Heme Catabolism: A New Look at Substrates and Enzymes

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Heme IX (iron(II) protoporphyrin IX) (**1**) is an important metabolite involved in the regulation of globin synthesis,[1] in the expression of human transferrin receptors,[2] in the regulation of the activities of guanylate cyclase[3] and adenylate cyclase,[4] in the inhibition of ATP-dependent proteolysis,[5] and in the regulation of its own in vivo synthesis.[6] As the prosthetic group of hemoproteins, it is involved in many of the most important biological reactions involving O_2 and oxidations.[7] In all these reactions, the heme prosthetic group is the catalytic site which is in contact with either O_2 or active oxidizing species, and it nevertheless remains unharmed by them, although there are exceptions to this rule (see below). Once detached from the proteins, heme is particularly susceptible to oxidative attack at the carbon bridges linking the pyrrole rings (meso positions). Its biological oxidation leads to the elimination of the α-meso carbon as carbon monoxide, which results in the cleavage of the tetrapyrrole macrocycle and in formation of a bilitriene known as biliverdin IXα (**2**) which is reduced in mammals to a bilidiene known as bilirubin IXα (**3**) (Figure 1).

The sequence of these reactions was clarified and established mainly by Lemberg and his co-workers, and the early work done in this field was summarized in 1949.[8] Later work on the different aspects of heme catabolism was summarized in several excellent reviews, and the reader is referred to them.[9] New experimental results on the nature of heme oxidation to biliverdin as well as on the nature of biliverdin reduction to bilirubin justify a new look at the substrates and enzymes involved in heme catabolism.

Specificity of Heme Oxygenase and α-Regioselective Oxidation

In the biological oxidation of heme IX only biliverdin IXα is formed, while during its chemical oxidation all the four possible biliverdin isomers [IXα (**2**), IXβ (**4**), IXγ (**5**), and IXδ (**6**); Figure 2] are formed, indicating a random oxidative attack at the four meso bridges. The relative amounts of the four biliverdin isomers[10] do not indicate any marked preference toward the α-meso bridge. When the chemical oxidation reaction was carried out on hemoproteins instead of free heme, biliverdins were also obtained but a selectivity in the oxidation of the meso bridges was found. The chemical oxidation of myoglobin formed only[9a] (or mainly[11]) biliverdin IXα (**2**), while hemoglobin formed about 60% of biliverdin IXα (**2**) and 40% of biliverdin IXβ (**4**). It was therefore suggested that the insertion of heme into a hemoprotein selectively shields some of the heme methine bridges from oxidation, thus resulting in the chemical oxidation of the unshielded bridges. This led to the proposal that the biological oxidation of heme is a chemical event which takes place on the hemoproteins by an "accidental" reaction, where active oxygen species produced by the hemoproteins oxidized the heme prosthetic group with preponderant formation of biliverdin IXα (**2**). Since hemoglobin is the main hemoprotein in vertebrates and provides most of the bilirubin IXα (**3**) formed in vivo (300 mg/day in humans), the absence of a major fraction of bilirubin IXβ was attributed to the specificity of the biliverdin reductase which was thought to be specific for the reduction of biliverdin IXα (**2**).[12] The idea that the biological degradation of heme is a chemical event controlled by the hemoprotein structure had to be abandoned when it was shown that microsomes contain an enzymatic heme oxygenase system which oxidizes heme to biliverdin while it consumes O_2 and requires NADPH as the reducing agent.[13] A soluble biliverdin reductase which reduces the biliverdin to bilirubin was also isolated[14]

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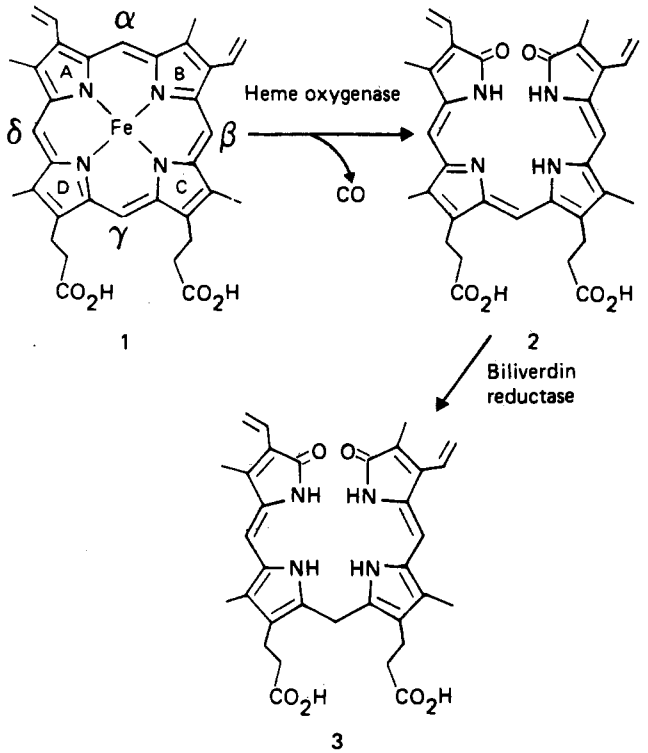


Figure 1. Enzymatic degradation of heme IX (1) to bilirubin IXα (3).

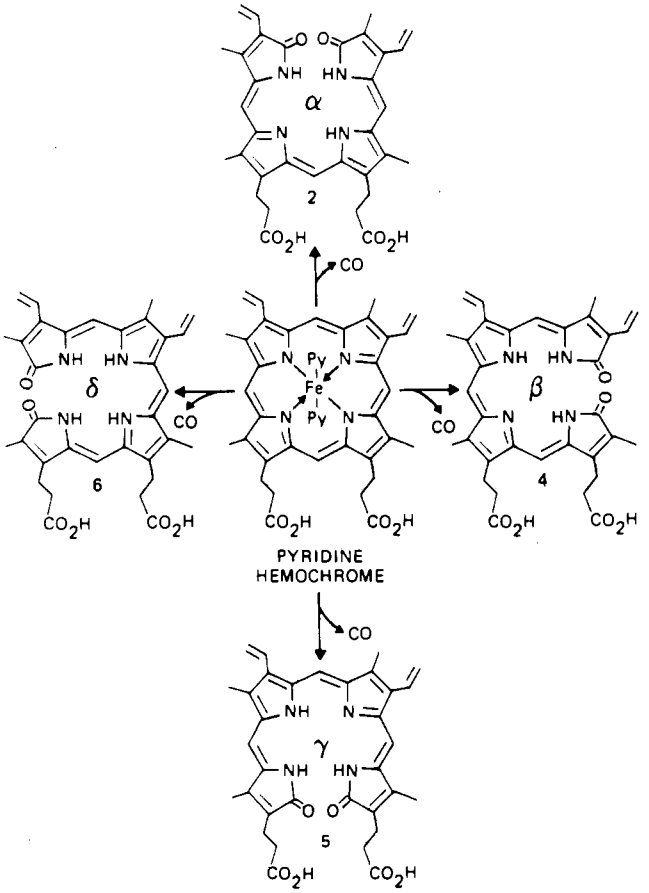
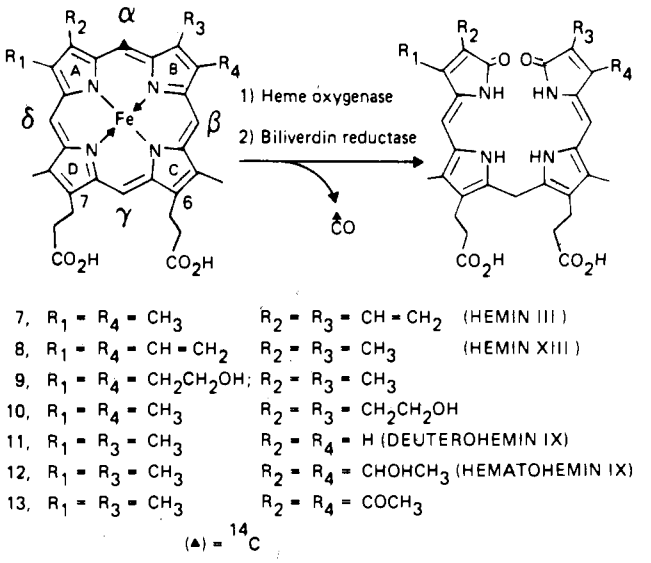


Figure 2. Chemical oxidation of heme IX in pyridine. (The solvent forms a stable hexacoordinated iron(II) derivative called hemochrome.)

and thus confirmed early work by Lemberg. The purified heme oxygenase is not a hemoprotein although in its purified form it always contains a small amount



7, $R_1 = R_4 = CH_3$ $R_2 = R_3 = CH=CH_2$ (HEMIN III)
8, $R_1 = R_4 = CH=CH_2$ $R_2 = R_3 = CH_3$ (HEMIN XIII)
9, $R_1 = R_4 = CH_2CH_2OH$; $R_2 = R_3 = CH_3$
10, $R_1 = R_4 = CH_3$ $R_2 = R_3 = CH_2CH_2OH$
11, $R_1 = R_3 = CH_3$ $R_2 = R_4 = H$ (DEUTEROHEMIN IX)
12, $R_1 = R_3 = CH_3$ $R_2 = R_4 = CHOHCH_3$ (HEMATOHEMIN IX)
13, $R_1 = R_3 = CH_3$ $R_2 = R_4 = COCH_3$

(▲) = ^{14}C

Figure 3. Enzymatic conversion of [α-^{14}C]hemin III (7), [α-^{14}C]hemin XIII (8), [α-^{14}C](hydroxyethyl)hemins (9 and 10), [α-^{14}C]deuterohemin (11), [α-^{14}C]hematohemin (12), and 2,4-diacetyl[α-^{14}C]deuterohemin (13) to the radioinactive α-bilirubins.

of heme.[15] When incubated with hemins,[16] the latter recombined with the oxygenase prior to their oxidation, giving rise to hemoproteins whose spectra resemble that of a true hemoprotein.

The existence of a membrane-bound heme oxygenase raised again the problem of the specific and regioselective biological oxidation of heme to biliverdin. The problems to be answered were the following: (a) Is heme oxygenase an enzymatic system which only oxidizes heme IX, and if not, what is its substrate specificity? (b) If hemes other than heme IX (1) are oxidized, is this oxidation always α-regioselective? (c) How does side chain substitution influence substrate specificity and α-regioselectivity, especially the substituents of rings A and B which are in close contact with the hydrophobic protein pocket? (d) If the heme oxygenase system is capable of generating a great diversity of biliverdins, will they be reduced by biliverdin reductase to bilirubins or is biliverdin reductase an enzyme with a narrow specificity which channels heme catabolism toward the exclusive formation of bilirubin IXα (3)?

To examine the substrate specificity of heme oxygenase and to explore the problem of the α-regioselective oxidation, the syntheses of hemins III (7)[17,18] and XIII (8) as well as of their [α-^{14}C] derivatives were carried out. These hemins are isomers of hemin IX (1) in which a permutation of the methyl and vinyl residues of rings A and B was introduced (Figure 3). The chemical oxidation of hemin XIII (8) gave biliverdins XIIIα (30%), XIIIβ-δ (55%), and XIIIγ (15%).[19] Each isomer was unambiguously identified by spectroscopic

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(16) Hemes are handled as hemin chlorides, i.e., as iron(III) porphyrin chlorides which are reduced in situ before the biological or chemical oxidations are carried out.
(17) Fischer's mesoporphyrin type notation is used. Fischer, H.; Orth, H. In *Die Chemie des Pyrrols*; Akademische Verlagsgesellschaft: Leipzig, 1937; Vol. II, p 434.
(18) Hemins were obtained by iron insertion into the corresponding synthetic porphyrins. Protoporphyrins III and XIII were obtained according to: Buldain, G.; Díaz, L.; Frydman, B. *J. Org. Chem.* **1977**, *42*, 2957.
(19) Frydman, R. B.; Awruch, J.; Tomaro, M. L.; Frydman, B. *Biochim. Biophys. Res. Commun.* **1979**, *87*, 928.

Table I.
Effect of the Substituents at C_2 and C_4 on the Substrate Activity of Heme IX Derivatives[a]

substrate	rel activity, %
hemin IX (1)	100
hemin XIII (8)	121
hemin III (7)	147
2-vinyl-4-methyldeuterohemin (14)	118
mesohemin (15)	83
2,4-dimethyldeuterohemin (16)	80
2,4-diisopentyldeuterohemin (17)	39
2,4-dilauryldeuterohemin (18)	36
2,4-diisopentenyldeuterohemin (19)	50
2,4-dilauryldeuterohemin (20)	45
2,4-diacetyldeuterohemin (13)	45
2,4-diformyldeuterohemin (21)	15
2-formyl-4-vinyldeuterohemin (22)	17
2-vinyl-4-formyldeuterohemin (23)	34
2,4-dipentanoyldeuterohemin (24)	37
2,4-dilauroyldeuterohemin (25)	45

[a] The data were obtained after 15-min incubation. The relative activities are expressed by relating them to hemin IX, which is taken as 100%.

data (^{1}H NMR and mass spectra) and was reduced to the corresponding bilirubin with sodium borohydride. The purity of each bilirubin XIII isomer was further secured by its transformation into azodipyrroles[20] for its unambiguous identification.[19] The chemical oxidation of hemin III (7) also afforded the three possible biliverdins: IIIα (27%), IIIβ–δ (60%), and IIIγ (13%), which were identified as mentioned above.

When hemins XIII (8) and III (7) were oxidized with the microsomal heme oxygenase system, they were found to be better substrates of the latter than hemin IX (1). The formed biliverdins were transformed in situ into bilirubins by the addition of biliverdin reductase. The formed bilirubins were identical with the α-bilirubin isomers. Hemin III (7) was the best substrate of the microsomal heme oxygenase, followed by hemin XIII (8) and then by the natural hemin IX (1) (Table I).

To examine whether hydrophobic residues on rings A and B were required for a heme to be a substrate of heme oxygenase, the bis(β-(hydroxyethyl))hemins **9** and **10** (Figure 3) were assayed as substrates of heme oxygenase. They were also very good substrates of the enzyme and also afforded α-bilirubins, indicating that the substrate specificity of heme oxygenase was always associated with α-regioselectivity. During these oxidations all the biliverdins formed by heme oxygenase were reduced to the bilirubins, suggesting that biliverdin reductase was capable of handling biliverdin types other than type IX. A further confirmation that the enzymatic oxidation was α-regioselective was given by the isolation of radioinactive α-bilirubins when the [α-^{14}C]hemins were incubated with the heme oxygenase system (Figure 3). To further expand these results, a new series of [α-^{14}C]hemins were prepared by a C-5 regiospecific synthesis.[21] They were [α-^{14}C]deuterohemin IX (11), [α-^{14}C]hematohemin IX (12), and 2,4-diacetyl[α-^{14}C]deuterohemin IX (13). Their enzymatic oxidation afforded the corresponding radioinactive α-bilirubins[22] (Figure 3).

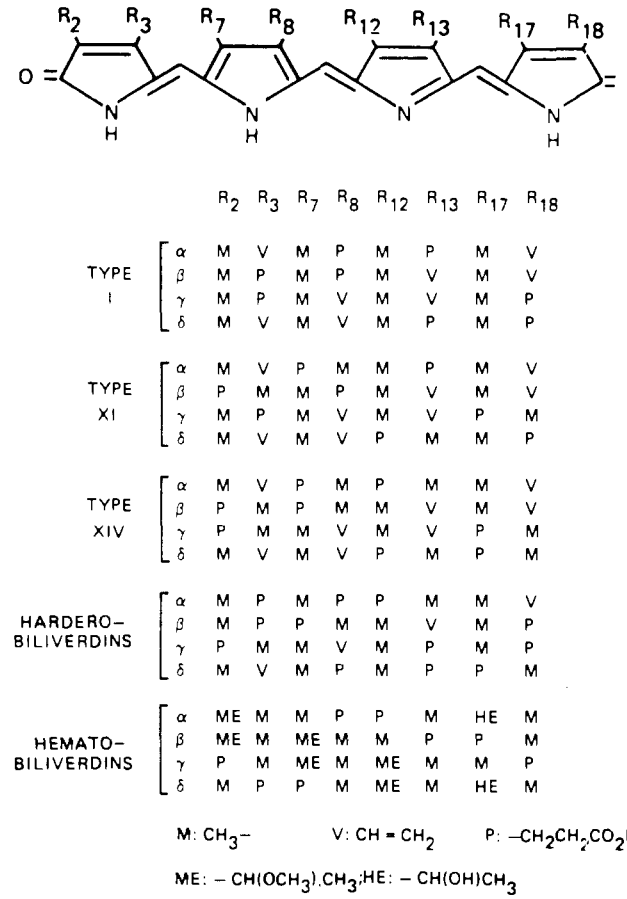
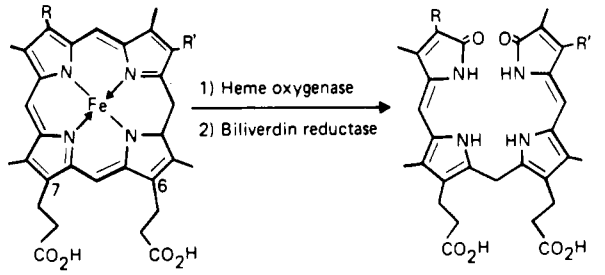


Figure 4. Biliverdin isomers obtained by the chemical oxidation of hemins I (27), XI (28), XIV (29), harderohemin (26), and hematohemin (12).

The amount of radioinactive bilirubin was stoichiometric with the amount of hemin radioactivity lost during the incubation, and this indicated a quantitative transformation of heme into biliverdin first and then into bilirubin. The enzymatic oxidation of hematohemin IX (12) was also examined. First, the four hematobiliverdin IX isomers formed in the chemical oxidation[23] were obtained and identified (Figure 4). Then it was found that hematobiliverdin IXα was the only isomer formed during the oxidation when the microsomal heme oxygenase system was used.[23] To further examine heme oxygenase specificity, deuteroporphyrin IX was acylated to give 2,4-diacylporphyrins. These were reduced to the corresponding homologues of hematoporphyrin, dehydrated to the corresponding 2,4-dialkenylporphyrins, and then finally reduced to 2,4-dialkylporphyrins[24] (Figure 5). Each porphyrin was transformed into its hemin, and together with other synthetic hemins such as mesohemin IX (15), hemin (16), as well as monovinylhemin (14), 2,4-diformyldeuterohemin IX (21), *Spirographis* hemin (22) (the prosthetic group of chlorocruorin), and *Isospirographis* hemin (23) (Figure 5) were assayed as substrates of the

(20) Blanckaert, M.; Every, J.; Heirwegh, K. P. M.; Compernolle, F. *Biochem. J.* **1977**, *164*, 237.
(21) Rezzano, I.; Buldain, G.; Frydman, B. *J. Org. Chem.* **1982**, 47, 3059.
(22) Rezzano, I.; Tomaro, M. L.; Buldain, G.; Frydman, R. B. *Biochim. Biophys. Acta* **1982**, *704*, 261.
(23) Buldain, G.; Frydman, R. B.; Tomaro, M. L.; Frydman, B. *Eur. J. Biochem.* **1986**, *156*, 179. An unexpected feature of the esterification of the free hematobiliverdin acids was the transetherification at several of the α-hydroxyethyl residues which resulted in the formation of the dimethyl ethers in the case of the β- and γ-isomers and of the monomethyl ethers in the case of the α- and δ-isomers.
(24) Buldain, G.; Díaz, L.; Frydman, B. *Anal. Asoc. Quím. Arg.* **1982**, *70*, 313.



14, R = CH = CH_2 ; R' = CH_3
15, R = R' = C_2H_5
16, R = R' = CH_3
17, R = R' = $CH_2CH_2CH(CH_3)_2$
18, R = R' = $CH_2(CH_2)_{10}CH_3$
19, R = R = CH = $CHCH(CH_3)_2$
20, R = R' = CH = $CH(CH_2)_9CH_3$
21, R = R' = CHO
22, R = CHO; R' = CH = CH_2
23, R = CH = CH_2; R' = CHO
24, R = R' = $CO(CH_2)_3CH_3$
25, R = R' = $CO(CH_2)_{10}CH_3$
26, R = CH = CH_2; R' = $CH_2CH_2CO_2H$

Figure 5. Synthetic hemins of type IX which are enzymatically transformed into α-bilirubins.

microsomal heme oxygenase system in the presence of biliverdin reductase. All of them were found to be substrates of heme oxygenase, and the α-biliverdins formed in these oxidations were reduced by biliverdin reductase to the corresponding α-bilirubins. As could be expected, they differed in their substrate activities (Table I).[25,26] Substitutions in rings A and B with methyl, vinyl, and ethyl residues gave hemins with the best substrate activity, while elongation of the alkyl or alkenyl side chains resulted in a marked decrease of these activities. It was clear that an increase in the hydrophobicity of hemin itself did not necessarily lead to an enhancement of substrate activity. When deactivating residues were present at C_2 and C_4, substrate activity was usually halved as compared to hemin IX, but oxidation still took place at the meso α-position.

All the aforementioned hemins had in common that they were diacid hemins carrying two propionates at C_6 and C_7. This appeared to be the main feature of all the hemins which were substrates of heme oxygenase, while the substituents in rings A and B played a minor role. This assertion was put to test by assaying a number of hemins isomeric with hemin IX such as hemin I (**27**), hemin XI (**28**), and hemin XIV (**29**), where the array of substituents in rings A and B was the same as in hemin IX (**1**) but the propionate residues in rings C and D were at different positions (Figure 6). None of them were a substrate of heme oxygenase.[26] Hemin IV (**30**), where the substituents of rings A and B were the same as in hemin XIII (**8**) but the propionates were at C_5 and C_8 (Figure 6), was not a substrate of the heme oxygenase system.[25] Nor was hemin **31**, which carried two β-hydroxyethyl residues in rings A and B (which were known to favor substrate activity when the two propionates were at C_6 and C_7) and two propionates at C_6 and C_8 (Figure 6).[25] The monopropionate hemin (**32**) also was not a substrate of heme oxygenase.[26]

The tricarboxylic harderohemin (**26**) (Figure 5) was a substrate of heme oxygenase (38% of α-harderobilirubin was formed[26]) while the tetracarboxylic copro-

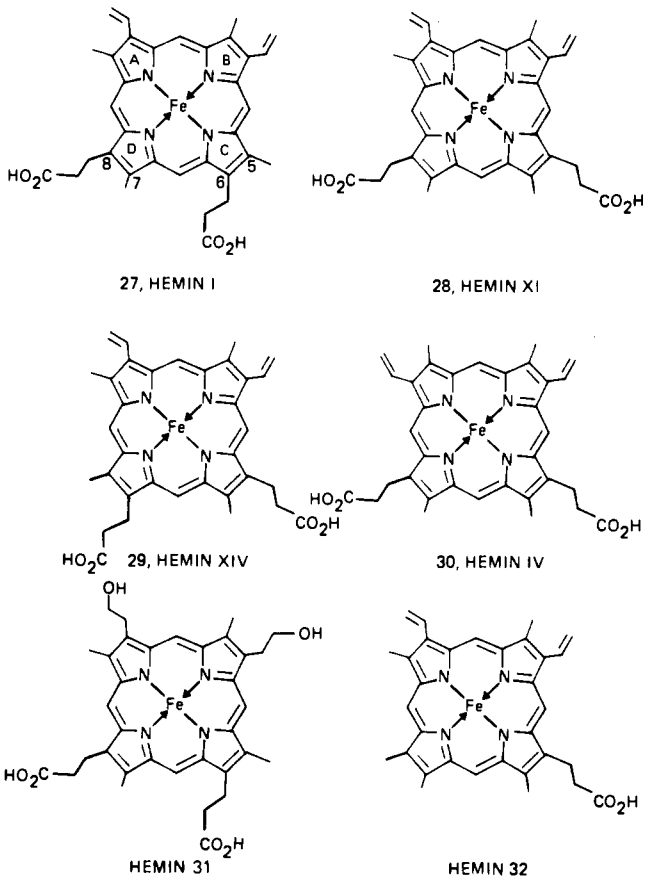


Figure 6. Synthetic hemins which are not substrates of heme oxygenase.

heme III and the octacarboxylic uroheme III[27] were not oxidized by the microsomal heme oxygenase.

The results obtained with the microsomal heme oxygenase system were further supported by experiments carried out in vivo where the synthetic hemins were perfused through a normal rat liver.[28] Under the conditions used, the bile fluid collected after hemin IX (**1**) perfusion contained bilirubin IXα–diglucuronide (56%), the mixture of bilirubin IXα–monoglucuronides (42%), and free bilirubin IXα (2%). These results are in general agreement with the known pathway of heme catabolism and bilirubin excretion in vivo. When hemin XIII (**8**) was perfused with the same technique, the bile contained the diglucuronide of bilirubin XIIIα (55%), the monoglucuronide of bilirubin XIIIα (43%), and free bilirubin XIIIα (2%). Similar results were obtained when the bis(hydroxyethyl)hemin (**9**) (Figure 3) and hematohemin IX (**12**) (Figure 3) were perfused.[28] These in vivo results fully validate the results obtained with the enzymatic incubations, and in addition they showed a braod specificity for the glucuronidation reaction involved in bilirubin excretion.

The requirement for two free propionate residues (the esters were inactive[23]) for substrate activity was further tested by using the 6,7-dibutyrate and 6,7-diacetate analogues of hemin IX which were prepared by synthesis.[29] While the diacetate was not oxidized, the

(25) Frydman, R. B.; Tomaro, M. L.; Buldain, G.; Awruch, J.; Díaz, L.; Frydman, B. *Biochemistry* **1981**, *20*, 5177.
(26) Tomaro, M. L.; Frydman, R. B.; Frydman, B.; Pandey, R. K.; Smith, K. M. *Biochim. Biophys. Acta* **1984**, *701*, 342.

(27) The uroporphyrins and coproporphyrins present in nature are of types III and I,[17] and they derive from the corresponding porphyrinogens. The porphyrinogens of type III are the metabolic precursors of heme IX. For a review see: Frydman, R. B.; Frydman, B.; Valasinas, A. In *The Porphyrins*; Dolphin, D., Ed.; Academic: New York, 1979; Vol. VI, p 1.
(28) Awruch, J.; Lemberg, A.; Frydman, R. B.; Frydman, B. *Biochim. Biophys. Acta* **1981**, *714*, 209.

dibutyrate was oxidized (50% as compared to hemin IX[26]).

Porphyrins whose hemins are substrates of heme oxygenase as well as metalloporphyrins other than hemins derived from the same porphyrins inhibited the enzymatic oxidation of heme IX (**1**).[25] Porphyrins whose hemins were not substrates of heme oxygenase did not inhibit the enzymatic reaction. This is very likely due to the formation of a porphyrin- or metalloporphyrin–heme oxygenase complex, where the heme pocket recognizes the favorable array of substituents of the porphyrin ring, but in the absence of iron no O_2 activation takes place.

The Mechanism of the Chemical and Enzymatic Oxidation of Heme

Although it is likely that the mechanism of the enzymatic oxidation of heme is similar to that of the chemical oxidation, there are important differences among them which shed light on the nature of the biological oxidation of heme. Lemberg and Fischer established[8] that the first intermediates in the chemical oxidation of heme are the mesohydroxyhemes [also called oxohemes or iron oxophlorins; **33** (Figure 7) is α-hydroxyheme bis(pyridine)]. Although it is conceivable that an α-hydroxyheme is the first intermediate in the biological oxidation of heme to biliverdin, evidence for the formation of such an intermediate in the enzymatic oxidation of heme is lacking. During the chemical oxidation of heme IX (**1**) a mixture of the four possible mesohydroxyhemes is formed, and they could be separated.[30] α-Hydroxyheme (**33**) was ligated to apomyoglobin, and the reconstituted apomyoglobin–α-hydroxyheme complex was chemically oxidized to biliverdin IXα (**2**).[30] However, to what extent this model mimics the heme oxygenase system is yet unknown.

It was also known that in the chemical oxidation of pyridine hemochrome an intermediate was formed between the hydroxyheme stage and the biliverdin stage which was called verdohemochrome and which was transformed into biliverdins by acid hydrolysis.[8,9] Since pyridine hemochrome is randomly oxidized at all the four methene bridges, verdohemochrome had to be a mixture of the four possible isomers, each giving rise to the corresponding biliverdin. However, the coupled oxidation of myoglobin allowed the isolation of α-verdohemochrome (**34**) (Figure 7), which was transformed by acid hydrolysis into biliverdin IXα (**2**).[31] α-Verdohemochrome (**34**) was also prepared by the chemical oxidation of **33**.[30] The formation of α-verdohemochrome (**34**) from α-hydroxyhemin implies a loss of CO from the α-meso bridge and the incorporation of an oxygen derived from O_2 into the oxonium bridge (Figure 7).

Experiments carried out using the microsomal heme oxygenase system and $^{18}O_2$ indicated that the oxygen liberated as CO as well as the oxygen found in each of the lactam carbonyls of biliverdin IXα (**2**) was derived from O_2 and not from water.[32] Moreover, when the enzymatic oxidation was carried out using $^{16}O_2$ enriched

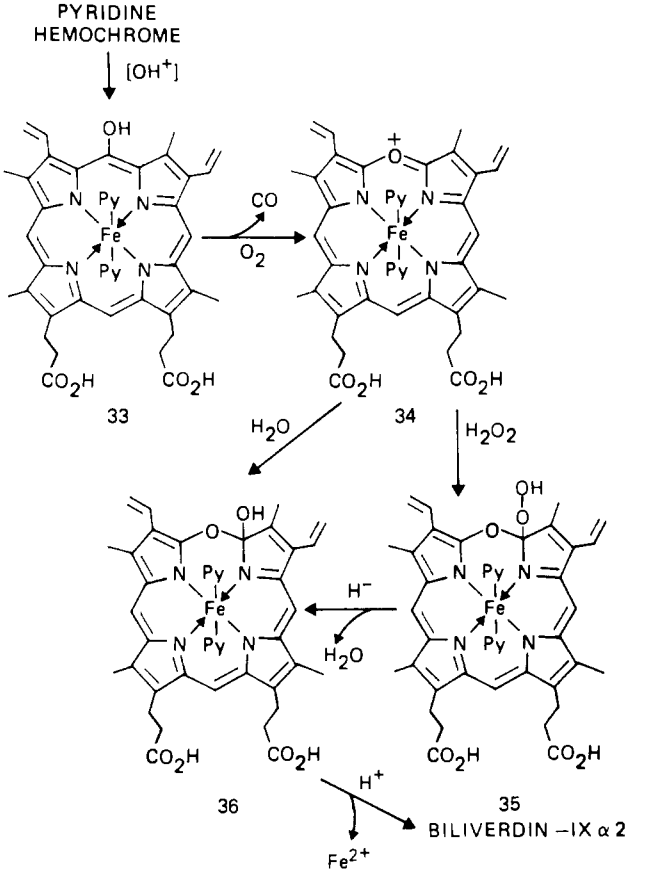


Figure 7. Formation of verdohemochrome IXα by oxidation of α-hydroxyheme bis(pyridine), followed by its hydrolytic (H_2O) or oxidative (H_2O_2) ring opening to biliverdin IXα.

with $^{18}O_2$, it was found that the terminal oxygens of bilirubin are derived from separate O_2 molecules.[33] Therefore, the possibility of an α-verdohemochrome (**34**) being an intermediate in the enzymatic reaction was ruled out[33c] since it was considered that its only possible transformation into biliverdin was by a hydrolytic reaction. However, the oxidative transformation of verdohemochrome (**34**) into biliverdin IXα (**2**) was shown to take place in the presence of hydrogen peroxide and a reducing agent (phenylhydrazine).[31] The α-verdoheme–apomyoglobin complex could also be oxidized with O_2 and ascorbate to biliverdin IX.[30] In this oxidative ring opening of verdoheme a peroxide (or dioxygen) very likely attacks the pyrrole α-carbon linked to the oxonium bridge to give **35**, and the latter is then reduced to the hemiketal form of iron biliverdin (**36**) which is demetalated as the last step of the reaction (Figure 7). This reaction sequence would reconcile the formation of a verdoheme as an intermediate in the enzymatic oxidation of heme and the presence in biliverdin of two oxygen atoms derived from two different O_2 molecules. In studies carried out with the reconstructed heme–heme oxygenase system, a protein complex (substance 688) was isolated which had a very similar spectrum to that of verdohemochrome.[34] Is is conceivable that an iron(II)-mediated oxidation leads from α-hydroxyheme to a verdoheme with an axial imidazole ligand, which again binds O_2, reduces it to an

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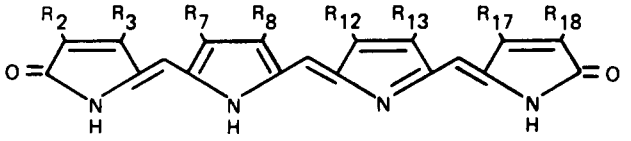

UROBILIVERDINS

I $R_2 = R_7 = R_{12} = R_{17} = A$; $R_3 = R_8 = R_{13} = R_{18} = P$ (Bactobilin)
IIIα $R_2 = R_7 = R_{13} = R_{17} = A$; $R_3 = R_8 = R_{12} = R_{18} = P$
IIIβ $R_2 = R_8 = R_{12} = R_{17} = A$; $R_3 = R_7 = R_{13} = R_{18} = P$
IIIγ $R_3 = R_7 = R_{12} = R_{17} = A$; $R_2 = R_8 = R_{13} = R_{18} = P$
IIIδ $R_2 = R_7 = R_{12} = R_{18} = A$; $R_3 = R_8 = R_{13} = R_{17} = P$

COPROBILIVERDINS

I $R_2 = R_7 = R_{12} = R_{17} = M$; $R_3 = R_8 = R_{13} = R_{18} = P$
IIIα $R_2 = R_7 = R_{13} = R_{17} = M$; $R_3 = R_8 = R_{12} = R_{18} = P$
IIIβ $R_2 = R_8 = R_{12} = R_{17} = M$; $R_3 = R_7 = R_{13} = R_{18} = P$
IIIγ $R_3 = R_7 = R_{12} = R_{17} = M$; $R_2 = R_2$ $R_{13} = R_{18} = P$
IIIδ $R_2 = R_7 = R_{12} = R_{18} = M$; $R_3 = R_8 = R_{13} = R_{17} = P$

37, $R_2 = R_8 = R_{12} = R_{18} = M$; $R_3 = R_{13} = R_{17} = E$; $R_7 = P$
38, $R_2 = R_7 = R_{12} = R_{18} = M$; $R_3 = R_{13} = R_{17} = E$; $R_8 = P$
39, $R_3 = R_8 = R_{12} = R_{18} = M$; $R_7 = R_{13} = R_{17} = E$; $R_2 = P$
40, $R_3 = R_8 = R_{12} = R_{18} = M$; $R_{13} = R_{17} = E$; $R_2 = P$; $R_7 = A$
41, $R_3 = R_8 = R_{12} = R_{17} = M$; $R_7 = R_{13} = E$; $R_2 = P$; $R_{18} = A$
42, $R_2 = R_7 = R_{13} = R_{17} = M$; $R_3 = R_{18} = E$; $R_8 = R_{12} = P$
43, $R_2 = R_7 = R_{13} = R_{17} = M$; $R_3 = R_{18} = E$; $R_8 = R_{12} = A$
44, $R_2 = R_7 = R_{13} = R_{17} = M$; $R_3 = R_{18} = E$; $R_8 = R_{12} = P$

M = CH_3; E = C_2H_5; A = CH_2CO_2H; P = $CH_2CH_2CO_2H$

Figure 8. Biliverdins obtained by total synthesis.

active peroxide form, and is self-oxidized by it to biliverdin. It is also possible that heme oxygenase binds more than one heme and that a catalytic hemoprotein center is present which activates O_2 for verdoheme degradation. The stoichiometry of the enzyme-mediated oxidation of heme indicated that 5–6 mol of NADPH and 3 mol of O_2 were consumed per mole of hemin oxidized.[35] There is therefore no lack of reducing equivalents in the enzymatic reaction.

The Specificity of Biliverdin Reductase

Biliverdins are found in nature free or bound to proteins. They are present in mammals, birds, amphibians, reptiles, fish, mollusks, and insects, in algae and higher plants, and in anaerobic bacteria.[36] Biliverdin IXα (2) and its derivatives are the most widely distributed. A notable exception is biliverdin IXγ (5) (Figure 2) (pterobilin) which is widely distributed either alone or bound to proteins in butterfly species, in beetles, and in insect hemolymph.[37] The only biliverdin found in bacteria was bactobilin, which was identified as urobiliverdin I[38] (Figure 8). It is obvious from what is known about heme oxygenase specificity (see above) that biliverdin IXγ (5) as well as bactobilin must be formed by other enzymatic systems.

Whenever a synthetic hemin was oxidized by the microsomal heme oxygenase system, the resulting biliverdin was reduced by biliverdin reductase to the corresponding bilirubin. The question was then raised as to the specificity of biliverdin reductase and its possible link with the specificity of heme oxygenase. Hemins I (27), XI (28), and XIV (29) (Figure 6), which were not substrates of heme oxygenase, were chemically oxidized to the four possible biliverdin isomers of each type (Figure 4). The latter were found to be reduced efficiently to the corresponding bilirubins by biliverdin reductase, and no major differences could be found in the reduction rates of the different biliverdin types. The biliverdins of type I and type XIV were reduced at higher rates than the mixture of the four biliverdin isomers of type IX.[39] Biliverdin reductase also reduced the four hematobiliverdin isomers (Figure 4)[23] as well as the mixture of the four tricarboxylic isomers derived from the chemical oxidation of harderohemin (26)[39] (Figure 4). To further explore the substrate activity of bilitrienes substituted with polar residues, the total synthesis of the urobiliverdin and coprobiliverdin isomers (Figure 8) was carried out.[40] All of them were enzymatically reduced to the corresponding bilirubins, although at different rates. The γ-isomers were reduced at slower rates than the other isomers although at higher rates than that of biliverdin IXγ (5). Unexpectedly, the biliverdin reductase proved to have a broad substrate specificity which included hydrophilic as well as hydrophobic biliverdins, and this required a new synthetic effect to establish the minimum requirements of a biliverdin to be reduced by biliverdin reductase. A series of eight biliverdins (37–44) were obtained,[41] (Figure 8) of which only mesobiliverdin IXα (44) was reduced by biliverdin reductase. Therefore, substrate activity of a biliverdin requires the presence of at least two propionate residues which may or may not be at vicinal carbons.

It can be concluded that the substrate specificity of heme oxygenase is much more restrictive than that of biliverdin reductase and that the latter will impose little selectivity on the heme catabolic pathway.

The Molecular Properties of Biliverdin Reductase

Biliverdin reductase from rat liver of normal animals could be separated into two molecular forms called molecular form 1 and molecular form 2.[42] The major molecular form 1 reduced biliverdin IXα (2) at a higher rate than the IXβ (4) isomer. The minor molecular form 2 reduced both isomers at similar rates. In livers of rats where hemoprotein degradation had been strongly accelerated and heme oxygenase activity highly enhanced by administration of $CoCl_2$ or phenyl-

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hydrazine, biliverdin reductase changed its pattern of molecular forms since molecular form 1 disappeared and a new molecular form 3 appeared. The latter reduced both biliverdins IXα and IXβ at similar rates.[42] The conversion of molecular form 1 into molecular form 3 was an enzyme-mediated process which required both mRNA and protein synthesis. This process was a consequence of the Co(II) or phenylhydrazine treatment which induced a NAD^+-dependent peroxysomal dehydrogenase which then oxidized molecular form 1 to molecular form 3.[43] Molecular form 3 was a dimer of molecular form 1 and resulted from the oxidation of a thiol group of form 1 to form the S–S linked dimer 3. Molecular form 3 could be reconverted to molecular form 1 by reduced thioredoxin in vitro. The conversion of molecular form 1 to molecular form 3 was a fast process in vivo since it was completed 3 h after the Co(II) treatment. The difference in the reduction rates of the α- and β-isomers by the molecular forms of biliverdin reductase was extensive to other biliverdin types such as the biliverdin XIII isomers,[44] the hematobiliverdin IX isomers, and the isomers of other synthetic biliverdin types. This is clearly a problem of enzymatic stereoselectivity originating in the remarkable high flexibility of the biliverdins,[45] and it depends on the configurational and conformational forms adopted by the different isomers.

The existence of molecular forms of biliverdin reductase (forms 2 and 3) with high affinities for biliverdin IXβ (**4**) suggests that this isomer is formed under normal conditions during hepatic breakdown processes and that its formation is enhanced when hemoprotein degradation is accelerated. Bilirubin IXβ was reported to be present in small amounts in normal bile.[46]

It is very likely that chemical oxidations of hemoproteins and especially of hemoglobin take place under conditions where oxidizing species are generated in vivo, as is the case when Co salts or phenylhydrazine is administrated. As mentioned above, biliverdin IXβ (**4**) was formed in the chemical oxidation of hemoglobin, and although this oxidation cannot account anymore for the mechanism of the biological degradation of heme, it might still be valid as a secondary path under conditions of oxidative stress. A recent crystallographic study of the structure of hepatic catalase[47] showed that apart from the heme prosthetic group a large amount of biliverdin IXβ was also present at the heme pocket, a fitting example of the above-mentioned "accidental" heme cleavage. The existence of molecular forms of biliverdin reductase with different reaction rates for the β-isomers might indicate that the metabolism is able to cope with a situation where a very minor reaction such as the chemical oxidation of hemoproteins can be accelerated under the influence of exogenous oxidation factors. It also vindicates the older view[9] that the chemical oxidation of hemoproteins may have a biological significance.

Concluding Remarks

Heme metabolism has been a subject of intense study for more than 50 years. During the past years new facts about heme oxidation to biliverdins and the latter's reduction to bilirubins opened new vistas in this field. Heme was found to be oxidized by a microsomal heme oxygenase system whose substrate specificity is restricted to hemins having vicinal propionates at the C_6 and C_7 positions, a fact which could be of pharmacological importance since synthetic porphyrins and hemins are starting to have a widespread use in therapeutics. The oxygenase was found to be α-regioselective in every case, and the formed α-biliverdins were efficiently reduced to the corresponding α-bilirubins by biliverdin reductase. The latter enzyme had a much broader specificity than heme oxygenase, although it only reduced bilitrienes which carried at least two propionate residues. The hepatic biliverdin reductase can be found in three molecular forms which have the capacity to reduce the biliverdin isomers formed not only in the enzymatic oxidation of heme but also in the minor and nonspecific chemical oxidations of hemoproteins which occur in vivo. Heme oxygenase is a protein which recombined with its substrate (heme) forms a hemoprotein where the prosthetic group is self-oxidized, and it is a very attractive model for further studies with synthetic hemins, where much can be learned on the nature of hemoproteins involved in oxidative processes.

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