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CONCERNING THE SPECIFICITY OF HEME OXYGENASE: THE ENZYMATIC OXIDATION OF SYNTHETIC HEMINS

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SUMMARY: Hemin XIII 4, hemin III 5, and iron 1,4-di(β-hydroxyethyl)porphyrin 6 were enzymatically oxidized by a microsomal heme oxygenase preparation from rat liver. These are all better substrates of the oxygenase than the natural substrate, hemin IX 1. The enzymatic oxidation was selective for the α-methine bridge and in every case only the α-biliverdins were obtained. The latter were readily reduced by biliverdin reductase to the corresponding α-bilirubins. The absence of isomers in addition to the α-bilirubins was established by preparing the derived azopigments and by using [α-^{14}C]6 and [α-^{14}C]4 as substrates. The chemical oxidation of 4, 5, and 6 gave the expected mixture of biliverdins. It is concluded that heme oxygenase is not specific for hemin IX. On the other hand, the enzyme is highly selective for the α-methine bridge, defined as the methine opposed to that flanked by the 6,7-propionic acid residues.

Heme 1 breakdown in algae and animals proceeds through an oxidative cleavage of the α-methine bridge and leads to the formation of biliverdin IXα 2 and of phycobilins (1). The enzymatic reduction of 2 by biliverdin reductase in mammals gives rise to bilirubin IXα 3, which is the overwhelming bulk of biliary bilirubin (Fig. 1). Although some minor traces of the IX β, IX δ and IX γ bilirubin isomers have been detected, the biological heme cleavage process has a high specificity for the α-methine bridge. Heme oxidation is catalyzed by a heme oxygenase (2) which has been recently purified to homogeneity (3), and has apparently an active binding site for heme where the oxidation takes place. The specificity of the enzymatic heme oxidation contrasts with the non-specificity of the chemical coupled oxidation of heme IX 1, where all the four possible biliverdin isomers are formed by oxidation at the four methine bridges (4). The α-specificity of the enzymatic heme cleavage has attracted the attention of many workers, and several proposals have been advanced to explain it. One proposal was that the α-specificity was due to an intrinsic lability of the α-methine bridge resulting from the structure of heme IX 1 itself (5); a second proposal was that the active site of heme-cleaving enzyme imposes the α-specificity on the cleavage system (2), while a third proposal advanced the suggestion that α-specificity is produced by an interaction of heme with the heme binding sites in the different hemoproteins in such a manner that only the α-bridge is exposed and then degraded by a chemical oxidation (4).

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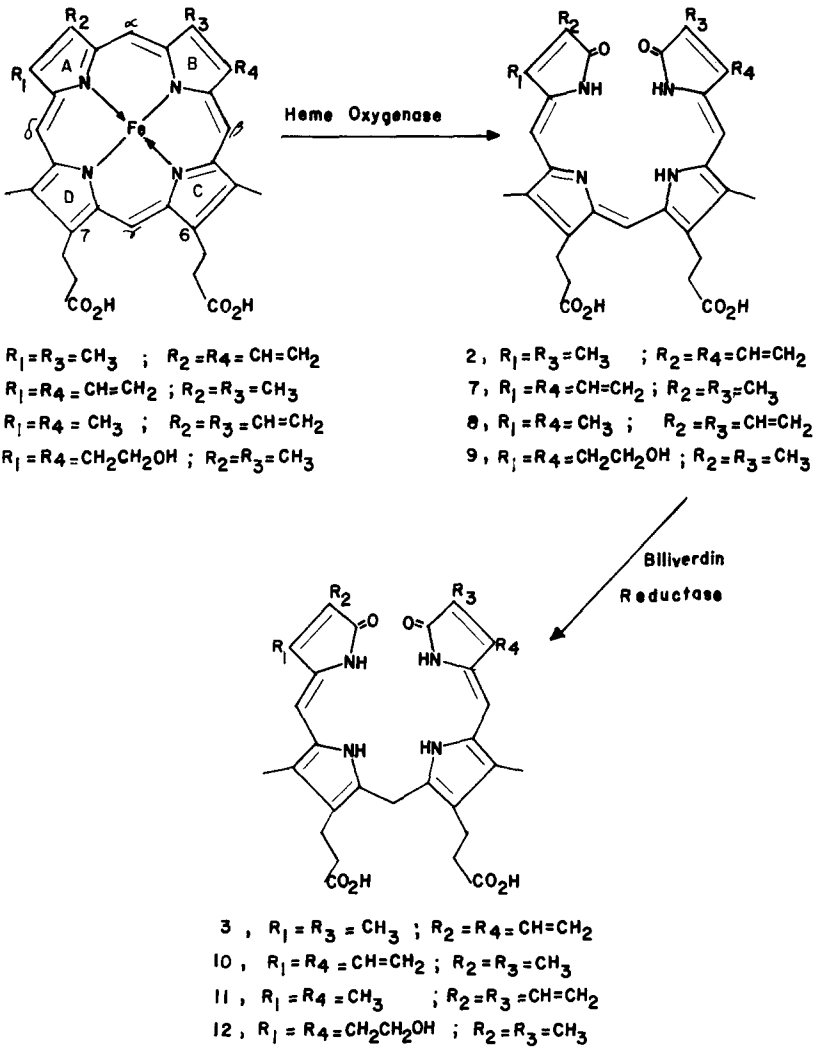

1, $R_1=R_3=CH_3$; $R_2=R_4=CH=CH_2$
4, $R_1=R_4=CH=CH_2$; $R_2=R_3=CH_3$
5, $R_1=R_4=CH_3$; $R_2=R_3=CH=CH_2$
6, $R_1=R_4=CH_2CH_2OH$; $R_2=R_3=CH_3$

2, $R_1=R_3=CH_3$; $R_2=R_4=CH=CH_2$
7, $R_1=R_4=CH=CH_2$; $R_2=R_3=CH_3$
8, $R_1=R_4=CH_3$; $R_2=R_3=CH=CH_2$
9, $R_1=R_4=CH_2CH_2OH$; $R_2=R_3=CH_3$

3, $R_1=R_3=CH_3$; $R_2=R_4=CH=CH_2$
10, $R_1=R_4=CH=CH_2$; $R_2=R_3=CH_3$
11, $R_1=R_4=CH_3$; $R_2=R_3=CH=CH_2$
12, $R_1=R_4=CH_2CH_2OH$; $R_2=R_3=CH_3$

Fig. 1. The enzymatic oxidation of iron porphyrins to biliverdins, and their reduction to bilirubins.

To test some of these mechanistic alternatives, we prepared the synthetic hemins XIII 4 and III 5 (6), and a type XIII iron porphyrin 6, devoid of vinyl substituents, and submitted these to enzymatic oxidation using the microsomal heme oxygenase. The array of substituents flanking the α-methine (defined as the methine opposed to the γ-methine, which is the one flanked by the 6,7-propionic acid residues) in hemins XIII 4 and III 5 is entirely different from the one present in natural heme IX 1. The rather surprising result was that the synthetic iron porphyrins 4-6 were better substrates of the enzymatic system than the natural heme IX, and that in all of these compounds the oxidative cleavage occurred exclusively at the α-methine bridge.

MATERIALS AND METHODS

Hemin IX 1 was purchased from Sigma Chemical Company. Hemin XIII 4, hemin III 5, and iron 1,4-di(β-hydroxyethyl)porphyrin 6 were prepared by incorporating iron into the synthetic porphyrins (7) following an established procedure (8). The purity of 4-6 was assured by tlc and by spectroscopic methods. Biliverdin IXα 2 was isolated from avian bile and its homogeneity was controlled by tlc methods (9). Biliverdins XIIIα 7, IIIα 8, and 2,17-di(β-hydroxyethyl)biliverdin 9 were prepared from 4, 5 and 6 by the coupled oxidation method (9). Their structures were confirmed (after tlc purification of their methyl esters) by mass spectroscopic methods (9). The compounds were converted into the corresponding bilirubins 3, 10, 11, and 12 by reduction of their dimethyl esters with sodium borohydride followed by a saponification step, or by the direct reduction of 2, 7, 8 and 9 with biliverdin reductase (see below). The homogeneity of the bilirubins 3, 10, 11, and 12 was checked by tlc methods (10), and by analysis of the azopigments obtained with diazotized ethyl anthranilate (11). [α-^{14}C]Iron-1,4-di(β-hydroxyethyl)porphyrin 6 and [α-^{14}C]hemin XIII 4 were prepared by synthesis (12). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADH, and NADPH were purchased from Sigma Chemical Co. Tlc was performed on precoated silica gel 60 F-254 plates (Merck, 0.25 mm layer thickness).

Microsomal heme oxygenase from rat liver. Wistar albino female rats (150-180 g) were injected subcutaneously with a single dose of $CoCl_2$ (200 mg/kg) and were later fasted for 18 hr. They were then anesthetized with ether and the partially bleached livers were excised and repeatedly washed with an ice-cold solution of 0.9% NaCl. All the further operations were carried out at 0-4°C. In a typical preparation, 4 g of liver were homogenized in 3 volumes of an ice-cold 0.25 M sucrose solution and 0.05 M phosphate buffer (pH 7.4) in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 20,000 g for 15 min, and the supernatant was further centrifuged at 105,000 g during 60 min. The microsomal pellet was then suspended in 1.5 volumes of 0.15 M KCl in 0.05 M phosphate buffer (pH 7.4) and was centrifuged again at the same speed for 30 min. This second pellet was then resuspended in 1.5 ml of a similar solution and was used as a source of heme oxygenase. The enzyme preparation was used within the first 24 hr.

Biliverdin reductase from rat liver. The 105,000 g supernatant obtained in the above microsomal preparation was fractionated by addition of ammonium sulfate (AS) and the 40-60% AS fraction was dissolved in 2 ml of 0.01 M phosphate buffer (pH 7.4) and dialyzed against the same buffer. This preparation was used as the source of biliverdin reductase (2).

Assay of microsomal heme oxygenase. The incubation mixture contained in a final volume of 150 µl: 10 µmol of phosphate buffer (pH 7.4), 0.5 µmol of glucose-6-phosphate, 1 µmol of $MgCl_2$, 20 µl (10 mg/ml) of glucose-6-phosphate dehydrogenase, 40 nmol of NADPH, 50 µl of the microsomal preparation (12-15 mg of protein/ml), 25 µl of biliverdin reductase, and 50 nmol of substrate. The incubations were carried out at 37°C during 15 min. Blanks were simultaneously run omitting either NADPH, enzyme or substrate. The latter were added at the end of the incubations to each of the respective blanks. Bilirubin formation was measured as follows after pooling two incubation mixtures: glycine-HCl buffer (pH 1.8, 2.4 ml) was added, followed by 0.6 ml of a saturated NaCl solution containing ascorbic acid (100 mg/ml), and 100 mg of solid NaCl. The mixture was extracted with chloroform (2 × 1 ml), the extracts were pooled, and the absorbance of the chloroform solution at 455 nm (peak of bilirubin absorption and at 520 nm was measured. The difference between the absorbance values at 455 and 520 nm was proportional to the bilirubin concentration. An $\varepsilon(CHCl_3) = 43\ mM^{-1}\ cm^{-1}$ was thus determined using a standard bilirubin solution. The absorbance values of the chloroform extracts of the blank runs were subtracted from those of the incubations to estimate the amount of bilirubin formed by the enzymatic oxidation. The chloroform extraction, performed as described above, recovered 85-90% of the bilirubins present in the incubation mixture whereas 25-30% of the remaining hemins present in the latter were also extracted. Bilirubins were also measured by evaporating the chloroform extracts followed by tlc analysis of the residues

on silica-gel plates (chloroform:methanol:water, 48:28:6). Bilirubins run with high R_f and different isomers could be distinguished. Tlc was performed under dim light, the bilirubin bands were eluted with 10% methanol in chloroform, and the bilirubin concentration in the eluates was determined by the absorbance measurements as described above.

RESULTS

Chemical oxidation of synthetic hemins: identification of biliverdin isomers. The synthetic hemins 4-6 were oxidized in aqueous pyridine with ascorbate as the "oxygen-activating" reductant using the procedure described for hemin IX 1 (9). The oxidation of hemin XIII 4 is representative of the procedure used to identify the isomers formed. Hemin XIII 4 gave biliverdins XIII α 7 (30%), XIII β and δ (55%) and XIII γ (15%). They were separated as the dimethyl esters using two dimensional tlc on silica-gel plates (Merck, 20 × 20 cm, 0.25 mm layer thickness; solvent A: 5% acetone in chloroform; solvent B: chloroform:acetone:propionic acid, 48:12:11). Biliverdin XIII α 7 (dimethyl ester) had R_f, 0.42 (5% acetone in chloroform); mass spectrum [see (9) for fragmentation pattern)]: (m/e), 610(M^+), 313 (30%), 300(56%). Biliverdin XIII γ (dimethyl ester) had R_f 0.49; (m/e), 610(M^+), 313(51%), 311(4%), 300(13%). Biliverdin β and δ (dimethyl ester) had R_f 0.55; (m/e), 610(M^+), 360(11%), 253(13%), 251(12%). Biliverdin XIII α 7 was reduced to bilirubin XIII α 10, biliverdin XIII β was reduced to bilirubin XIII β 13, biliverdin XIII γ was reduced to bilirubin XIII γ 14, and the bilirubins were transformed into their azopigments (Fig. 2) using a known procedure (11) with the following modifications. The reaction with the diazotized ethyl anthranilate was carried out during 90 min. The azopigments were separated from the unreacted bilirubin by tlc using 15% methanol in chloroform as solvent. For separating azopigment 15, the latter solvent was used until the solvent front advanced 6 cm above the starting line. The plate was dried and was then placed in chloroform: methanol:water, 62:25:3, for a second full run (18 cm). The azopigment bands were eluted from the silica with methanol, esterified with diazomethane, and purified by tlc on silica using 10% methanol in chloroform as a first solvent (solvent front, 1 cm above starting line), followed by 10% ethyl acetate in benzene (solvent front, 18 cm above starting line). R_f and m/e values were as follows: Azopigment 16, R_f 0.84; mass spectrum, m/e 416(M^+). Azopigment 15, R_f 0.54; m/e, 476(M^+), identical with a sample obtained from bilirubin IX α (11). Azopigment 17, R_f 0.47; m/e 476(M^+), identical with a sample obtained from biliverdin IX γ (11). Azopigment 18, R_f 0.21; m/e 536(M^+), identical with a sample obtained from bilirubin IX δ (11).

The analogous chemical oxidation of hemin III 5 and of iron-porphyrin 6 also gave three biliverdin isomers derived from each of them. Isomer identity was established following the procedures described above.

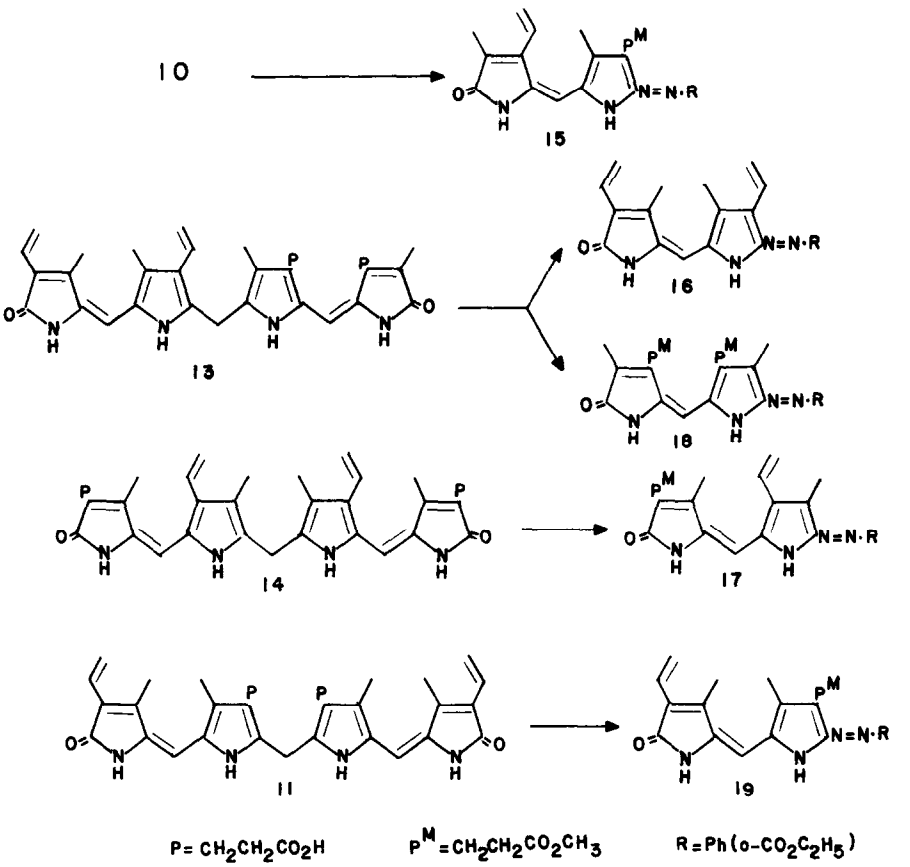

Fig. 2. Azopigment analysis of bilirubins XIII α, XIII β, XIII γ, and III α.

TABLE I: Enzymatic oxidation of hemin IX and synthetic analogs by microsomal heme oxygenase

Substrate	Reducing agent	Bilirubin formed (nmol/min)	Substrate transformed (%)
Hemin IX 1	NADPH	13.5	27
	NADH	5.6	11
Hemin XIII 4	NADPH	23.7	47
	NADH	13.5	27
Hemin III 5	NADPH	35	70
	NADH	16.4	32
Iron-porphyrin 6	NADPH	31.3	63

The incubation mixture and conditions were as described in Materials and Methods. Bilirubins were assayed by using the tlc procedure (see Methods).

Enzymatic oxidation of hemins XIII 4 and III 5 and of iron-porphyrin 6. Heme oxygenase was found to oxidize not only the natural hemin IX 1, but also a number of synthetic analogs (Table I). Hemins XIII 4 and III 5, and even the

iron-porphyrin 6 were better substrates than the natural hemin IX 1. Thus, substrate specificity of heme oxygenase is not limited to the substituent array found in heme IX 1 and does not require the presence of vinyl residues in the substrate. Although the three synthetic substrates share the natural array of substituents in rings C and D, the array of substituents of rings A and B differs from the natural pattern. Therefore, the inductive and steric effects exerted by the substituents on the methine bridges α, β, and δ of the synthetic analogs greatly differ from those exerted by the natural substrate. Biliverdin reductase also has no great substrate specificity, since in every case the corresponding bilirubins were obtained. Biliverdins IX α-δ were all found to be substrates of the reductase, and biliverdins XIII α, XIII β, and XIII γ were even better substrates. The methyl esters of biliverdins were not substrates of the reductase.

When NADH replaced NADPH in the incubation mixture the hemins were also oxidized, although to a lesser extent (Table I).

Selectivity of the enzymatic bridge cleavage. The bilirubins obtained by the enzymatic oxidation of 1, 4, 5 and 6 were transformed into their azopigments as described above. The resulting azopigments were compared with those prepared from the bilirubins obtained from 4-6 by the chemical coupled oxidation method (see above). From bilirubin IX α 3, azopigments 15 and 19 were obtained (see Fig. 2). The bilirubin obtained from hemin XIII 4 gave only azopigment 15 and was therefore bilirubin 10. The bilirubin obtained from hemin III 5 gave only azopigment 19 and was therefore bilirubin 11. It is evident that heme oxygenase cleavage of the isomeric hemins takes place at the methine bridge α. A possible oxidation at the other methine bridges would give a mixture of azopigments derived from the isomeric bilirubins (Fig. 2).

To confirm further the bridge selectivity of the enzymatic oxidation, the oxidation of [α-^{14}C]6 was assayed (Table II). The bilirubin 12 was isolated and was devoid of radioactivity, whereas the recovered iron porphyrin 6 (30%

TABLE II: Specificity of the enzymatic bridge cleavage

Enzymatic system	Incubation time (min)	Total radioactivity in the incubation (dpm/min)	CO loss (%)	Bilirubin 12 (nmol)	Bilirubin 12 (dpm/min)
-NADPH	10	17,600	--	none	13*
Complete	10	7,050	60	28	10
-NADPH	30	17,700	--	none	10
Complete	30	4,170	76	38	14

The incubations were performed as described in Materials and Methods using [α-^{14}C]6 (50 nmol, sp.act., 3.5×10^5 dpm/ mol).

*Background dpm. Radioactivity was measured in a liquid scintillation counter using Bray's solution.

recovery) retained its original specific activity. The structure of bilirubin 12 was verified by the azopigment analysis following the described procedures. Similar results were obtained when [α-^{14}C]hemin XIII 4 was oxidized.

DISCUSSION

The enzymatic oxidation of 4 to 6 indicate that heme oxygenase has no strict substrate specificity for iron porphyrins, at least when the array of substituents on rings C and D are those of the natural substrate 1. Of special significance is the oxidation of 5, which is the "type II" hemin. It is well known that coproporphyrinogen II (the formal metabolic precursor of 5) is not oxidized by coproporphyrinogen oxygenase, which thus restricts metabolic heme formation to the type III and IV porphyrins (13). This limitation is apparently not valid for heme degradation. The lack of substrate specificity of biliverdin reductase suggests that any biliverdin isomer present in the biological media in mammals will be readily excreted as its bilirubin derivative. In support of this assertion, we found that the $CoCl_2$ treatment also induced the activity of biliverdin reductase

The enzymatic oxidation of heme has an α-bridge selectivity, which is independent of the flanking substituents. It is the array of substituents which in the normal substrate (hemin IX 1) provides the structural environment of the α-methine, that is present in the synthetic hemin XIII 4 around the β- and δ-methines. In spite of this structural resemblance of the β and δ methines in 4, the enzymatic oxidation of 4 takes place at the α-methine. The chemical oxidation of 4 to 6 gives all the possible biliverdin isomers and is therefore not different from that of 1.

The hemins 1, 4, 5, and 6 were enzymatically oxidized at the α-methine bridge although they were not part of a hemoprotein.

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