Participation of Carboxylate Amino Acid Side Chain in Regiospecific Oxidation of Heme by Heme Oxygenase

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Heme oxygenase (HO) catalyzes the degradation of heme to biliverdinIXα (α-biliverdin), CO, and free iron by three sequential reactions which require O_2, NADPH, and cytochrome P-450 reductase.[1−4] The enzyme regiospecifically oxidizes the α-meso position of heme to form α-biliverdin isomer (see Figure 1). This regioselective oxidation is quite unique, in contrast to the non-enzymatic heme degradation which forms four possible α, *β*, *γ*, *δ*-biliverdin isomers.[5] The X-ray crystal structure of the heme−HO-1 complex has revealed that steric regulation is critical in effecting the α-regioselectivity.[6] The distal helix approaches within 4 Å across the entire width of the heme, thereby sterically restricting access of the heme ligands to the *β*-, *γ*-, and *δ*-meso carbon atoms. Possibly because of this streic effect, there has been no report of the formation of biliverdin isomers other than α-biliverdin from the HO reaction even by HO mutants. To explore the other factors that control the α-regioselectivity, we have mutated amino acids located near the HO active site and have found that the α-regioselectivity of the HO heme degradation can be altered by mutations of the highly conserved arginine-183 (R183) of HO-1. Here we report that the side chain of R183 plays a key role in the α-regiospecificity.

R183 in rat HO-1 has been changed to glutamic acid (E) by site-directed mutagenesis and the mutant protein has been expressed in *Escherichia coli*. The purified R183E-HO mutant binds heme with a 1:1 ratio to form a heme complex that exhibits absorption and EPR spectra very similar to those of the heme complex of wild-type HO.[7] This indicates that, as the wild-type heme complex,[7] histidine-25 remains the proximal ligand in the heme−R183E-HO complex. R183E rapidly degrades heme to biliverdin upon addition of ascorbic acid and desferrioxamine, suggesting that the heme−R183E complex is properly folded.[8] The pK_a value of the acid/base transition of the iron-bound water is changed from 7.9 to 9.2 upon R183-to-E mutation, suggesting

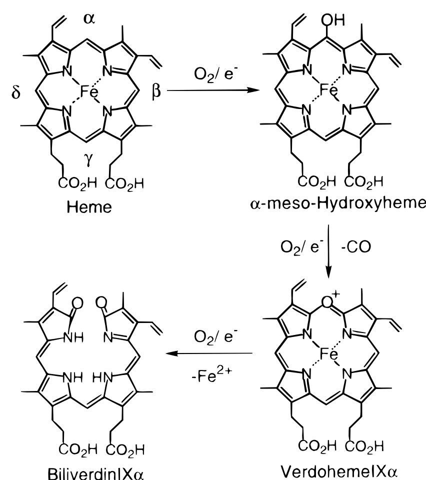

Figure 1. Reaction intermediates in the heme oxygenase-catalyzed oxidation of heme to biliverdinIXα.

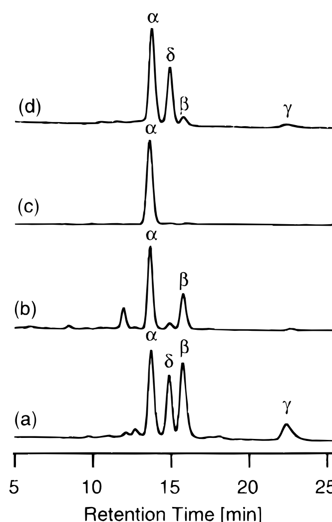

Figure 2. HPLC analysis of products isolated from the HO reactions. HPLC condition; column: Capcell Pak C18 (SG 120, SHISEIDO CO., Ltd.) 4 × 150 mm, solvent: acetonitrile: water (3:2, v/v), temperature: 35 °C, flow rate: 1 mL/min, monitor: absorption at 320 nm. Biliverdin products isolated from HO reactions were esterified in 5% HCl−methanol for 16 h at 4 °C before HPLC analysis. (a) mixture of α, *β*, *γ*, *δ*-biliverdin dimethyl esters. (b) From hemoglobin. (c) From wild-type HO. (d) From R183E-HO.

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(8) R183E- and R183D-HO could degrade heme to biliverdin with NADPH and NADPH-cytochrome P450 reductase, but the reactions were much slower than those of the wild-type complex. The electron transfer rate from the reductase is drastically decreased for R183E and R183D, but not for R183N, R183A, R183Y, and R183T (See Supporting Information). The electronic repulsion formed by the negative charge introduced at position 183 in R183E and R183D would weaken the interaction with the reductase and decrease in electron-transfer rate.

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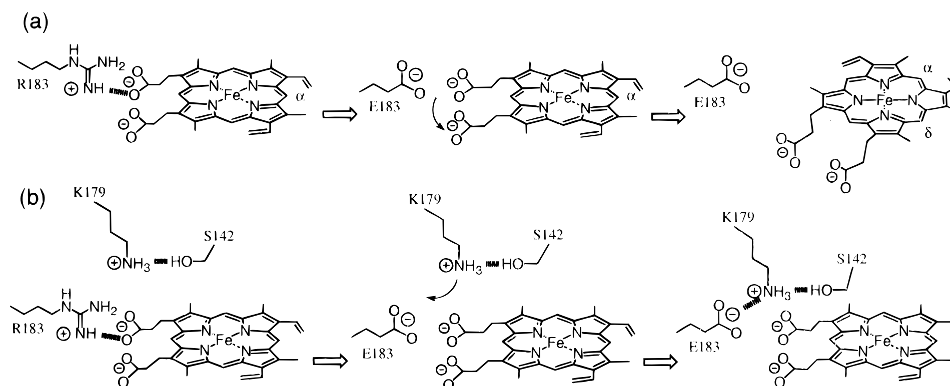
Scheme 1



that changes in the hydrogen-bonding networks in the distal heme pocket. To examine the formation of biliverdin isomers other than α-biliverdin, we have performed HPLC analysis of biliverdins extracted from the final solution of the heme degradation reactions. Interestingly, as shown in Figure 2, while only α-biliverdin is produced from the heme oxygenase reaction of wild-type HO-1, δ-biliverdin (35%) and small amount of β- and γ-biliverdins are found in addition to the normal α-biliverdin from that of R183E-HO. This is the first observation of HO-catalyzed formation of biliverdin isomers other than the α-isomer from the physiological substrate, iron protoporphyrinIX.

Lysine (K)-179 and arginine (R)-185 are highly conserved amino acids located near R-183 in HO-1.[9] The α-regioselectivity is fully retained in both K179E-HO and R185E-HO mutants. There are six more conserved arginine residues in HO-1.[9] We have replaced each of them by glutamic acid (E) and examined their heme oxygenase reaction products. None of these mutants exhibit changes in the α-regioselectivity.

To examine the effect of the carboxylate of E183 on the regioselectivity, we have prepared a mutant substituted R183 of HO-1 with aspartic acid (D), and its HO catalytic products have been examined. A significant amount (20%) of the δ-biliverdin was formed from the reaction of R183D-HO. We have also prepared mutants substituted R183 with asparagine (N) and glutamine (Q). These mutations introduce amide groups instead of the carboxylate groups of R183E-HO and R183D-HO. The heme oxygenase reactions of R183N-HO and R183Q-HO do not produce other biliverdin isomers except for the normal α-biliverdin. Furthermore, the α-regioselectivity is also retained when R183 is replaced with alanine having a non-polar side chain, threonine having polar side chain, and tyrosine having a weak anionic side chain. All of these results lead to a clear conclusion that only a carboxylate group at position 183 is able to change the α-regioselectivity of the heme oxygenase reaction.

The formation of δ-biliverdin by R183E-HO and R183D-HO could be explained by changes of the distal pocket structure with these mutations. The crystal structure of heme−HO-1 complex shows that the R183 residue interacts with a carboxylate moiety of the heme propionate through a hydrogen-bond interaction.[6] Since the amino acid residue at position 183 is farther than 10 Å from the heme iron center, the carboxylates introduced at position 183 of R183E and R183D mutants cannot modulate the α-regioselectivity by directly changing the geometry of iron-bound dioxygen. A possible explanation of the δ-biliverdin formation would be the rotation of heme by the mutations (Scheme 1a). The original positive charge of arginine at position 183 is changed to negative by introduction of the carboxylate, resulting in the electronic repulsion with the carboxylates of the heme propionate group. The electronic repulsion might rotate the heme along the heme iron−proximal histidine axis to keep the carboxylate of the heme propionate away from the carboxylate at the position 183. This rotation places the δ-meso position to the position originally occupied by the α-meso carbon, resulting in formation of the α- and δ-biliverdin mixture. However, the similarities of ^{1}H NMR shifts of heme methyl signals for wild type-HO, R183D-HO, and R183E-HO indicate that the heme in R183D and R183E is not rotated drastically with respect to the heme in its wild-type counterpart.[10] Another possible mechanism for the change in regioselectivity is formation of a new hydrogen-bonding network with the mutation (Scheme 1b). There are many lysines (K) around R183, such as K179, K18, and K22.[9] The newly introduced carboxylate at position 183 may interact with K179 residue which forms a hydrogen-bonding interaction with serine-142 (S142). Thus, the formation of the hydrogen bond between E183 and K179 shifts S142 as well as K179. The shift of S142 would induce the change in the distal pocket structure since S142 is located in the distal helix. This is consistent with the drastic change in the pK_a value of the iron-bound water by the mutation.[11] The change in the distal protein structure with the mutation makes it possible to oxidize the δ-meso position, leading to the δ-biliverdin formation.

In summary, we found that the mutation of R183 to E or D changes the α-regioselectivity of the HO catalysis. The result shows the importance of the hydrogen-bonding interaction between the arginine at position 183 and the carboxylates of the heme propionate group, as well as steric effect of the distal helix, for the α-regioselectivity.

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Supporting Information Available: ^{1}H NMR spectra of cyanide forms of heme−HO complexes and apparent reduction rates of heme complexes of wild type-HO and R183 mutants by NADPH and NADPH cytochrome P450 reductase (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) As observed for the wild-type complex, heme−R183D and −R183E complexes exhibit two sets of heme methyl signals due to the heme orientational disorder (isomer X and Y). Interestingly, the heme methyl signals of the isomer X are significantly broadened than those of the isomer Y and the wild-type complex. This appears to indicate that heme of the isomer X rapidly fluctuates due to the electronic repulsion of the carboxylates in R183D and R183E, and this fluctuation may change the regioselectivity.

(11) When alanine and glutamate were introduced instead of lysine at 179, the pK_a values of iron-bound water drastically shifted 9.6 and 10.6 from the original pK_a of 7.9, respectively.