Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 338 (2005) 558–567
BBRC
www.elsevier.com/locate/ybbrc

Review

Heme oxygenase and heme degradation ☆

Goro Kikuchi [a,b,*], Tadashi Yoshida [c], Masato Noguchi [d]

[a] Tohoku University School of Medicine, Sendai, Japan
[b] Nippon Medical School, Bunkyo-ku, Tokyo, Japan
[c] Department of Biochemistry, Yamagata University School of Medicine, Yamagata, Japan
[d] Department of Medical Biochemistry, Kurume University School of Medicine, Kurume, Japan

Received 25 July 2005
Available online 15 August 2005

Abstract

The microsomal heme oxygenase system consists of heme oxygenase (HO) and NADPH–cytochrome P450 reductase, and plays a key role in the physiological catabolism of heme which yields biliverdin, carbon monoxide, and iron as the final products. Heme degradation proceeds essentially as a series of autocatalytic oxidation reactions involving heme bound to HO. Large amounts of HO proteins from human and rat can now be prepared in truncated soluble form, and the crystal structures of some HO proteins have been determined. These advances have greatly facilitated the understanding of the mechanisms of individual steps of the HO reaction. HO can be induced in animals by the administration of heme or several other substances; the induction is shown to involve Bach1, a translational repressor. The induced HO is assumed to have cytoprotective effects. An uninducible HO isozyme, HO-2, has been identified, so the authentic HO is now called HO-1. HOs are also widely distributed in invertebrates, higher plants, algae, and bacteria, and function in various ways according to the needs of individual species.

Keywords: Heme; Heme oxygenase; Heme degradation; α-*meso*-Hydroxyheme; Verdoheme; Biliverdin; Carbon monoxide

Heme oxygenase (HO) is the first and the rate-limiting enzyme of the microsomal heme degradation pathway that yields biliverdin, carbon monoxide (CO), and iron as the final products. In 1968, Schmid and his associates discovered HO activities in microsomes of the spleen, liver, kidney, and bone marrow of the rat [1–3], and now it is widely accepted that microsomal HO plays a key role in physiological heme catabolism in animals.

The microsomal HO system consists of HO and NADPH–cytochrome P450 reductase, and HO was initially supposed to involve cytochrome P450 as a terminal oxidase [4]. However, we have shown that HO is independent of any type of cytochrome P450 by examining purified heme oxygenase from pig spleen microsome [5–11].

HO is not a heme protein by nature, but it binds heme to form a 1:1 complex [8,10]. The absorption spectrum of the complex of ferric heme and HO is very similar to those of metmyoglobin and methemoglobin [8,10].

The heme degradation reaction catalyzed by the HO system is essentially very similar to that of heme decomposition by the so-called coupled oxidation of myoglobin or hemoglobin with ascorbic acid [12–15].

HO protein can now be prepared in large amounts by employing bacterial overexpression technology [16,17]. Moreover, the crystal structures of human [18] and rat [19] HOs, and also those in complex with heme [20–22] have been determined. These studies have led to important progress in clarifying the mechanisms of individual

☆ *Abbreviations:* HO, heme oxygenase; CO, carbon monoxide; NO, nitric oxide; HemO, HO of *Neisseria meningitidis*; HmuO, HO of *Corynebacterium diphtheriae*; PigA, HO of *Pseudomonas aeruginosa*.

* Corresponding author. Fax: +81 942 31 4377.
E-mail address: mnoguchi@med.kurume-u.ac.jp (G. Kikuchi, c/o M. Noguchi).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.08.020

steps of the HO reaction, although further investigation is needed to resolve some remaining controversy concerning the detailed mechanisms of some steps of the reaction [23–25].

An isozyme of HO, referred to as heme oxygenase-2 (HO-2) by Maines' group [26–28], has been identified. HO-2 is constitutively expressed in the brain, testis, and vascular systems, and the amino acid sequence of HO-2 shares 43% similarity with that of authentic HO, now designated as heme oxygenase-1 (HO-1).

HO has been suggested to function as a defense system against oxidative stress, since biliverdin or bilirubin produced locally in the body may act as physiological antioxidants [29–31]. In addition, CO is assumed to function, like nitric oxide (NO), as a physiological regulator of cGMP by activating guanylyl cyclase [32,33], although some questions still remain to be answered [34].

HO is widely distributed not only in vertebrates but also in invertebrates such as insects. HO has also been identified in higher plants [35,36], cyanobacteria [35,36], algae [35,36], fungi [37], and some pathogenic bacteria [38–40]; these HOs are soluble proteins, owing to the lack of a C-terminal hydrophobic region.

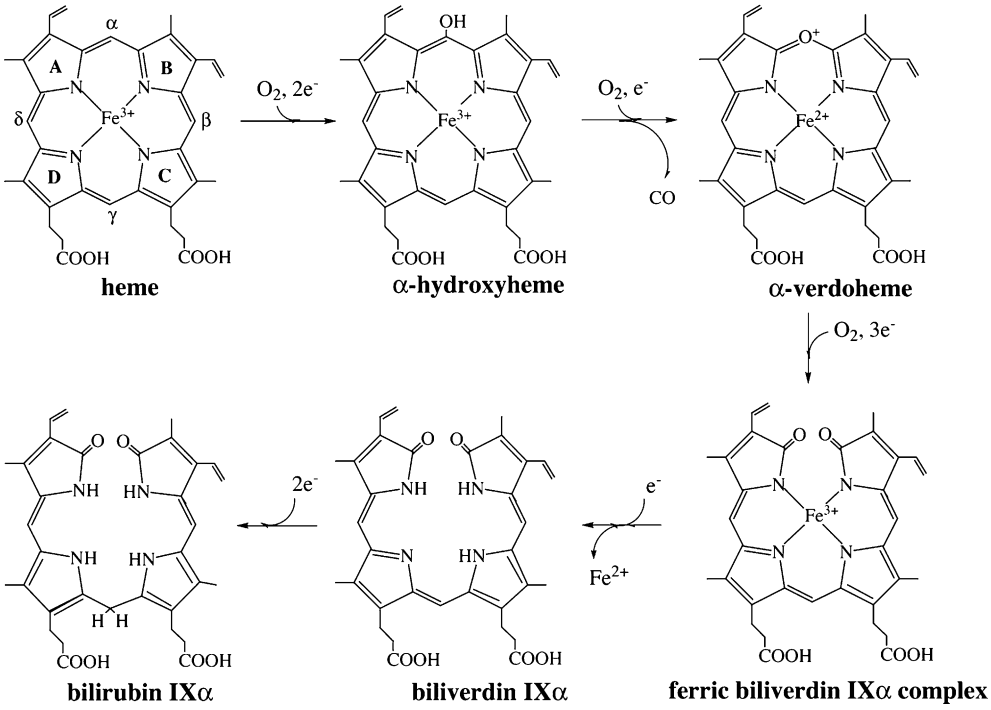
In the present article, we will describe the nature of HO and the heme degradation reaction catalyzed by HO, mainly in vertebrates. Some biological aspects of HO will also be described.

Heme degradation reaction catalyzed by HO

Properties of HO

HO is a simple protein that does not have any of the prosthetic groups necessary for O_2 activation [8–10]. Instead, O_2 activation is performed by the substrate heme and its two intermediates, α-*meso*-hydroxyheme and verdoheme (Scheme 1). HO binds substrate heme at the specific position of its pocket to form heme–enzyme complex. This complex behaves as a kind of heme protein, whose spectrophotometric properties closely resemble those of myoglobin [8,10], suggesting that histidine is a fifth ligand of heme–iron.

HO exists as two isoforms, a 33 kDa inducible HO-1 and a 36 kDa constitutive HO-2 [28], which has an extra N-terminal stretch of 20 amino acids compared to HO-1. Both enzymes have hydrophobic sequences at their C-terminal ends that are involved in binding to microsomal membrane. Interestingly, a trypsin-treated HO lacking the C-terminal hydrophobic region is fully active and is water-soluble [41]. As described later, the successful expression of these truncated HO-1 [16,17] and HO-2 [42] in *Escherichia coli* has greatly facilitated the characterization of HO and the understanding of the reaction mechanism by HO at the molecular level.



Scheme 1. Heme degradation pathway. Heme to biliverdin IXα is catalyzed by HO and biliverdin IXα to bilirubin IXα is catalyzed by biliverdin reductase. A, B, C, and D represent pyrrole rings A, B, C, and D of heme, respectively, and α, β, γ, and δ, α-, β-, γ-, and δ-*meso*-carbons of heme, respectively.

The ferric HO–heme complex is in a six-coordinate high spin state at neutral pH, while it converts to a low spin state at basic pH [43]. Resonance Raman study suggested that the sixth ligands of the high and the low spin forms are water and hydroxide, respectively.

Findings from various spectroscopic studies and a site-directed mutagenesis study indicate that in HO-1 a neutral imidazole of His-25 residue serves as the proximal ligand [16,43]. Most heme proteins participating in O_2 activation have imidazolate of histidine or thiolate of cystein as proximal ligands and a few have a neutral imidazole as the proximal ligand. In this sense, HO falls into a special class of heme proteins.

Mechanism of heme degradation by HO

The HO reaction starts with the formation of the ferric heme–HO complex and ferric heme–iron is then reduced to a ferrous state by the first electron donated from NADPH–cytochrome P450 reductase [9]. Next, molecular oxygen binds with the complex to form a metastable oxy-form [44]. The iron-bound oxygen converts to a hydroperoxide intermediate (Fe^{3+}–OOH), by receiving another electron from the reductase and a proton from the distal pocket water. Lastly, the terminal oxygen of Fe^{3+}–OOH attacks the α-*meso*-carbon of the porphyrin ring to form ferric α-*meso*-hydroxyheme [45].

Involvement of ferric-hydroperoxide species in the reaction sequence was supported by the fact that H_2O_2 but not *meta*-chloroperbenzoic acid leads to the degradation of heme to verdoheme [17]. These observations also suggested that the distal of the two oxygen atoms attacks the α-*meso* carbon of heme to yield α-*meso*-hydroxyheme. Therefore, this process is different from cytochrome P450-directed reactions, in which the O–O bond of the hydroperoxide is heterolytically cleaved to generate a ferryl-oxo intermediate (Fe^{4+}=O).

The resonance Raman spectra of the oxy-form of the ferrous heme–HO complex provided strong evidence that the Fe–O–O angle is highly bent to about 110° [46]. The terminal oxygen atom is located within a van der Waals contact with the α-*meso* carbon of the porphyrin ring, as evidenced by the EPR measurement of the oxy-form of the cobalt(II)–porphyrin–HO complex [47] and from X-ray analysis of the crystal structure of dioxygen-bound HmuO, a HO of *Corynebacterium diphtheriae* [48]. A recent study on HmuO, which has Asp-136 corresponding to Asp-140 of HO-1, provided evidence that the nearby water molecule located in the vicinity of the Fe^{3+}–OOH plays an important role in controlling the reactivity of Fe^{3+}–OOH [49] (see Mechanism of oxygen activation and its implication for catalysis).

At the stage where ferric α-*meso*-hydroxyheme is in complex with HO, the heme–iron exists in a five-coordinate high spin state and also as an oxophlorin resonance structure including a ferrous porphyrin π neutral radical [23]. This species of heme then reacts with molecular oxygen and yields the ferrous verdoheme–HO complex and CO. Mansfield Matera et al. [23] reported that this reaction consumed oxygen and one electron simultaneously, while other groups proposed different reaction mechanisms. Liu et al. [24] insisted that the oxygen molecule alone converts α-*meso*-hydroxyheme to ferric verdoheme and that one electron is used to reduce the ferric verdoheme to the ferrous species. In contrast, Sakamoto et al. [25] reported that conversion of α-*meso*-hydroxyheme to ferrous verdoheme requires only an oxygen molecule without an electron. At any rate, in this reaction, molecular oxygen is thought to react directly on the porphyrin macrocycle rather than on heme–iron. This proposal is supported by the fact that this step is not inhibited by CO and that the other three isomers of *meso*-hydroxyheme are effectively and quantitatively converted to their corresponding isomers of verdoheme [50].

The ferrous form of the verdoheme–HO complex has unique properties as compared to other heme proteins. The ferrous complex is in a six-coordinate high spin state with a proximal imidazole ligand on the protein and a possible axial hydroxide ligand [51]. It is inferred that the positive charge of the verdoheme ring yields the partial ferric character in the ferrous verdoheme–HO complex. In fact, the ferrous verdoheme–HO complex binds typical ferric heme ligands, such as cyanide and azide [51,52], in addition to the CO that binds to ferrous heme iron.

The mechanism of the verdoheme degradation to ferric biliverdin is not well understood. Conversion of ferrous verdoheme to ferric biliverdin–iron chelate requires both oxygen and reducing equivalents [17,52]. Although there is no evidence for the presence of an oxy-verdoheme–HO complex, we infer that the oxygen molecule likely binds to the iron of ferrous verdoheme and is activated by a mechanism similar to that in the first step. This hypothesis is supported by the fact that only verdoheme IXα is converted to biliverdin IXα, while the other three verdoheme isomers are not [50]. Then, the oxy-verdoheme–HO complex is converted to ferric iron-biliverdin chelate via hydroperoxide intermediate, which is still bound to HO protein. For the liberation of free iron and biliverdin to complete the total HO reaction, the iron of the ferric biliverdin is further reduced to the ferrous state by the reductase [9]. In the in vitro reaction, the release of biliverdin from the enzyme is the rate-limiting step [53].

It is noteworthy that the overall reaction of HO is not severely inhibited by CO. In the first step of the reaction, the affinity of ferrous iron of heme for molecular oxygen is extremely high, thirty times higher than that of myoglobin, whereas the CO-affinity is almost the same as that of myoglobin [54]. This is the main reason that CO failed to inhibit the first step of the reaction. The detailed molecular mechanism of the evasion of CO inhibition at this

step will be discussed under Mechanisms of the discrimination between CO and O_2, and the evasion of CO inhibition.

As for the second step, ferrous iron of *meso*-hydroxyheme does not participate in O_2 activation, as already mentioned. In the third step, CO binding to the verdoheme–HO complex is extremely weak and the CO dissociation constant is very large, so that CO has no influence at all on the conversion of verdoheme into ferric biliverdin [54].

Regioselectivity of the HO reaction

All HOs so far reported, except PigA of *Pseudomonas aeurginosa* [40] and a HO from *Drosophila melanogaster* [55], regiospecifically oxidize the α-*meso*-carbon of the heme, resulting in the formation of biliverdin IXα. Coupled oxidation of hemoglobin with ascorbic acid gives a mixture of α- and β-isomers, and coupled oxidation of pyridine–hemin with ascorbic acid forms all four possible biliverdin isomers at nearly identical yields. These observations have suggested that the heme pocket structure of HO should determine the α-specificity of the HO reaction [56]. This proposal was confirmed by the recent X-ray analysis of the crystal structure of the heme–HO complexes, as will be described in detail under Structure of HO.

α-Specificity is also controlled by the interactions of propionate side chains of heme and basic amino acid residues of HO. In fact, the deletion of the interaction by the mutation of Arg-183 to Glu-183 in rat HO-1 [57] induced heme rotation in plane, leading to the formation of α- and δ-isomers of biliverdin IX. Similar observations were reported for mutants of HmuO in which Arg-177 (corresponding to Arg-183 of HO-1) was changed to Glu or Asp [58]. Recently, another example has been found in PigA, which does not produce biliverdin IXα at all but forms mixtures of β- and δ-isomers of biliverdin IX at a ratio of 3:7 [40]. The crystal structure of heme–PigA complex revealed that this unique regioselectivity is due to rotation of the heme by about 100° [59]. Interestingly, replacement of Lys-34 and/or Lys-132 with Ala increases the formation of α-isomer of biliverdin, suggesting that these basic amino acid residues play an essential role in holding the heme at the rotated position by means of hydrogen-bonding interactions [60]. These observations indicate that the regioselectivity of the HO reaction is primarily under steric control.

Structure of HO and molecular mechanism of the HO reaction

Structure of HO

Thus far, the structures of seven HOs have been determined by X ray crystal analysis. These include human [20] and rat [22] HO-1s of mammalian origin, three HOs of bacterial origin, HemO of *Neisseria meningitidis* [61], HmuO of *C. diphtheriae* [62], and PigA of *Pseudomonas aeruginosa* [59], and two HO isoforms of cyanobacterial origin, Syn HO-1 [63] and Syn HO-2 [64]. For crystallization of the mammalian HO-1 (~33 kDa), the truncated and soluble versions (~28 kDa) lacking the membrane-binding domain have been employed. On the other hand, bacterial HOs are slightly smaller (24–26 kDa) and are naturally soluble because they have no membrane-binding domain. Although the homology in amino acid sequence between mammalian and bacterial HOs is low, their crystal structures are quite similar.

The structure of rat HO-1–heme complex is shown in Fig. 1A; rat HO-1 protein consists mainly of α-helices, and the heme is sandwiched between two helices; the proximal helix (helix A) includes His-25 as the proximal ligand to the heme–iron, and the distal helix (helix F) bending at Leu-141 and Ser-142 lies above the β-, γ-, and δ-*meso* carbon atoms of the porphyrin macrocycle. These structural constraints brought about by helix F direct the α-regiospecificity of heme degradation by HO-1. Half of the heme molecule is exposed to the solvent, and hydroxide at basic pH or water at neutral pH is present as the distal ligand.

The inner segment in the F helix (Pro-126-Lys-149 in rat HO-1), called the heme oxygenase signature, is highly conserved among HOs from various sources and has been assumed to play an important role in HO activity. Interestingly, amino acids with a dissociable side chain, which are often seen at the distal side in common heme enzymes, do not exist in mammalian HO-1s. Instead, in rat HO-1, a Gly-139 carbonyl group and a Gly-143 amide group, which are localized at the kinking point of the F helix, are close enough to hydrogen-bond to the proximal hydroxide or water ligand; the distances between them are 2.9 and 2.6 Å, respectively. With human HO-1, there are two forms: the closed form in which the distal helix is draped across the heme, and the open form in which the distal helix is further away from heme compared to the closed form. Thus, it has been pointed out that the distal helix is able to assume flexible conformations [20].

With rat HO-1, the propionate groups of heme electrostatically interact with Lys-18, Lys-179, and Arg-183, and these interactions determine the direction of heme within the heme pocket. Exceptionally PigA shows β- and δ-regiospecificity, instead of α-regiospecificity as described under Regioselectivity of the HO reaction.

For human [18] and rat [19] HO-1s, the crystal structures of heme-free forms (apoHO) have been determined. A comparison of the overall structure of rat apoHO-1 with that of rat heme–HO-1 complex (holo form) shows that the helices that form heme pocket

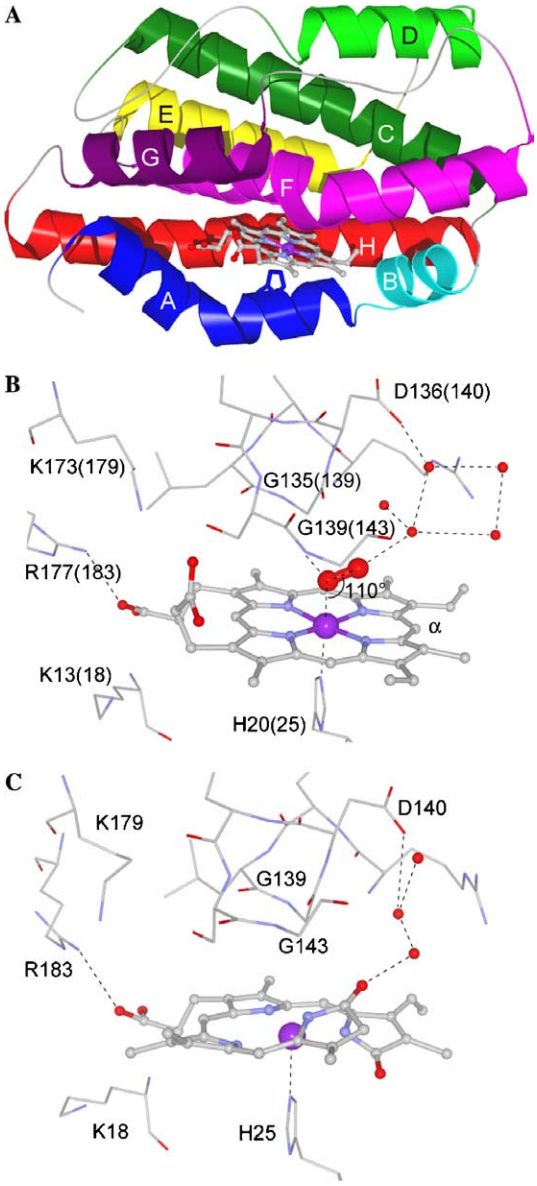


Fig. 1. The whole HO structure and the heme pocket structure. (A) Rat HO-1–heme complex (PDB code: 1DVE). (B) The oxy form of HmuO–heme complex (1V8X). In parentheses are presented the corresponding human and rat HO-1 amino acid residue numbers. (C) Rat HO-1–biliverdin–iron chelate complex (1J2C). Dotted lines stand for hydrogen bonds or coordination bonds. (Reproduced by permission of *Seikagaku*.)

(i.e., A, B, and F helices) fluctuate or are disordered. However, the rest of the structure in the apo state is similar to that of heme–HO-1 and the side chain structures of Lys-179 and Arg-183 are preserved. From these findings, we have proposed an induced fit model for heme binding to rat apoHO-1; at first, the binding starts by electrostatic interactions of propionate groups with basic residues such as Lys-179 and Arg-183, then the orientation of the proximal helix and the position of His-25 are fixed to form a heme pocket.

Mechanism of oxygen activation and its implication for catalysis

A distinct feature of the HO reaction is that hydroperoxide species (Fe^{3+}–OOH), instead of ferryl-oxo species (Fe^{4+}=O) as seen in the cytochrome P450 reaction, is the activated oxygen in the first hydroxylation reaction of α-*meso* carbon, leading to the formation of α-hydroxyheme, through which the electrophilic attack on the α-*meso* carbon by the terminal oxygen atom of Fe^{3+}–OOH takes place.

A relatively stable Fe^{3+}–OOH species is generated in the HO reaction, while heterolytic cleavage of the O–O bond easily occurs in such heme enzymes as peroxidases. To search for the structural factors behind this, we analyzed the structures of the azide-bound form (N_3^-–heme–HO-1) [65] and the nitric oxide-bound form (NO–heme–HO-1) [66], and clarified that (1) several water molecules within the heme pocket form a hydrogen-bonding network involving Asp-140 that is a critical residue for HO activity and its neighboring residues, (2) the ligands (N_3^- or NO) are accommodated in a relatively narrow space between heme and the distal helix, and are directed toward the α-*meso* carbon, and (3) there are hydrophilic interactions of the ligands with the water molecules and the surrounding residues.

According to the crystal structure of the oxy form (Fig. 1B) of heme–HmuO complex, which Unno et al. [48] reported recently, an oxygen molecule binds heme–iron in an end-on fashion (with an Fe–O–O angle of 110°) and is pointed in the direction of the α-carbon. The closest residues to the heme are Gly-135 and Gly-139 (corresponding to Gly-139 and Gly-143 of rat HO-1, respectively) and interactions of dioxygen with the water molecules and the surrounding residues are also observed, as seen in N_3^-– and NO–heme–HO-1. Thus, the supply of proton from the hydrogen-bonding network should be important for the stable formation of Fe^{3+}–OOH. In HmuO, this hydrogen-bonding network is connected to the solvent through several amino acid residues so the proton can be supplied from the outside.

The next question is what kind of structural factors are responsible for the oxidative cleavage of the porphyrin ring after α-*meso* carbon hydroxylation. We have succeeded in determining the structure of biliverdin–iron chelate–HO-1 complex (Fig. 1C) [67], the direct precursor of biliverdin (Scheme 1), and this may provide an answer to the question, though indirectly.

Biliverdin–iron chelate is located at the position originally occupied by heme in heme–HO-1 complex, the electrostatic interactions with the protein are maintained, and the water molecules constituting the hydrogen-bonding network in the pocket are also conserved, though partially. The α-*meso* bridge has been split and the pyrrole rings, A and B, have turned to the up and down directions, respectively, so that a collision of the

lactam oxygens is avoided. The water on the heme–iron is lost, and the iron assumes a five-coordinate distorted pyramidal structure. Consequently, the iron can easily dissociate from heme. Because the side chains of biliverdin–iron chelate are exposed to the solvent, their electron densities are not observed, indicating that its binding to HO-1 would be unstable compared to heme. The chelate has been slightly relocalized outside, and the heme pocket is expanded. This seems to facilitate the release of biliverdin from HO. That the distance between the lactam oxygen of pyrrole A and a water molecule is about 2.6 Å, a hydrogen-bond forming distance, raises a possibility that the hydrogen-bonding network might also participate in the ring opening of verdoheme.

Mechanisms of the discrimination between CO and O_2, and the evasion of CO inhibition

In HO, O_2 bound to the heme–iron is stabilized by the hydrogen-bonding network and by polar interactions with the surrounding amino acids. This also coincides with the fact that the O_2 dissociation rate is very slow.

This being the case, how is the affinity of CO suppressed? We analyzed the structure of a CO-bound form of rat HO-1–heme complex (Fig. 2A) and discovered that the heme accompanying the CO ligand is shifted 1 Å toward the α-*meso* carbon along the α–γ axis. Further, the distal helix F is shifted in the opposite direction. Such a shift must occur in order to avoid the steric collision between CO and the distal helix, because CO binds to heme in a tilted fashion (with an Fe–C–O angle of 158°) (cf. O_2 and NO bind to heme in a bent fashion). Consequently, the interactions of CO with the surrounding residues become weak and a part of the electrostatic binding of propionate groups and basic residues is also lost. Further, the surrounding hydrophilic environment should be disadvantageous for a hydrophobic CO molecule. Thus O_2 binding in HO is clearly distinguished from that of CO.

In order to investigate the avoidance mechanism of CO inhibition, we collected X-ray diffraction data from a crystal of the CO-bound form of ferrous heme–HO-1 complex in the dark and under illumination by a red laser at ~35 K [68]. On the difference Fourier map, photodissociated CO electron densities appeared at two sites (Fig. 2B). Site-1 was above the porphyrin ring; this CO trapping site was also observed in a similar experiment using myoglobin. Site-2 was a hydrophobic space about 10 Å distant from heme–iron and surrounded by such hydrophobic residues as Leu, Phe, and Met. In a control study employing Xe, a hydrophobic gas, we revealed that Xe was also trapped at site-2. Taking into consideration that the affinity of verdoheme for CO is remarkably low compared to that for heme, CO molecules generated in the HO reaction will be temporarily trapped at site-2 via site-1 and will then be released from HO after the generation of biliverdin.

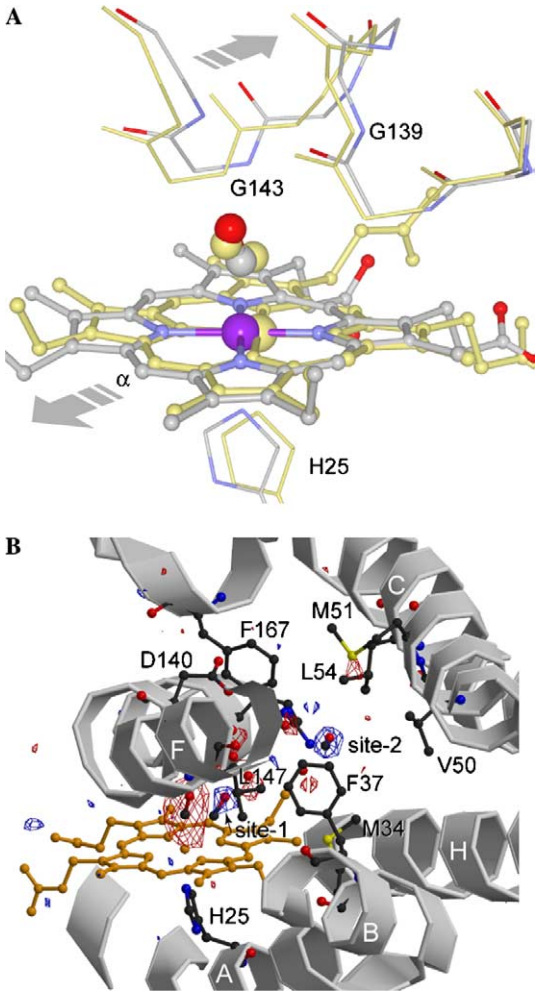


Fig. 2. Differences in binding by ligands and CO trapping sites in HO-1. (A) Rat NO–heme–HO-1 complex (yellow, 1J02) and CO–heme–HO-1 complex (carbon, gray; oxygen, red; nitrogen, blue; and iron, purple (1IX4)). (B) CO–heme–HO-1 complex that partially dissociates CO after laser irradiation (1ULX). The electron densities that increased or decreased after photolysis are indicated in blue and red, respectively. (Reproduced by permission of *Seikagaku*.)

Interaction between heme oxygenase and cytochrome P450 reductase

HO and NADPH–cytochrome P450 reductase need to associate with each other for transfer of the electrons required for heme degradation. Moreover, since the release of biliverdin from HO is promoted by biliverdin reductase [53], HO must react directly with biliverdin reductase to deliver biliverdin. That is, for the heme degradation system consisting of NADPH–cytochrome P450 reductase/HO/biliverdin reductase, there should be an elaborate mechanism whereby the three enzymes associate and dissociate in an orderly fashion.

The molecular surface around the heme binding site of human and rat HO-1s is positively charged, whereas the surface of NADPH–cytochrome P450 reductase is negatively charged. Recently, Wang et al. [69] showed, using fluorescence energy transmission, that NADPH–cytochrome P450 reductase and biliverdin reductase combine competitively with human HO-1, and demonstrated, by the amino acid mutation, that some common basic residues near the heme binding site of human HO-1 interact with NADPH–cytochrome P450 reductase and biliverdin reductase; binding sites of NADPH–cytochrome P450 reductase and biliverdin reductase to human HO-1 are mostly overlapped.

We also examined the interaction of rat HO-1 and NADPH–cytochrome P450 reductase by surface plasmon resonance technique [70] and found that combination of HO-1 and NADPH–cytochrome P450 reductase is reinforced about five times by addition of NADP(H). Since the HO-1 affinity for NADPH–cytochrome P450 reductase increases by NADP(H) addition even in the presence of biliverdin reductase, it seems likely that NADPH–cytochrome P450 reductase combines with substrate NADP(H) to achieve a conformation with higher affinity to HO-1

However, NADPH–cytochrome P450 reductase does not combine with apo HO-1 whose heme pocket has collapsed, irrespective of the presence of NADP(H). This implies that there should be a molecular recognition mechanism through which HO-1 and NADPH–cytochrome P450 reductase, guided by binding of the respective substrates, will associate together after recognizing these structural changes, and then carry out the electron transfer. Lys-149 and Arg-185 of rat HO-1 are found to be important in combination with NADPH–cytochrome P450 reductase from the site-specific mutation and the docking model analysis. It was suggested that Lys-149 and Arg-185 interact with the acidic cluster which resides at the FMN binding site of NADPH–cytochrome P450 reductase and the 2′-phosphate group of NADP(H), respectively. In this model, it is expected that the electrons from FMN are transferred from the distal side of the heme pocket to heme. In P450s, electrons are supplied from the proximal side. This difference between HO and P450 may be the prime cause of HO's peculiar O_2 activation mechanism.

Biological aspects of HO

The activity of HO-1 in liver and other organs is markedly increased by the administration of hemin or hemoglobin to animals [71–73]. Induction of HO-1 is now shown to be due to de-repression of the HO-1 gene by heme through direct binding to a translational repressor, Bach1 [74]. HO-1 in rat liver is also increased by a number of non-heme substances such as endotoxin, bromobenzene, hormones, and certain metal ions [75]. Cadmium ion was shown to induce heme oxygenase also by way of the Bach1 mechanism [76].

Expression of HO is assumed to act in a cytoprotective manner in a variety of cell types. The beneficial effects of the induction seem to be due to the increased production of biliverdin and bilirubin, which are potent antioxidants. It was shown that oxidative stress caused enhanced endothelial cell injury in a patient with HO-1 deficiency [77]. HO-related mechanisms seem to play important roles in several aspects of different diseases [78–80].

In reptiles, fishes, insects, and birds, biliverdin is used directly for pigmentation (cf. [36]).

The primary function of plant HO is to form biliverdin IXα as a starting material for the synthesis of phytobilin, an open-chain tetrapyrrole compound that acts as a sensor for red and far-red light [35,36]. Cyanobacteria do not possess chloroplasts but have phycobilisomes as machinery for photosynthesis.

Some pathogenic bacteria use iron both as an essential nutrient and for the production of proteinaceous poisons [81]. As the free iron content in their host is very limited, they developed HO, by which they acquire iron from the heme of the host hemoglobin.

Insects do not use hemoprotein as a transport vehicle or as a storage vessel for oxygen. However, they possess a variety of heme proteins such as cytochromes in the respiratory chain and cytochrome P450, and to degrade heme *Drosophila melanogaster* has a HO. This HO has a C-terminal hydrophobic domain, and a truncated form lacking this region, tentatively named DmΔHO, catalyzes heme degradation to biliverdin, CO, and iron in the presence of NADPH–cytochrome P450 reductase [55].

Despite these similarities, DmΔHO is distinctly different from other HOs, namely, the heme catabolism in DmΔHO is not α-specific and yields three isomers of biliverdin: biliverdin IXα, Xβ, and IXδ [55]. The structure and hydrogen-bonding of the DmHO active site must be quite different from those of other HOs.

Recently, a third type of heme oxygenase (HO-3) was reported in the rat brain [82]. HO-3 shares 90% homology with HO-2. However, no functional HO-3 gene could be found in rats; the HO-3 gene may be processed genes derived from HO-2 transcripts [83].

Acknowledgments

We thank Drs. M. Ikeda-Saito, D.L. Rousseau, S. Takahashi, M. Sato, H, Fujii, Z. Hong, C.T. Migita, J.S. Olson, B.M. Hoffman, X. Zhang, S. Shibahara, K. Fukuyama, G. Palmer, H. Sakamoto, M. Sugishima, and Y. Higashimoto for their collaboration in this study. This work was supported in part by Grants-in-

Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, and by a grant for the Scientific Promotion Fund from the Promotion and Mutual Corporation for Private Schools of Japan.

References

[1] R. Tenhunen, H.S. Marver, R. Schmid, The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase, Proc. Natl. Acad. Sci. USA 61 (1968) 748–755.
[2] R. Tenhunen, H.S. Marver, R. Schmid, Microsomal heme oxygenase. Characterization of the enzyme, J. Biol. Chem. 244 (1969) 6388–6394.
[3] B.A. Schacter, E.B. Nelson, H.S. Marver, B.S.S. Masters, Immunochemical evidence for an association of heme oxygenase with the microsomal electron transport system, J. Biol. Chem. 247 (1972) 3601–3607.
[4] R. Tenhunen, H.S. Marver, N.R. Pimstone, W.F. Trager, D.Y. Cooper, R. Schmid, Enzymatic degradation of heme. Oxygenative cleavage requiring cytochrome P-450, Biochemistry 11 (1972) 1716–1720.
[5] T. Yoshida, S. Takahashi, G. Kikuchi, Partial purification and reconstitution of the heme oxygenase system from pig spleen microsomes, J. Biochem. (Tokyo) 75 (1974) 1187–1191.
[6] T. Yoshida, G. Kikuchi, Sequence of the reaction of heme catabolism catalyzed by the microsomal heme oxygenase system, FEBS Lett. 48 (1974) 256–261.
[7] G. Kikuchi, T. Yoshida, Heme catabolism by the reconstituted heme oxygenase system, Ann. Clin. Res. 8 (1976) 10–17.
[8] T. Yoshida, G. Kikuchi, Purification and properties of heme oxygenase from pig spleen microsomes, J. Biol. Chem. 253 (1978) 4224–4229.
[9] T. Yoshida, G. Kikuchi, Features of the reaction of heme degradation catalyzed by the reconstituted microsomal heme oxygenase system, J. Biol. Chem. 253 (1978) 4230–4236.
[10] T. Yoshida, G. Kikuchi, Purification and properties of heme oxygenase from rat liver microsomes, J. Biol. Chem. 254 (1979) 4487–4491.
[11] G. Kikuchi, T. Yeshida, Heme degradation by the microsomal heme oxygenase system, Trends Biochem. Sci. 5 (1980) 323–325.
[12] R. Lemberg, The chemical mechanism of bile pigment formation, Rev. Pure Appl. Chem. 6 (1956) 1–23.
[13] R. Lemberg, J.W. Legge, Hematin Compounds and Bile Pigments, Interscience Publishers, New York, 1949, 453–501.
[14] K. Kajiro, G. Kikuchi, C. Hanaoka, Verdohemoglobin, an immediate precursor of biliverdin in the model reaction of hemoglobin breakdown in vitro, J. Biochem. (Tokyo) 42 (1955) 423–437.
[15] T. Yoshida, G. Kikuchi, Sequence of heme decomposition by the coupled oxidation of myoglobin with ascorbic acid, Tohoku J. Exp. Med. 115 (1975) 67–74.
[16] K. Ishikawa, M. Sato, M. Ito, T. Yoshida, Importance of histidine residue 25 of rat heme oxygenase for its catalytic activity, Biochem. Biophys. Res. Commun. 182 (1992) 981–986.
[17] A. Wilks, P.R. Ortiz deMontellano, Rat liver heme oxygenase. High level expression of a truncated soluble form and nature of the *meso*-hydroxylating species, J. Biol. Chem. 268 (1993) 22357–22362.
[18] L. Lad, D.J. Schuller, H. Shimizu, J. Friedman, H. Li, P.R. Ortiz de Montellano, T.L. Poulos, Comparison of the heme-free and -bound crystal structures of human heme oxygenase-1, J. Biol. Chem. 278 (2003) 7834–7843.
[19] M. Sugishima, H. Sakamoto, Y. Kakuta, Y. Omata, S. Hayashi, M. Noguchi, K. Fukuyama, Crystal structure of rat apo-heme oxygenase (HO-1): mechanism of heme binding in HO-1 inferred from structural comparison of the apo and heme complex form, Biochemistry 41 (2002) 7293–7300.
[20] D.J. Schuller, A. Wilks, P.R. Ortiz de Montellano, T.L. Poulos, Crystal structure of human heme oxygenase-1, Nat. Struct. Biol. 6 (1999) 860–867.
[21] Y. Omata, S. Asada, H. Sakamoto, K. Fukuyama, M. Noguchi, Crystallization and preliminary X-ray diffraction studies on the water soluble form of rat heme oxygenase-1 in complex with heme, Acta Cryst. D 54 (1998) 1017–1019.
[22] M. Sugishima, Y. Omata, Y. Kakuta, H. Sakamoto, M. Noguchi, K. Fukuyama, Crystal structure of rat heme oxygenase-1 in complex with heme, FEBS Lett. 471 (2000) 61–66.
[23] K. Mansfield Matera, S. Takahashi, H. Fujii, H. Zhou, K. Ishikawa, T. Yoshimura, D.L. Rousseau, T. Yoshida, M. Ikeda-Saito, Oxygen and one reducing equivalent are both required for the conversion of α-hydroxyhemin to verdoheme in heme oxygenase, J. Biol. Chem. 271 (1996) 6618–6624.
[24] Y. Liu, P. Moënne-Lcccoz, T.M. Loehr, P.R. Ortiz de Montellano, Heme oxygenase-1, intermediates in verdoheme formation and the requirement for reduction equivalents, J. Biol. Chem. 272 (1997) 6909–6917.
[25] H. Sakamoto, Y. Omata, G. Palmer, M. Noguchi, Ferric α-hydroxyheme bound to heme oxygenase can be converted to verdoheme by dioxygen in the absence of added reducing equivalents, J. Biol. Chem. 274 (1999) 18196–18200.
[26] M.D. Maines, G.M. Trakshel, R.K. Kutty, Characterization of two constitutive forms of rat liver microsomal heme oxygenase. Only one molecular species of the enzyme is inducible, J. Biol. Chem. 261 (1986) 411–419.
[27] G.M. Trakshel, R.K. Kutty, M.D. Maines, Purification and characterization of the major constitutive form of testicular heme oxygenase. The noninducible isoform, J. Biol. Chem. 261 (1986) 1131–1137.
[28] M.D. Maines, Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications, FASEB J. 2 (1988) 2557–2568.
[29] R. Stocker, A.N. Glazer, B.N. Ames, Antioxidant activity of albumin-bound bilirubin, Proc. Natl. Acad. Sci. USA 84 (1987) 5918–5922.
[30] R. Stocker, Y. Yamamoto, A.F. McDonagh, A.N. Glazer, B.N. Ames, Bilirubin is an antioxidant of possible physiological importance, Science 235 (1987) 1043–1046.
[31] D.E. Baranano, M. Rao, C.D. Ferris, S.H. Snyder, Biliverdin reductase: a major physiological cytoprotectant, Proc. Natl. Acad. Sci. USA 99 (2002) 16093–16098.
[32] D.E. Baranano, S.H. Snyder, Neural roles for heme oxygenase: contrasts to nitric oxide synthase, Proc. Natl. Acad. Sci. USA 98 (2001) 10996–11002.
[33] M.D. Maines, The heme oxygenase system: a regulator of second messenger gases, Annu. Rev. Pharmocol. Toxicol. 37 (1997) 517–554.
[34] S.P.L. Cary, M.A. Marletta, The case of CO signaling: why the jury is still out, J. Clin. Invest. 107 (2001) 1071–1073.
[35] S.I. Beale, Biosynthesis of phycobilins, Chem. Rev. 93 (1993) 785–802.
[36] M.J. Terry, P.J. Linley, T. Kohchi, Making light of it: the role of plant haem oxygenases in phytochrome chromophore synthesis, Biochem. Soc. Trans. 30 (2002) 604–609.
[37] M.L. Pendrak, M.P. Chao, S.S. Yan, D.D. Roberts, Heme oxygenase in *Candida albicans* is regulated by hemoglobin and is necessary for metabolism of exogenous heme and hemoglobin to α-biliverdin, J. Biol. Chem. 279 (2004) 3426–3433.
[38] A. Wilks, M.P. Schmitt, Expression and characterization of a heme oxygenase (Hmu O) from *Corynebacterium diphtheriae*. Iron

acquisition requires oxidative cleavage of the heme macrocycle, J. Biol. Chem. 273 (1998) 837–841.
[39] W. Zhu, A. Wilks, I. Stojiljkovic, Degradation of heme in gram-negative bacteria: the product of the *hemO* gene of Neisseriae is a heme oxygenase, J. Bacteriol. 182 (2000) 6783–6790.
[40] M. Ratliff, W. Zhu, R. Deshmukh, A. Wilks, I. Stojiljkovic, Homologues of Neisserial heme oxygenase in gram-negative bacteria: degradation of heme by the product of the *pigA* gene of *Pseudomonas aeruginosa*, J. Bacteriol. 183 (2001) 6394–6403.
[41] T. Yoshida, K. Ishikawa, M. Sato, Degradation of heme by a soluble peptide of heme oxygenase obtained from rat liver microsomes by mild trypsinization, Eur. J. Biochem. 199 (1991) 729–733.
[42] K. Ishikawa, K. Mansfield Matera, H. Zhou, H. Fujii, M. Sato, T. Yoshimura, M. Ikeda-Saito, T. Yoshida, Identification of histidine 45 as the axial heme–iron ligand of heme oxygenase-2, J. Biol. Chem. 273 (1998) 4317–4322.
[43] S. Takahashi, J. Wang, D.L. Rousseau, K. Ishikawa, T. Yoshida, J.R. Host, M. Ikeda-Saito, Heme-heme oxygenase complex. Structure of the catalytic site and its implication for oxygen activation, J. Biol. Chem. 269 (1994) 1010–1014.
[44] T. Yoshida, M. Noguchi, G. Kikuchi, Oxygenated form of heme. Heme oxygenase complex and requirement for second electron to initiate heme degradation from the oxygenated complex, J. Biol. Chem. 255 (1980) 4418–4420.
[45] R.M. Davydov, T. Yoshida, M. Ikeda-Saito, B.M. Hoffman, Hydroperoxy-heme oxygenase generated by cryoreduction catalyzes the formation of α-*meso*-hydroxyheme as detected by EPR and ENDOR, J. Am. Chem. Soc. 121 (1999) 10656–10657.
[46] S. Takahashi, K. Ishikawa, N. Takeuchi, M. Ikeda-Saito, T. Yoshida, D.L. Rousseau, Oxygen-bound heme-heme oxygenase complex: evidence for a highly bent structure of the coordinate oxygen, J. Am. Chem. Soc. 117 (1995) 6002–6006.
[47] H. Fujii, Y. Dou, H. Zhou, T. Yoshida, M. Ikeda-Saito, Cobalt porphyrin heme oxygenase complexes. EPR evidences for the distal heme pocket hydrogen bonding, J. Am. Chem. Soc. 120 (1998) 8251–8252.
[48] M. Unno, T. Matsui, G.C. Chu, M. Couture, T. Yoshida, D.L. Rousseau, J.S. Olson, M. Ikeda-Saito, Crystal structure of the dioxygen-bound heme oxygenase from *Corynebacterium diphtheriae*: implications for heme oxygenase function, J. Biol. Chem. 279 (2004) 21055–21061.
[49] T. Matsui, M. Furukawa, M. Unno, T. Tomita, M. Ikeda-Saito, Roles of distal Asp in heme oxygenase from *Corynebacterium diphtheriae*, HmuO: a water-driven oxygen activation mechanism, J. Biol. Chem. 280 (2005) 2981–2989.
[50] X. Zhang, H. Fujii, K. Mansfield Matera, C.T. Migita, D. Sun, M. Sato, M. Ikeda-Saito, T. Yoshida, Stereoselectivity of each of the three steps of the heme oxygenase reaction: hemin to *meso*-hydroxyhemin, *meso*-hydroxyhemin to verdoheme, and verdoheme to biliverdin, Biochemistry 42 (2003) 7418–7426.
[51] S. Takahashi, K. Mansfield Matera, H. Fujii, H. Zhou, K. Ishikawa, T. Yoshida, M. Ikeda-Saito, D.L. Rousseau, Resonance Raman spectroscopic characterization of α-hydroxyheme and verdoheme complexes of heme oxygenase, Biochemistry 36 (1997) 1402–1410.
[52] T. Yoshida, M. Noguchi, Features of intermediary steps around the688-nm substance in the heme oxygenase reaction, J. Biochem. (Tokyo) 96 (1984) 563–570.
[53] Y. Liu, P.R. Ortiz de Montellano, Reaction intermediates and single turnover rate constants for the oxidation of heme by human heme oxygenase-1, J. Biol. Chem. 275 (2000) 5297–5307.
[54] C.T. Migita, K. Mansfield Matera, M. Ikeda-Saito, J.S. Olson, H. Fujii, T. Yoshimura, H. Zhou, T. Yoshida, The oxygen and carbon monoxide reaction of heme oxygenase, J. Biol. Chem. 273 (1998) 945–949.
[55] X. Zhang, M. Sato, M. Sasahara, C.T. Migita, T. Yoshida, Unique features of recombinant heme oxygenase of *Drosophila melanogaster* compared with those of other heme oxygenases studied, Eur. J. Biochem. 271 (2004) 1713–1724.
[56] S.B. Brown, A.A. Chabot, E.A. Enderby, A.C.T. North, Orientation of oxygen in oxyhaemoproteins and its implications for haem catabolism, Nature 289 (1981) 93–95.
[57] H. Zhou, C.T. Migita, M. Sato, D. Sun, X. Zhang, M. Ikeda-Saito, H. Fujii, T. Yoshida, Participation of carboxylate amino acid side chain in regiospecific oxidation of heme by heme oxygenase, J. Am. Chem. Soc. 122 (2000) 8311–8312.
[58] Y. Zeng, R. Deshmukh, G.A. Caignan, R.A. Bunce, M. Rivera, A. Wilks, Mixed regioselectivity in the Arg-177 mutants of *Corynebacterium diphtheriae* heme oxygenase as a consequence of in-plane heme disorder, Biochemistry 43 (2004) 5222–5238.
[59] J. Friedman, L. Lad, H. Li, A. Wilks, T.L. Poulos, Structural basis for novel δ-regioselective heme oxygenation in the opportunistic pathogen *Pseudomonas aeruginosa*, Biochemistry 43 (2004) 5239–5245.
[60] H. Fujii, X. Zhang, T. Yoshida, Essential amino acid residues controlling the unique regioselectivity of heme oxygenase in *Pseudomonas aeruginosa*, J. Am. Chem. Soc. 126 (2004) 4466–4467.
[61] D.J. Schuller, W. Zhu, I. Stojiljkovic, A. Wilks, T.L. Poulos, Crystal structure of heme oxygenase from the gram-negative pathogen *Neisseria meningitidis* and a comparison with mammalian heme oxygenase-1, Biochemistry 40 (2001) 11552–11558.
[62] S. Hirotsu, G.C. Chu, M. Unno, D.S. Lee, T. Yoshida, S.Y. Park, Y. Shiro, M. Ikeda-Saito, The crystal structures of the ferric and ferrous forms of the heme complex of HmuO, a heme oxygenase of *Corynebacterium diphtheriae*, J. Biol. Chem. 279 (2004) 11937–11947.
[63] M. Sugishima, C.T. Migita, X. Zhang, T. Yoshida, K. Fukuyama, Crystal structure of heme oxygenase-1 from cyanobacterium *Synechocystis* sp. PCC 6803 in complex with heme, Eur. J. Biochem. 271 (2004) 4517–4525.
[64] M. Sugishima, Y. Hagiwara, X. Zhang, T. Yoshida, C.T. Migita, K. Fukuyama, Crystal structure of dimeric heme oxygenase-2 from *synechocystis* sp. PCC 6803 in complex with heme, Biochemistry 44 (2005) 4257–4266.
[65] M. Sugishima, H. Sakamoto, Y. Higashimoto, Y. Omata, S. Hayashi, M. Noguchi, K. Fukuyama, Crystal structure of rat heme oxygenase-1 in complex with heme bound to azide. Implication for regiospecific hydroxylation of heme at the α-*meso*carbon, J. Biol. Chem. 277 (2002) 45086–45090.
[66] M. Sugishima, H. Sakamoto, M. Noguchi, K. Fukuyama, Crystal structures of ferrous and CO-, CN^--, and NO-bound forms of rat heme oxygenase-1 (HO-1) in complex with heme: structural implications for discrimination between CO and O_2 in HO-1, Biochemistry 42 (2003) 9898–9905.
[67] M. Sugishima, H. Sakamoto, Y. Higashimoto, M. Noguchi, K. Fukuyama, Crystal structure of rat heme oxygenase-1 in complex with biliverdin–iron chelate. Conformational change of the distal helix during the heme cleavage reaction, J. Biol. Chem. 278 (2003) 32352–32358.
[68] M. Sugishima, H. Sakamoto, M. Noguchi, K. Fukuyama, CO-trapping site in heme oxygenase revealed by photolysis of its CO-bound heme complex: mechanism of escaping from product inhibition, J. Mol. Biol. 341 (2004) 7–13.
[69] J. Wang, P.R. Ortiz de Montellano, The binding sites on human heme oxygenase-1 for cytochrome p450 reductase and biliverdin reductase, J. Biol. Chem. 278 (2003) 20069–20076.
[70] Y. Higashimoto, H. Sakamoto, S. Hayashi, M. Sugishima, K. Fukuyama, G. Palmer, M. Noguchi, Involvement of NADP(H) in the interaction between heme oxygenase-1 and cytochrome P450 reductase, J. Biol. Chem. 280 (2005) 729–737.

[71] R. Tenhunen, H.S. Marver, R. Schmid, The enzymatic catabolism of hemoglobin: stimulation of microsomal heme oxygenase by hemin, J. Lab. Clin. Med. 75 (1970) 410–421.
[72] S. Shibahara, T. Yoshida, G. Kikuchi, Induction of heme oxygenase by hemin in cultured pig alveolar macrophages, Arch. Biochem. Biophys. 188 (1978) 243–250.
[73] S. Shibahara, T. Yoshida, G. Kikuchi, Mechanism of increase of heme oxygenase activity induced by hemin in cultured pig alveolar macrophages, Arch. Biochem. Biophys. 197 (1979) 607–617.
[74] K. Ogawa, J. Sun, S. Taketani, O. Nakajima, C. Nishitani, S. Sassa, N. Hayashi, M. Yamamoto, S. Shibahara, H. Fujita, K. Igarashi, Heme mediates derepression of Maf recognition element through direct binding to transcription repressor Bach1, EMBO J. 20 (2001) 2835–2843.
[75] G. Kikuchi, T. Yoshida, Function and induction of the microsomal heme oxygenase, Mol. Cell. Biol. 53/54 (1983) 163–183.
[76] H. Suzuki, S. Tashiro, J. Sun, H. Doi, S. Satomi, K. Igarashi, Cadmium induces nuclear export of Bach1, a transcriptional repressor of heme oxygenase-1 gene, J. Biol. Chem. 278 (2003) 49246–49253.
[77] A. Yachie, Y. Niida, T. Wada, N. Igarashi, H. Kaneda, T. Toma, K. Ohta, Y. Kasahara, S. Koizumi, Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency, J. Clin. Invest. 103 (1999) 129–135.
[78] A. Tosaki, D.K. Das, The role of heme oxygenase signaling in various disorders, Mol. Cell. Biochem. 232 (2002) 149–157.
[79] S. Shibahara, T. Kitamuro, K. Takahashi, Heme degradation and human disease: diversity is the soul of life, Antioxid. Redox. Signal 4 (2002) 593–602.
[80] S. Shibahara, The heme oxygenase dilemma in cellular homeostasis: new insights for the feedback regulation of heme catabolism, Tohoku J. Exp. Med. 200 (2003) 167–186.
[81] M.P. Schmitt, Utilization of host iron sources by *Corynebacterium diphtheriae*: identification of a gene whose product is homologous to eukaryotic heme oxygenases and is required for acquisition of iron from heme and hemoglobin, J. Bacteriol. 179 (1997) 838–845.
[82] W.K. McCoubrey Jr., T.J. Huang, M.D. Maines, Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3, Eur. J. Biochem. 247 (1997) 725–732.
[83] S. Hayashi, Y. Omata, H. Sakamoto, Y. Higashimoto, T. Hara, Y. Sagara, M. Noguchi, Characterization of rat heme oxygenase-3 gene. Implication of processed pseudogenes derived from heme oxygenase-2 gene, Gene 336 (2004) 241–250.