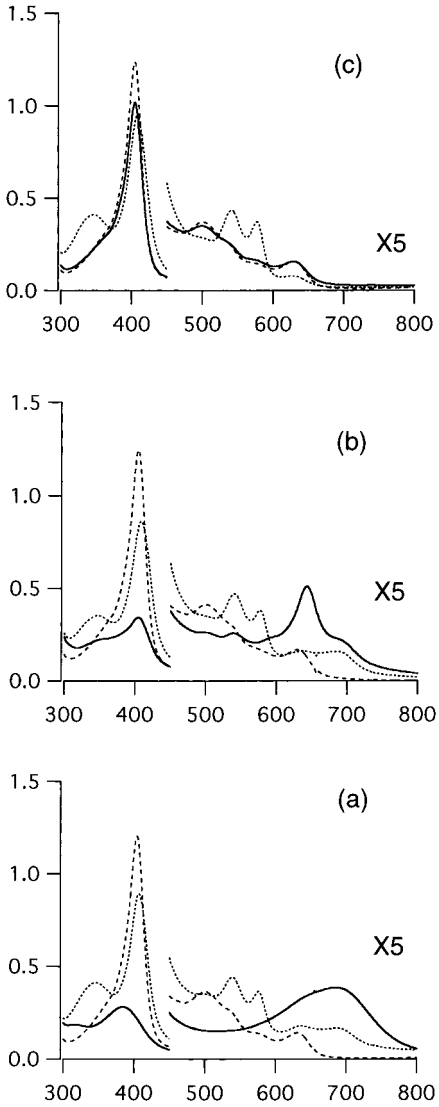

Figure 9. Absorption spectral changes in the HO reactions by D140 mutants with NADPH and NADPH cytochrome P450 reductase in 0.1 M phosphate buffer, pH = 7.0, at 23 °C. Broken line: ferric resting state, concentration: 10 μM, dotted line: immediate after addition of 3.5 equiv of NADPH and 0.08 μM of the reductase, solid line: after 20 min. (a) D140E. (b) D140N. (c) D140F.

heme complex is recovered after consumption of NADPH. Ten percent of heme in D140F would be decomposed nonenzymatically in the reaction. NADPH would be consumed by the reduction of the ferric complex of D140F formed by the autoxidation of the oxy-form, as the case of D140A and D140N. Heme degradation activity is lowered with increase in the size of side chain of D140. All of the present findings clearly demonstrate a crucial role of the carboxylate anion at position 140 in HO activity.

Characterization of D140A Mutant Heme Complexes. To investigate the active-site structure of the heme complex of D140A, ^{1}H NMR spectrum of the cyanide form of D140A heme complex was examined. As in the previous report, two sets of heme methyl proton signals are observed for both wild-type and D140A heme complexes. These two sets of signals are interpreted by the rotational disorder of heme in HO around its $\alpha-\nu$ axis.[26] The chemical shifts of the heme methyl signals for the D140A heme complex are close to those of the wild-type heme complex, suggesting that the coordination structure and the electronic state of the heme in D140A are similar to those of the wild-type complex.

Structural similarity between D140A and the wild-type has been observed for the ferrous heme complex. The resonance Raman spectrum shows that the iron−histidine-stretching mode of ferrous deoxy complex of D140A is observed at 214 cm^{-1}, which is close to that (218 cm^{-1}) for the wild-type deoxy complex.[17] The resonance Raman spectrum of ferrous CO form of the D140A heme complex also resembles that of the wild-type CO complex. The Fe−CO and C−O stretching bands and the Fe−CO bending band are observed at 502, 1962, and 570 cm^{-1}, respectively. These are close to those (503, 1958, and 576 cm^{-1}) of the wild-type.[19] We have examined resonance Raman spectrum of the oxy-form of D140A complex, but could not observe the spectrum because of low stability.

The active-site structure of the D140A heme complex is further investigated by EPR spectroscopy. The EPR spectrum of the D140A heme complex exhibits a typical ferric high-spin spectrum, but it is a mixture of axial-type spectrum (∼70%) and rhombic-type spectrum (∼30%), suggesting the equilibrium of two conformations at low temperatures.

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Discussion

Heme Oxygenase Activities of the HO Mutants. We have mutated polar amino acid residues, T135, R136, D140, and S142, in the distal helix of HO-1 to nonpolar alanine. All mutant proteins examined in this study are folded properly and bind heme normally. The single-turnover HO reactions and the stopped flow measurements clearly show that T135 and S142 residues are not essential to the HO reaction.

R136A degrades heme normally with H_2O_2, but slowly with NADPH and NADPH-cytochrome P450 reductase. The stopped flow results show that the slow HO catalytic reaction for the reductase system results from the decrease in the k_1 and k_2 values. Since O_2 binding rate to the ferrous heme complex is much faster than the reduction rate of the ferric heme complex to ferrous heme complex, the decrease in the k_1 value for R136A is due to the slow reduction of the ferric heme to ferrous heme by the reductase. The slow reduction of the ferric heme complex for R136A would come from the low affinity of R136A to the reductase rather than the decrease in the reduction potential of the ferric heme, because the k_1' value for R136A is slightly larger than that of the wild-type. This is consistent with the slow consumption of NADPH for R136A. With rapid autoxidation, the oxy-form formed during the HO reaction is oxidized to the ferric complex before reduction to a hydroperoxide complex by the electron from the reductase. The electron from NADPH would be consumed to re-reduce the ferric complex to the oxy-form. Therefore, more than 3.5 equiv of NADPH is required to degrade a molecule of heme to biliverdinIXα. Although overall HO reaction for R136A is slower than that of the wild-type, the rate of the oxygen activation process (k_2) is not changed by the R136-to-A mutation. This is supported by the finding that the ratio of k_1 and k_2 for R136A is close to that for the wild-type; the k_1 and k_2 are both decreased at the same fraction with the mutation. The present results show that, as well as the T135 and S142 residues, the R136 residue is not involved in the activation of the iron-bound oxygen directly but is important for the electron transfer from the reductase.

On the other hand, the mutation of D140 to alanine causes drastic changes in the HO activity. D140A cannot degrade heme but forms an oxo ferryl species with H_2O_2. The same result has been observed for D140N and D140F. These results indicate that the hydroperoxide complex formed in the D140 mutants cleaves its O−O bond before reacting with the heme normally. The formation of the oxo ferryl species with H_2O_2 has been also reported for G139L, G139F, and G139D mutants and explained by the steric effect of the amino acid residue introduced at position 139.[27] However, this is not the case for D140A because G139A degrades heme normally and D140 residue is located much farther than G139 residue from the heme. Furthermore, the D140E, having a longer side chain at position 140 than D140A, retains normal HO activity. Thus, the present results including D140N and D140F clearly indicate the functionality of the carboxylate at position 140 to activate iron-bound hydroperoxide properly. The details of the function of the carboxylate will be discussed in the next section.

D140A also changes the HO reaction mediated by NADPH-cytochrome P450 reductase. The single turnover HO reaction shows that D140A degrades only 50% of heme with 3.5 equiv of NADPH. The absorption spectra of the HO reaction for D140A suggest that the oxy-form is accumulated in the reaction,and this is confirmed by the stopped-flow experiments. In contrast to R136A, the k_1 value for D140A is increased with the mutation. This is not due to the increase in the reduction potential of ferric heme in D140A since the k_1' is decreased with the mutation. The increase in the k_1 value would result from efficient electron transfer from the reductase, probably due to high affinity of D140A to the reductase. Although the k_1 value is drastically increased with the D140A mutation, the k_2 value is not increased at the same fraction, leading to the decrease in the ratio of the k_1 and k_2 values. This results in the accumulation of the oxy-form, as observed. The drastic decrease in the ratio of the k_1 and k_2 for D140A indicates that the reaction process from the ferrous oxy complex to verdohemeIXα is hampered by the D140-to-A mutation. The electron from the reductase would be consumed with the re-reduction of the ferric heme formed from the autoxidation of the oxy-form. Thus, large excess of NADPH is required to convert all of heme in D140A to biliverdin.

Functionality of Distal Hydrogen-Bonding Network through D140 Residue. The present mutations of polar amino acid residues in the distal helix clearly demonstrate the intrinsic function of the carboxylate anion of D140. The present findings for D140 mutants would be explained by an oxygen activation mechanism proposed in Figure 10. We have modeled a bound dioxygen into the crystal structure of the ferric heme−HO complex.[13] Although the D140 side chain is not close enough to a modeled heme-bound dioxygen to interact with it directly, its side chain allows interaction with the bound dioxygen via a bridging water molecule that is found in the distal pocket on the crystal structure (Figure 2).[13] For example, the oxygen atom of the bridging water is found at 2.78 and 2.76 Å away from the D140 side chain and 2.70 and 3.15 Å far from the carbonyl oxygen of T135 main chain in molecule A and B, respectively. Furthermore, another water molecule is placed in the hydrogen-bonding distance from this water. The hydrogen-bonding interaction of the bound dioxygen has been shown by our previous EPR results of cobalt-substituted oxy-HO.[28] This hydrogen-bonding network stabilizes the bound dioxygen against autoxidation and also allows the bridging water to donate a proton to the bound dioxygen. Thus, the oxy-form of the wild-type complex is efficiently reduced to a hydroperoxide intermediate, which further oxidizes α-meso carbon to form α-meso-hydroxyheme. However, when D140 is mutated to A, N, or F, the bridging water moves its position due to the loss of the hydrogen-bond interaction with D140 side chain and the change in the polarity in the distal pocket. Therefore, the proton donation from the bridging water would be unfavorable and would only happen slowly. The loss of the hydrogen bond with the bound dioxygen leads to increase in the autoxidation rate of D140A and to decrease in the reduction rate of the bound dioxygen to hydroperoxide. This idea is supported by the present finding that the HO activity is decreased with increase in the hydrophobicity of the D140 side chain, F > A > N. The hydrogen-bonding network in the distal side would be disrupted partially for D140A and D140N and completely for D140F.

The hydrogen-bonding effects of the bridging water would also explain the altered reactions of D140A, D140N, and D140F with H_2O_2. H_2O_2 binds ferric heme iron to form a hydroperoxide intermediate. The iron-bound hydroperoxide would be fixed at the position suitable to oxidize the α-meso carbon by the hydrogen-bonding interaction with the bridging water, as the

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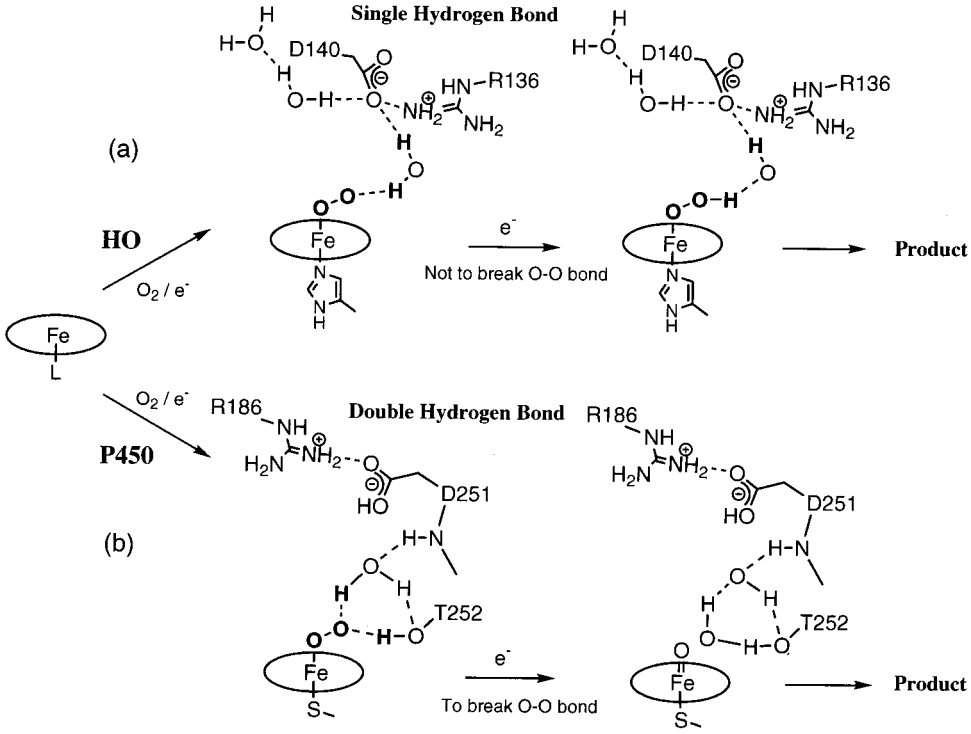

Figure 10. Schematic view of proposed mechanisms for the oxygen activation in HO and cytochrome P450. (a) HO, (b) cytochrome P450CAM.

case of the reduction of the oxy-form. Since the formation of the oxo ferryl species does not lead to the heme degradation, the hydrogen-bonding interaction with the bridging water would stabilize the hydroperoxide not to break the O−O bond. The hydrogen-bond interaction of D140 with the bridging water would decrease its acidity, which suppresses the formation of the oxo ferryl species. On the other hand, the bound hydroperoxides in the D140A, D140N, and D140F are not fixed at the proper position due to the loss of the hydrogen-bonding interaction with the bridging water. Thus, the O−O bond of the bound hydroperoxide is cleaved with the aid of the proton donation from a free water before the oxidation of the α-meso carbon takes place, which results in the formation of the oxo ferryl species.

The hydrogen-bonding network proposed for HO in Figure 10 is contrasted to that for cytochrome P450-CAM. The mutations of polar amino acids in the active site of cytochrome P450-CAM showed two functional key amino acids, threonine (T)-252 and aspartic acid (D)-251, to carry out the proton donation.[11,29−31] T252 side chain has been proposed to form a hydrogen bond to the iron-bound oxygen moiety,[28] and D251 side chain has been thought to function as a carboxylate switch between solvent accessible residue and catalytic water molecules.[29,30] The recent crystallographic study of the oxy-form demonstrates the hydrogen bonds of the iron-bound dioxygen with T252 side chain and a water molecule that interacts with amido N−H of D251, not with D251 carboxylate side chain (Figure 10b).[32] D251 side chain, rather, directs opposite to the heme active site. In HO, T135, and D140 are placed at similar positions to those of T252 and D251 in cytochrome P450-CAM, respectively. However, D140 carboxylate side chain interacts with the bridging water, and the T135 alcohol residue in HO directs opposite to the heme pocket. Thus, it is quite reasonable that the mutation of T135 to alanine for HO does not affect heme degradation activity, while the drastic change in the enzyme activity is observed by the mutation of T252 to alanine for cytochrome P450-CAM.

The function of the hydrogen bonds of the bound oxygen molecule in HO is also contrasted to that in cytochrome P450. The hydrogen-bond interaction of the bound dioxygen in cytochrome P450 assists a heterolytic O−O bond cleavage of the peroxide formed by the reduction of the bound dioxygen,[11,31] but in HO it does not. This difference would result from the number of hydrogen bonds to the iron-bound dioxygen and the acidity of the bridging water. In cytochrome P450, the bound dioxygen interacts both with the protons of T252 side chain and the bridging water (Figure 10b). The double hydrogen bonds would enhance the heterolytic O−O bond cleavage of the iron-bound peroxide due to the favorable structure for the elimination as a water molecule. Furthermore, the acidity of the bridging water is increased with the interaction of amido N−H; the acidic water also assists the O−O bond cleavage through acid catalysis. The double hydrogen-bond interactions of the iron-bound hydroperoxide with the T252 residue and the acidic water result in the formation of high-valent iron oxo species, an active species of substrate oxygenation by P450. On the other hand, the bound dioxygen in HO interacts only with the bridging water, and the bridging water is lowered its acidity by the interactions with D140 carboxylate side chain (Figure 10a). Although this hydrogen bond assists to reduce the iron-bound dioxygen to peroxide, it does not lead to the O−O bond cleavage of the peroxide due to the single hydrogen bond and lower acidity of the bridging water. This single hydrogen bond in HO would rather stabilize the bound hydroperoxide, not to break the O−O bond before the meso oxidation, and allows the

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oxidization of the α-meso carbon by the terminal oxygen of the bound hydroperoxide to form α-meso hydroxyheme.

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Supporting Information Available: Absorption spectra of ferric heme complexes of HO-1 mutants in 0.1 M phosphate buffer between pH 6 and 10 (Figure S1), resonance Raman spectra of ferric heme complexes of rat-D140A and human-D140A mutants (Figure S2) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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