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Essential Amino Acid Residues Controlling the Unique Regioselectivity of Heme Oxygenase in *Pseudomonas aeruginosa*

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Heme oxygenase (HO), an amphipathic microsomal protein, catalyzes the oxygen-dependent degradation of heme (iron-protoporphyrinIX) to α-biliverdin, CO, and free iron ion.[1,2] HO has been found in a wide array of organisms, such as vertebrates, photosynthetic organisms, and pathogenic bacteria.[2−5] Interestingly, all of these HO regiospecifically oxidize the α-meso position of the heme to form α-biliverdin isomer, while nonenzymatic heme degradation forms all four possible α-, *β*-, *γ*-, *δ*-biliverdin isomers at nearly identical yield.[2,4−6] The α-regioselectivity of HO suggests that HO controls the reactivity of an active oxygen species formed in the reaction, and the mechanism of the α-regioselectivity has been studied to understand protein machinery of HO. Previously, we showed an essential role of a hydrogen-bonding interaction between a positively charged residue and the heme propionate to the α-regioselectivity.[7,8] Deletion of the interaction by the mutation of Arg183 in rat-HO-1 induces heme rotation in plane in the active site, leading to the formation of α- (65%) and *δ*- (35%) biliverdins. Recently, a more interesting example has been found in HO (PigA) of the Gram-negative bacterium *Pseudomonas aeruginosa*, which does not produce α-biliverdin at all, but forms the mixture of *β*- and *δ*-biliverdins at a ratio of 3:7.[9] NMR studies suggest the rotation of the heme in PigA is due to the absence of the hydrogen-bonding interaction at the normal heme position,[10] as the case of the Arg183 mutant.[7] However, the drastic change in the regioselectivity of PigA led us to expect participation of more unique mechanisms for controlling the regioselectivity. While studying the mechanism of the unique regioselectivty of PigA, we found essential amino acid residues controlling the unique regioselectivity of PigA. In this communication, we show a mechanism of regioselectivity of PigA, in which Lys34 and/or Lys132 are essential amino acid residues to hold the rotated heme in the active site of PigA via hydrogen-bonding interaction with the heme propionate and in which Phe189 controls the product ratio of *β*- and *δ*-biliverdins via steric interaction with heme substituents.

On the basis of the amino acid sequence alignments and the available X-ray crystal structures of bacterial HOs,[9,11] we expected that Lys34 and Lys132 in PigA are located in suitable positions to stabilize the rotated heme by hydrogen-bonding interaction with the heme propionates. To investigate the function of these amino acids, we have changed Lys34 and Lys132 in PigA to alanine by site-directed mutagenesis. If these amino acids interact with the propionates of the rotated heme, the mutations of these amino acids to nonpolar alanine liberate the heme from the rotated position, leading the change in the regioselectivity of these mutants. The mutant proteins, Lys34Ala and Lys132Ala, have been expressed in *Escherichia coli*. The purified mutants bind heme with a 1:1 ratio to form heme complexes that exhibit absorption and EPR spectra very close to those of the heme complex of wild-type PigA.[9] The heme degradation activities of these mutants are also similar to that of the wild-type PigA.[9] These indicate that the heme complexes of these alanine mutants are properly folded as the heme complex of the wild type. To examine regioselectivity of these mutants, we have performed HPLC analysis of biliverdins extracted from the final solution of the HO reactions of these mutants (see Table 1). As Table 1 reports, the wild-type PigA forms *β*- (28%) and *δ*- (72%) biliverdins. On the other hand, Lys34Ala produces 11% of α-biliverdin in addition to the formation of normal *β*- (34%) and *δ*- (55%) biliverdins. Lys132Ala also produces 12% of α-biliverdin in addition to the formation of normal *β*- (26%) and *δ*- (62%) biliverdins. The formation of α-biliverdin is not observed from the HO reactions of other single mutants of PigA. Therefore, the formation of α-biliverdin from Lys34Ala and Lys132Ala indicates that Lys34 and Lys132 are involved in stabilizing the rotated heme in PigA. Partial or complete loss of the hydrogen-bonding interaction with the heme propionates by these mutations would liberate the heme from the rotated position and allow the α-meso position of the heme to place at the oxidation site.

To further investigate the function of these amino acids, we prepared a double mutant that replaced both Lys34 and Lys132 with alanine: Lys34Ala/Lys132Ala. If both Lys34 and Lys132 are essential to hold the rotated heme, the double mutant forms more α-biliverdin than the single mutants. The double mutant binds heme normally and degrades it to ferric biliverdin complex as the wild type. The product analysis for the double mutant shows the formation of 17% of α-biliverdin, 27% of *β*-biliverdin, and 56% of *δ*-biliverdin. The double mutant forms more α-biliverdin than the single mutants, indicating that both Lys34 and Lys132 are essential to hold the heme at the rotated position with the hydrogen-bonding interactions in the active site of PigA.[12]

We have also explored the amino acid residue affecting the ratio of *β*- and *δ*-biliverdins. The formation of *β*- and *δ*-biliverdin from the wild-type PigA results from the orientational disorder of the heme in the active site:[10] the rotation of the heme around the α−*γ* meso axis in the active site places the *β*-meso position, instead of the *δ*-meso position, at the normal oxidation site. Therefore, we assumed that the steric interaction of amino acid residue with heme substituents controls the ratio of *β*- and *δ*-biliverdins. From the amino acid sequence alignments and the X-ray crystal structure of bacterial HOs,[9,11] we searched amino acids interacting with the heme, and we have found drastic change in the ratio of *β*- and *δ*-biliverdin formation with mutation of Phe189. To study the steric effect, we mutated Phe189 to glycine, alanine, tyrosine, leucine, and tryptophan. All of the Phe189 mutants listed in Table 2 produce only *β*- and *δ*-biliverdins, indicating that these mutants are folded properly as the wild type. Interestingly, as the amino acid residue introduced at position 189 of the mutant is bulkier than that

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Table 1. Regioselectivity of PigA Mutants

PigA mutant	biliverdin isomer ratio (%)[a] α	β	γ	δ
WT	0	28(23)[b]	0	72(77)[b]
Lys34Ala	11(5)[b]	34(34)	0	55(64)
Lys132Ala	12(4)	26(26)	0	62(70)
Lys34Ala/Lys132Ala	18(10)	27(23)	0	56(67)

[a] The percentage of each isomer was obtained by integration of the peaks within each chromatogram. The standard deviation is ±5%. Conditions: Heme complex, 30 μM; CPR, 0.3 μM; NADPH, 150 μM. [b] The numbers in parentheses show the product ratios when sodium ascorbate is used as a reductant. Conditions: Heme complex, 30 μM; ascorbate, 10 mM.

Table 2. Regioselectivity of PigA Phe189 Mutants

Phe189 mutant	isomer ratio (%)[a] biliverdin α	β	γ	δ	disorder of heme
Gly	0	70(76)[b]	0	30(24)[b]	70:30
Ala	0	55(59)	0	45(41)	66:34
Phe(WT)	0	28(23)	0	72(77)	26:74
Tyr	0	33(26)	0	67(74)	29:71
Leu	0	87(84)	0	13(16)	77:23
Trp	0	83(90)	0	17(10)	82:18

[a] The percentage of each isomer was obtained by integration of the peaks within each chromatogram. The standard deviation is ±5%. [b] The numbers in parentheses show the product ratios when sodium ascorbate is used as a reductant. The experimental conditions are the same shown in Table 1.

(phenylalanine) of the wild type, the ratio of β-biliverdin is increased. In an extreme case, 90% of β-biliverdin is produced with introduction of tryptophan at position 189, Phe189Trp. This result means that wild-type PigA is altered to a β-selective enzyme by the single mutation of Phe189. On the other hand, as the amino acid at position 189 is smaller than that of the wild type, the ratio of β-biliverdin is also increased. For Phe189Gly, 70% of β-biliverdin is produced. To investigate the change in the ratio of the orientational disorder of the heme in each mutant, we measured ^{1}H NMR spectra of ferric cyanide complexes of Phe189 mutants (see Figure S1). The ratio of orientational disorder estimated from the intensities of the heme methyl signals for each mutant is summarized in Table 2. The ratio of the orientational disorder of each mutant is very close to the ratio of β- and δ-biliverdins produced from the mutant. The results clearly show that the change in the product ratio with the mutation of Phe189 results from the change in the ratio of the orientational disorder of the heme. The steric interaction of amino acid residue at position 189 with the heme substituents would change the ratio of the orientational disorder of the heme, leading to the change in the product ratio of β- and δ-biliverdins.

In summary, the present study suggests the active site structure of PigA shown in Figure 1. The hydrogen-bonding interaction of the heme propionate with Lys34 and/or Lys132 holds the heme at the rotated position, and the steric interaction of heme substituents with Phe189 affects the ratio of orientational disorder of heme. These interactions put the β- or δ-meso position at the oxidation site, resulting in the unique regioselectivity of PigA. The present study also suggests the strategy to manipulate the regioselectivity of HO. Alternation of regioselectivity of HO based on mutations of amino acids in the active site is under investigation in our laboratory.

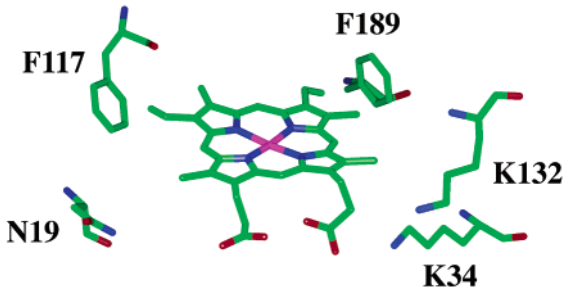

Figure 1. Active-site structure of PigA heme complex estimated from the present study. This model structure was made based on the crystal structure of HO of *Neisseria meningitidis*: PDB accession code 1J77.[11a]

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Supporting Information Available: Figure of ^{1}H NMR spectra of cyanide forms of ferric heme complexes of PigA Phe189 mutants (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) The present results suggest two possibilities as functions of Lys34 and Lys132 in PigA. One is that both Lys34 and Lys132 interact with the heme propionate to hold the heme at the rotated position and the other is that either Lys34 or Lys132 interacts with the heme propionate and the other stabilizes the interaction. In the latter case, the mutation of the lysine stabilizing the interaction would destabilize the interaction, leading to partial break of the interaction. On the other hand, the mutation of the lysine interacting with the heme propionate would break the interaction, but partially form a new interaction between the other lysine and the heme propionate.

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