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Specificity of Heme Oxygenase: A Study with Synthetic Hemins†

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ABSTRACT: A large number of synthetic iron porphyrins were enzymatically oxidized by a microsomal heme oxygenase preparation from rat liver. They all had in common two vicinal propionic acid residues at C_6 and C_7. Iron porphyrins of type I were not substrates of the enzyme. Iron porphyrins that carried electron-withdrawing substituents (acyl residues) at C_2 and C_4 were substrates of heme oxygenase, although the product yields were reduced. Several iron porphyrins, such as hemin XIII (**4**) and hemin III (**5**), were better substrates of heme oxygenase than the natural substrate hemin IX (**1**). The enzymatic oxidation was selective for the α-methine bridge, and the α-biliverdins obtained were reduced by Biliverdin reductase to the corresponding α-bilirubins. Preincubation of the enzymatic system with hemin IX (**1**) and hemin XIII (**4**) in the absence of NADPH resulted in an inhibition of their oxidation. The iron-free porphyrins which carried two vicinal propionic acid residues at C_6 and C_7 were also found to be inhibitors of the enzymatic system when preincubated with the latter. The presence of hematochemin IX (**18**) suppressed the enzymatic oxidation of hemin IX (**1**).

Heme IX (**1**) (Figure 1), the prosthetic group of hemoglobin and of a large number of hemoproteins, is one of the most abundant compounds of the general metabolism. It is degraded by a heme oxygenase which oxidizes the α-methine bridge of heme IX with the formation of biliverdin IX-α (**2**) (Tenhunen et al., 1969). Heme oxygenase is a membrane-bound enzyme, and it has been recently solubilized and purified (Yoshida & Kikuchi, 1979; Maines et al., 1977).

The enzymatic reduction of biliverdin IX-α (**2**) by a biliverdin reductase gives rise in mammals to bilirubin IX-α (**3**), which is the overwhelming bulk of biliary bilirubin (Figure 2). The enzymatic oxidation of the α-methine bridge is highly specific, although some minor traces of the other bilirubin isomers (IX-β, IX-γ, and IX-δ) have been detected (Blumenthal et al., 1977). The enzymatic oxidation of **1** poses two questions, the first with regard to the substrate specificity of heme oxygenase and the second with regard to the mechanism which leads to the specific oxidation of the α-methine bridge. We have recently shown (Frydman et al., 1979) that heme oxygenase oxidizes not only heme IX but also several isomeric hemins and that the oxidation was specific for the α-methin bridge. We therefore prepared by synthesis a series of iron porphyrins to make a systematic study of both problems, substrate specificity and methine bridge selective oxidation.

The question of the selective oxidation at the α-methine bridge has been extensively discussed (O'Carra, 1975). It stands in striking contrast with the chemical oxidation of heme IX which gives rise to all four possible isomers of biliverdin IX (Bonnett & McDonagh, 1973). In the chemical oxidation, the biliverdin IX-α is formed in a slight excess over the other isomers. It has been proposed that the enzymatic α specificity arises as a result of intrinsic electronic features of the heme IX molecule itself (Lemberg, 1956; Pullman & Perault, 1959), which would favor a preferential attack at the α bridge. A second interesting proposal was that the α specificity is a result of the interaction of heme with the heme binding sites in the different hemoproteins in such a manner that only the α bridge is oxidized (O'Carra, 1975). Support for this proposal was found by carrying out chemical oxidations of anomalous hemoglobins which resulted in biliverdin IX isomers of various types (Brown & Docherty, 1978). The third proposal suggested that the active site of heme oxygenase imposes the α specificity of the cleavage (Yoshida & Kikuchi, 1978). Our preliminary work (Frydman et al., 1979) indicated that hemins XIII (**4**) and III (**5**) (Figure 3), which are isomers of hemin IX (**1**) (Figure 1), were substrates of heme oxygenase. Both **4** and **5** carried the two vicinal propionic acid residues at C_6 and C_7. The present study was therefore performed by using synthetic iron porphyrins which carried two vicinal propionic acid residues at C_6 and C_7 (Figures 1 and 3). The iron porphyrins **6** and **7** (Figure 3), which lacked this feature, were also prepared and assayed as substrates. The iron 2,4-diacetyl-, 2,4-dipentanoyl-, 2,4-diisopentanoyl-, and 2,4-dilauroyldeuteroporphyrins IX (**8**, **9**, **10**, and **11**, respectively), which carried electron-withdrawing substituents, enabled us to examine the possible electronic effects on the substrate of heme oxygenase.

The enzymatic oxidations of these hemins were carried out with a rat liver microsomal heme oxygenase which was induced by cobaltous chloride. The use of the synthetic iron porphyrins also allowed us to examine the specificity of biliverdin reductase on the different biliverdins formed during the oxidation of the former.

Materials and Methods

Materials. Hemin IX and bilirubin IX-α were purchased from Sigma Chemical Co. and were purified before use. Protoporphyrin XIII (**4**) (iron-free porphyrins will have the same numbering as their iron dervatives), protoporphyrin III

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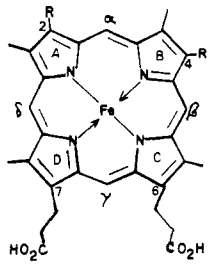
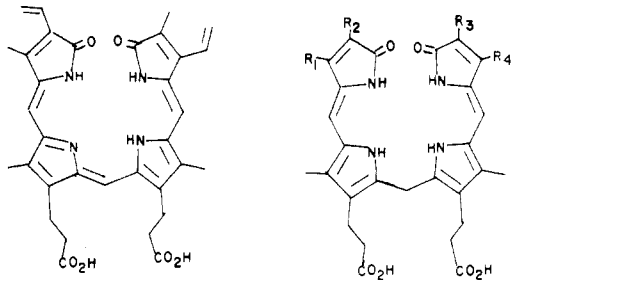


1, R = $CH{=}CH_2$
8, R = $COCH_3$
9, R = $CO(CH_2)_3CH_3$
10, R = $COCH_2CH(CH_3)_3$
11, R = $CO(CH_2)_{10}CH_3$
13, R = H
16, R = $CH_2CH_2CO_2H$
18, R = $CHOHCH_3$
19, R = $CHOH(CH_2)_3CH_3$
20, R = $CHOHCH_2CH(CH_3)_2$
21, R = $CHOH(CH_2)_{10}CH_3$
22, R = $CH{=}CH(CH_2)_2CH_3$
23, R = $CH{=}CHCH(CH_3)_2$
24, R = $CH{=}CH(CH_2)_9CH_3$
25, R = C_2H_5
26, R = $CH_2(CH_2)_3CH_3$
27, R = $CH_2CH_2CH(CH_3)_2$
28, R = $CH_2(CH_2)_{10}CH_3$

FIGURE 1: Hemin IX synthetic analogues.



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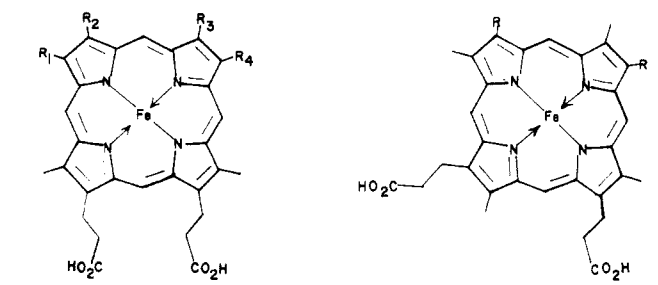
3, $R_1 = R_3 = CH_3$; $R_2 = R_4 = CH{=}CH_2$

29, $R_1 = R_4 = CH{=}CH_2$; $R_2 = R_3 = CH_3$

FIGURE 2: Biliverdins and bilirubins.



4, $R_1 = R_4 = CH{=}CH_2$; $R_2 = R_3 = CH_3$
5, $R_1 = R_4 = CH_3$; $R_2 = R_3 = CH{=}CH_2$
12, $R_1 = R_4 = CH_2CH_2OH$; $R_2 = R_3 = CH_3$
15, $R_1 = R_4 = CH_3$; $R_2 = R_3 = CH_2CH_2CO_2H$
17, $R_1 = R_4 = CH_2CH_2CO_2H$; $R_2 = R_3 = CH_3$

7, R = CH_2CH_2OH
14, R = $CH_2CH_2CO_2H$

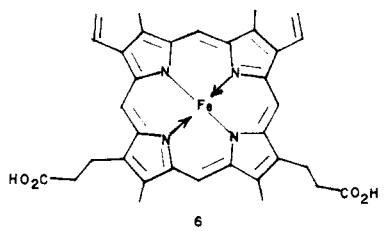


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FIGURE 3: Synthetic hemins.

(**5**), and 1,4-di(β-hydroxyethyl)porphyrin (**12**) were prepared as described elsewhere (Buldain et al., 1977). Deuteroporphyrin IX (**13**) and 2,4-diacetyldeuteroporphyrin IX (**8**) were prepared by synthesis (Brockmann et al., 1968).

Coproporphyrin isomers I (**14**), II (**15**), III (**16**), and IV (**17**) were obtained by synthesis (Díaz et al., 1979). 2,4-Dipentanoyldeuteroporphyrin IX (**9**), 2,4-diisopentanoyldeuteroporphyrin IX (**10**), and 2,4-dilauroyldeuteroporphyrin IX (**11**) were prepared by acylation of deuteroporphyrin IX (**13**) under Friedel–Crafts reaction conditions. They were reduced to the corresponding hematoporphyrins with sodium borohydride. Hematoporphyrin IX (**18**) was obtained by reduction of 2,4-diacetyldeuteroporphyrin IX (**8**). The hemato derivatives **19**–**21** were dehydrated to the corresponding alkenylporphyrins **22**–**24** by treatment with benzoyl chloride in dimethylformamide. The alkenyl side chains were reduced with hydrogen over 10% palladium on charcoal, affording the corresponding alkylporphyrins **25**–**28**.

2,3,6,7-Tetramethyl-1,4-divinyl-5,8-bis(β-carboxyethyl)-porphyrin (**6**) was prepared by condensation of 5,5′-dicarboxy-3,3′-dimethyl-4,4′-bis(β-hydroxyethyl)dipyrrylmethane (Buldain et al., 1977) with 5,5′-diformyl-3,3′-dimethyl-4,4′-bis(β-methoxycarbonylmethyl)dipyrrylmethane following the synthetic outline used to prepare the other protoporphyrins (Buldain et al., 1977). 1,3,5,7-Tetramethyl-2,4-bis(β-hydroxyethyl)-6,8-bis(β-carboxyethyl)-porphyrin (**7**) was prepared by condensation of 5′-(*tert*-butyloxycarbonyl)-5-formyl-3,4′-dimethyl-4,3′-bis(β-acetoxyethyl)dipyrrylmethane with 5-(*tert*-butyloxycarbonyl)-3,4′-dimethyl-4,3′-bis(β-methoxycarbonylethyl)dipyrrylmethane to give the corresponding b-bilene, following the synthetic approach outlined elsewhere (Díaz et al., 1981). The latter was then cyclized with triethyl orthoformate in acid medium to give porphyrin **7** after suitable transesterifications (A. Cellino, A. Valasinas, and B. Frydman, unpublished results).

The hemins of the above-mentioned porphyrins were prepared by incorporating iron following an established procedure (Cavaleiro et al., 1974), with the following modifications. When the hemin dimethyl ester was obtained, it was purified by low-pressure liquid chromatography on a column packed with TLC silica gel 60 (Merck) using 5% methanol in chloroform as a solvent. After saponification with 1 M KOH in methanol, the hemin was again purified by the same procedure using a mixture of chloroform/methanol/water (48:28:6 v/v).

[α-^{14}C]Protoporphyrin XIII (**4**) was prepared by synthesis following the described method (Buldain et al., 1977). The necessary [2-^{14}C]ethyl 2,3-dimethyl-4-(β-ethoxycarbonylethyl)-5-pyrrolecarboxylate was obtained by reductive alkylation of the α-unsubstituted pyrrole with [^{14}C]paraformaldehyde.

All the reagents and solvents used were of analytical grade. TLC was performed on precoated silica gel 60 F254 plates (Merck, 0.25-mm-layer thickness).

Mass spectra were determined at UMIMFOR (Buenos Aires) with a CH-7 mass spectrometer. All the new porphyrins and hemins were identified by their mass spectra, their NMR spectra, and, when necessary, their analytical composition.

Methods. Microsomal Heme Oxygenase from Rat Liver. Wistar albino female rats (180–200 g) were injected subcutaneously with a single dose of cobaltous chloride (160 mg/kg) and were later fasted for 19 h. They were then anesthetized with ether, and the bleached livers were excised and repeatedly washed with an ice-cold saline solution. All further operations were carried out at 0–4 °C. The livers were homogenized in a Potter–Elvehjem-type glass homogenizer in 3 volumes of an ice-cold 0.25 M sucrose solution containing 50 mM phosphate buffer (pH 7.4). The homogenate was centrifuged at 20000*g* for 15 min, and the supernatant was further centrifuged at 105000*g* during 60 min. The microsomal pellet was then

resuspended in 1.5 volumes of 0.15 M KCl in 50 mM phosphate buffer (pH 7.4) and was again centrifuged at 105000*g* for 30 min. This second pellet was then resuspended in the same buffer to a volume so as to obtain a protein concentration of 15 mg/mL of suspension. This enzyme preparation was used as the microsomal heme oxygenase. It was usually used during the first 48 h when its activity with the synthetic iron porphyrin was assayed. Its activity usually decreased to about 50% after 5-days storage at −20 °C.

Biliverdin Reductase from Rat Liver. The 105000*g* supernatant obtained from the microsomal preparation was fractionated by addition of ammonium sulfate (AS), and the 40–60% AS fraction was dissolved in 10 mM phosphate buffer (pH 7.4) and dialyzed against the same buffer. It was used as the biliverdin reductase (Tenhunen et al., 1970).

Assay of Microsomal Heme Oxygenase. The incubation mixture contained (unless otherwise stated), in a final volume of 225 μL, 10 μmol of either phosphate or Tris-HCl buffer (pH 7.4), 100 nmol of NADPH, 50 μL of the microsomal heme oxygenase, 50 μL of biliverdin reductase (0.42 mg of protein, 44 nmol of bilirubin formed/5 min), and 32 nmol of the substrate iron porphyrin. The incubation was usually carried out for 15 min at 37 °C. This incubation time afforded good yields of bilirubin, although with some of the substrates the time courses were not linear. Blanks omitting either NADPH, enzyme, or substrate were run simultaneously. The latter were added at the end of the incubation. The incubations were stopped by adding 1 mL of glycine–HCl buffer (pH 1.8) to each tube, followed by 0.3 mL of a NaCl saturated solution containing 100 mg/mL ascorbic acid and 50 mg of solid NaCl. The mixture was extracted with 1 mL of chloroform and centrifuged, and the clear chloroform extract was separated. The absorption of this chloroform solution was read at 455 (peak of most bilirubins absorption) and 520 nm. The difference between the absorbance values at 455 nm (440 nm in the case of hematobilirubin IX) and the absorbance at 520 nm was proportional to the bilirubin concentration. The blanks gave no Δ455–520 nm. A standard concentration curve of bilirubin was obtained by using variable amounts of bilirubin IX-α (**3**) and submitting the bilirubin solutions to the same extraction procedure as that described above. The concentration of bilirubin in the different chloroform extracts gave a linear plot which was used as the standard plot. The above-mentioned method of extraction recovered around 90% of the bilirubin present in the incubation mixtures.

Bilirubins were also measured by evaporating the chloroform extracts, followed by TLC analysis on silica gel plates (solvent chloroform/methanol/water, 48:28:6; runs of 5 cm were performed). Bilirubin IX-α ran with an R_f of 0.90 whereas hematobilirubin IX-α had an R_f of 0.70. The bilirubins were eluted from the silica with 10% methanol in chloroform, and the bilirubin concentration was measured by the difference between the absorbance of the maximum peak of the corresponding bilirubin and the absorbance at 520 nm. The determinations of bilirubins carried out by both procedures (direct measurement in the chloroform extracts and elution of TLC) gave similar values. The TLC analysis also indicated that no biliverdins remained among the reaction products. Therefore biliverdin reductase was not rate limiting when the heme oxygenase activity on the synthetic iron porphyrins was measured. On the other hand, the biliverdins obtained by the chemical oxidation of the latter were good substrates of biliverdin reductase.

The assay was carried out by using freshly prepared solutions of the iron porphyrins. Aging or storage of the iron porphyrin solutions resulted in a strong decrease of their substrate capacity. All the compounds listed in a specific table were assayed with the same enzyme preparation.

Table I: Structural Requirements of the Substrate of Heme Oxygenase[a]

substrate	bilirubin formed [nmol (mg of protein)$^{-1}$ (15 min)$^{-1}$]	rel act. (%)
hemin IX (**1**)	21.1	100
hemin XIII (**4**)	25.6	121
hemin III (**5**)	31.0	147
deuterohemin IX (**13**)	10.0	48
iron bis(β-hydroxyethyl)porphyrin XIII (**12**)	37.0	175
iron porphyrin (**6**)	0	
iron 6,8-bis(β-carboxyethyl)porphyrin (**7**)	0	
iron coproporphyrin III (**16**)	2.1	10
iron coproporphyrin IV (**7**)	0	
iron coproporphyrin I (**14**)		
zinc protoporphyrin IX	0	

[a] The incubation mixtures were prepared as described under Materials and Methods. The relative activities are related to hemin IX (taken as 100% oxidation).

Results

Substrate Specificity of Heme Oxygenase. Heme oxygenase resembles a mixed-function oxidase in its requirement for a reducing agent and O_2 to oxidize its substrate. Since mixed-function oxidases have a rather broad substrate specificity, the latter was examined for heme oxygenase with the help of a number of synthetic hemins and closely related iron porphyrins. It was found that a relatively large number of the latter are substrates of heme oxygenase. As can be seen from the data summarized in Table I, several synthetic hemins such as hemin XIII (**4**) and hemin III (**5**), were better substrates than the natural hemin IX (**1**). Iron (β-hydroxyethyl)-porphyrin (**12**) [a type XIII porphyrin in Fischer's notation; see Fischer & Orth (1937)] was also a better substrate than hemin IX (**1**).

All these hemins have in common the vicinal propionic acid residue at C_6 and C_7, flanked by the methyl groups at C_5 and C_8. It was conceivable that this array of the propionic acid side chains in the hemin nucleus is the essential structural requirement of the substrates. Two iron porphyrins were prepared by synthesis to put this suggestion to test. Iron porphyrin (**6**) (Figure 3) has the two propionic acid side chains at C_5 and C_8, while the substituents in rings A and B are identical with those of hemin XIII (**4**). Synthetic iron (β-hydroxyethyl)porphyrin (**7**) is a type I hemin. Neither was a substrate of heme oxygenase (Table I), thus lending support to the aforementioned suggestion that the vicinal propionic acid residues are essential structural requirements for a hemin to be a substrate of heme oxygenase.

Iron coproporphyrin III (**16**) (Figure 1) was a very poor substrate of heme oxygenase, while iron coproporphyrin IV (**17**) (Figure 3) was not. Therefore heme oxygenase is rather specific for dicarboxylic iron porphyrins. Iron coproporphyrin I (**14**) was not a substrate.

The methyl or ethyl esters of all the above-mentioned iron porphyrins as well as the iron-free porphyrins were not substrates of the enzyme. Zinc protoporphyrin IX, although it is a natural metalloporphyrin (Lamola & Yamane, 1974), was not oxidized by heme oxygenase.

The oxidation products of the aforementioned iron porphyrins were the corresponding bilirubins (Table I). This was

Table II: Oxidation of Iron Acylporphyrins by Heme Oxygenase[a]

substrate	bilirubin formed [nmol (mg of protein)$^{-1}$ (15 min)$^{-1}$]	rel act. (%)
hemin IX (1)	37.5	100
iron 2,4-diacetyldeuteroporphyrin IX (8)	17	45
iron 2,4-dipentanoyldeuteroporphyrin IX (9)	13.8	37
iron 2,4-diisopentanoyldeuteroporphyrin IX (10)	10.3	27
iron 2,4-dilauroyldeuteroporphyrin IX (11)	17	45

[a] The incubation mixtures were prepared as described under Materials and Methods. The relative activities are related to hemin IX (taken as 100% oxidation).

due to the presence of biliverdin reductase in the incubation mixture and indicated that the specificity of the reductase is not confined to the biliverdin IX isomers. For examination of whether the electronic effects affect the substrate specificity, a number of iron porphyrins of type IX carrying acyl substituents were assayed as substrates of heme oxygenase (Table II). The somewhat surprising result was that all served as substrates of heme oxygenase and formed the corresponding bilirubins. Although they were poorer substrates than the natural hemin IX, they were comparable to 2,4-deuterohemin IX (**13**) (Table I). Iron 2,4-diacetyldeuteroporphyrin IX (**8**) and the 2,4-dilauroyl homologue **11** were as good substrates as detuerohemin IX (**13**), while the iron 2,4-dipentanoyl- and 2,4-diisopentanoylporphyrins (**9** and **10**, respectively) were failry good substrates. Therefore, although the electron-withdrawing substituents decrease the capacity of the iron porphyrins to undergo electrophilic oxidation, their effect is not more decisive than the absence of the vinyl side chains of hemin IX.

The data of Table II suggested that the presence of hydrophobic side chains in the iron porphyrin structure favored its oxidation by heme oxygenase. The results obtained with deuterohemin IX (**13**) also indicated that the absence of substituents at C_2 and C_4 resulted in a poorer substrate of the enzyme. For evaluation of the relative contributions of the substituents at C_2 and C_4, the substrate capacities of hemin IX (**1**), its meso derivative **25**, hematohemin IX (**18**), and iron 2,4-diacetylporphyrin (**8**) (Figure 1) were compared (Table III). The results showed that the reduction of the vinyl residues decreased substrate capacity, that hematohemin **18** was a good substrate, and that the 2,4-diacetyl derivative **8** was the poorest substrate. In the 2,4-diisopentyl series as well as in the 2,4-dipentyl series, the alkenyl derivatives were also the best substrates when compared to their meso, hemato, or acyl analogues. This effect was not evident in the 2,4-dilauryl series (Table III). The 2,4-dipentyl and 2,4-dilauryl homologues of hematoporphyrin were not substrates of the enzymatic system. A comparison of the 2,4-dilauryl series and the 2,4-dipentyl series showed that the hydrophobic side chains favored the substrate capacity of the iron porphyrins. This could be due to the fact that a membrane-bound enzyme (the membrane also contained the electron transport chain) was being assayed.

Specific Inhibition of Heme Oxygenase by Hemins and Porphyrins. When the microsomal heme oxygenase preparation was preincubated with hemin IX (**1**) [or with hemin XIII (**4**)], in the absence of NADPH, and the latter was added after the preincubated period, the enzymatic oxidation of the hemin was found to be inhibited (Table IV). This inhibitory

Table III: Effect of the Substituents at C_2 and C_4 on the Substrate Capacity of Hemin IX Derivatives[a]

substrate	bilirubin formed [nmol (mg of protein)$^{-1}$ (15 min)$^{-1}$]	rel act. (%)
hemin IX (1)	37.5	100
mesohemin IX (**25**)	31.0	83
hematohemin IX (**18**)	32.0	86
2,4-diacetyldeuterohemin IX (**8**)	16.9	45
2,4-dipentenyldeuterohemin IX (**22**)	17	45
2,4-dipentyldeuterohemin IX (**26**)	10.5	28
2,4-bis(α-hydroxypentyl)deuterohemin IX (19)	1.7	4
2,4-dipentanoyldeuterohemin IX (9)	13.9	37
2,4-diisopentenyldeuterohemin IX (**23**)	19.0	50
2,4-diisopentyldeuterohemin IX (**27**)	14.6	39
2,4-bis(α-hydroxyisopentyl)deuterohemin IX (**20**)	18.0	48
2,4-diisopentanoyldeuterohemin IX (10)	10.1	27
2,4-dilaurenyldeuterohemin IX (**24**)	17	45
2,4-dilauryldeuterohemin IX (**28**)	13.6	36
2,4-bis(α-hydroxylauryl)deuterohemin IX (**21**)	0	
2,4-dilauroyldeuterohemin IX (11)	16.9	45

[a] The incubation mixtures were prepared as described under Materials and Methods. The relative activities are expressed by relating them to hemin IX (taken as 100% oxidation). The buffer used was Tris-HCl (pH 7.4).

Table IV: Inhibitory Effect of Hemin IX (1) and of Hemin XIII (4) on Their Enzymatic Oxidations[a]

system preincubated with hemin	nmol	added after preincubation	bilirubin formed [nmol (mg of protein)$^{-1}$ (15 min)$^{-1}$]	inhibition (%)
		hemin IX	34	
IX	3.2	hemin IX	23	30
IX	8.1	hemin IX	11	67
IX[b]	8.1	hemin IX	6.8	80
XIII	3.2	hemin IX	3.4	90
XIII	8.1	hemin IX	0	100
		hemin XIII	37	
IX	3.2	hemin XIII	26	30
XIII	3.2	hemin XIII	3.4	90
XIII	8.1	hemin XIII	0	100

[a] In a final volume of 150 μL, the preincubated system contained phosphate buffer, pH 7.4 (10 μmol), microsomal heme oxygenase (50 μL), biliverdin reductase (50 μL), and hemin. Preincubations were carried out for 5 min at 37 °C. After preincubation, the system was completed by addition of NADPH (100 nmol) and the indicated hemins (32 nmol), and the incubations were carried out for 15 min at 37 °C. The same results were obtained if biliverdin reductase was added during the incubation. [b] Preincubated for 15 min at 37 °C.

effect exerted by the hemins by preincubation was dependent on the concentration of the hemin used (higher hemin concentrations produced stronger inhibitory effects) and of the duration of the preincubation (longer preincubation periods produced stronger inhibitions). Preincubations with hemin IX (**1**) inhibited its own enzymatic oxidation as well as that of hemin XIII. Hemin XIII (**4**) also inhibited its own oxidation and that of hemin IX (**1**). The inhibitory effect of hemin XIII (**4**) was stronger than that exerted by hemin IX (**1**) at the same concentration (Table IV).

The inhibition in the oxidation of a hemin produced by its preincubation with the microsomal enzymatic system in the

Table V: Specific Inhibitory Effect of Porphyrins on Heme Oxygenase[a]

addition	nmol	preincubation: bilirubin formed [nmol (mg of protein)$^{-1}$ (15 min)$^{-1}$]	preincubation: inhibition (%)
		21.2	
protoporphyrin IX (1)	1.8	3.4	84
	3.6	0	100
zinc protoporphyrin IX	1.6	5.0	76
	3.2	1.7	90
2,4-diacetyldeuteroporphyrin IX (8)	1.7	5.1	76
	3.4	5.1	76
deuteroporphyrin IX (13)	2.0	12.0	43
	10.0	6.8	68
coproporphyrin III (16)	2.8	15.3	27
	10.0	6.8	68
coproporphyrin IV (17)	5.6	10.2	52
	10.0	5.1	76
coproporphyrin II (15)	2.8	10.2	52
	10.0	3.4	84
coproporphyrin I (14)	10.0	21.0	0
2,4-bis(β-hydroxyethyl)porphyrin (7)	18	21.0	0

[a] The conditions used were the same as those described for Table IV, except that Tris-HCl buffer was used and preincubations were carried out for 15 min at 37 °C. Hemin IX (32 nmol) was used as the substrate. The incubations were carried out in the dark.

absence of NADPH indicates that both hemin and the reducing cofactor must be present simultaneously for the enzymatic reaction to take place. The simplest explanation for this inhibitory effect is that the hemin binds to the active site of the enzyme and remains inaccessible to the reducing agent. If it is accepted that (a) the reducing agent not only produces the reactive oxygen species (as is the case with mixed function oxidases) but also reduces the hemin (Fe^{3+}) to a heme form (Fe^{2+}) which is stabilized by electron-donating residues on the enzyme and (b) the oxidation takes place on that heme-bound protein, then the inhibition obtained by the preincubation with hemin can be explained by its binding to the active site of the enzyme. The presence of the hemin in the enzyme's crevice hinders its reduction to heme (Fe^{2+}) and thus interferes with the conformational change that must take place on the membrane-bound enzyme when heme is formed. Support for this assertion was found in the fact that the inhibition by hemins was highly specific. While deuterohemin IX (**13**), hematohemin IX (**18**), iron 2,4-diacetylporphyrin IX (**8**), iron 2,4-dilauroylporphyrin IX (**11**), and iron 2,4-diisopentanoylporphyrin IX (**10**) inhibited the enzymatic oxidation of hemin IX (**1**), the biologically inert hemin **6** and iron 2,4-bis(β-hydroxyethyl)porphyrin (**7**) (Table I) did not affect the enzymatic oxidation of hemin IX (**1**).

The structural feature that confers to a hemin its inhibitory property on heme oxygenase is not even the presence of the metal but the array of the substituents on the porphyrin nucleus. All the porphyrins which carry the vicinal propionic acid residues at C_6 and C_7 are also inhibitors of the enzymatic oxidation of heme IX. Thus, protoporphyrin IX (**1**), zinc protoporphyrin IX, coproporphyrins II (**15**), III (**16**), and IV (**17**), 2,4-diacetylprotoporphyrin IX (**8**), and deuteroporphyrin IX (**13**) are good inhibitors of heme oxygenase either by preincubation or by direct incubation (Table V). Coproporphyrin I and 2,4-bis(β-hydroxyethyl)porphyrin (**7**) (a type I porphyrin) were noninhibitory. Therefore, the inhibitory effect on heme oxygenase is confined to those porphyrins whose dervatives are usually substrates of the enzyme.

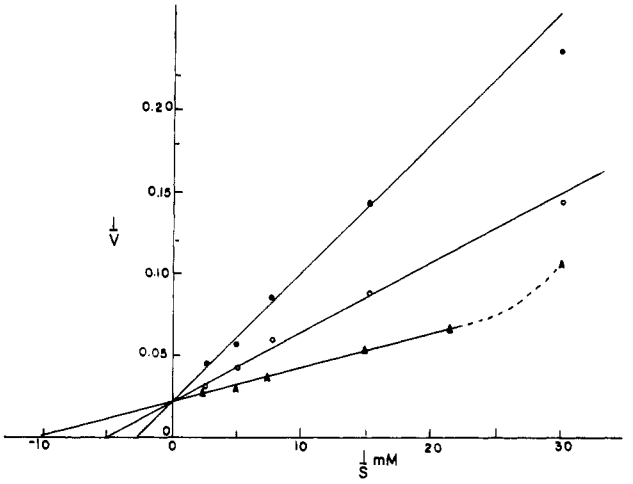

FIGURE 4: Lineweaver–Burk plots showing the reaction rate of heme oxygenase as a function of substrate concentration under standard assay conditions (▲) and in the presence of the competitive inhibitor hematohemin IX (**18**) [6.4 nmol (○) and 12.8 nmol (●)]. Velocities are expressed as nmol of bilirubin formed (mg of protein)$^{-1}$ (15 min)$^{-1}$.

Table VI: Specificity of the Enzymatic Bridge Cleavage of [α-^{14}C]Hemin XIII (4)[a]

system	incubation time (min)	total radioactivity in incubation (dpm/min)	CO loss (%)	bilirubin XIII: nmol	bilirubin XIII: dpm/min
NADPH	10	25 460			28[b]
complete		12 400	51	36	50
NADPH	20	24 500			29
complete		7 800	68	45	15

[a] The incubations were performed as described under Materials and Methods by using [α-^{14}C]hemin XIII (4) (72 nmol, specific activity 3.5×10^5 dpm/μmol). [b] Background disintegrations per minute. Ratioactivity was measured in a liquid scintillation counter using Bray's solution.

A special reference should be made here to the inhibitory effect of hematohemin IX. The inhibitory effect of hematohemin IX on the oxidation of hemin IX is of a competitive nature at low concentrations of hematohemin IX (Figure 4). When higher hematohemin concentrations were used, the bilirubin composition of the products (hematobilirubin IX was separated from bilirubin IX by TLC as described under Methods) indicated that it was almost exclusively hematobilirubin IX and that no oxidation of hemin IX took place.

Specific Oxidation of the α-Methine Bridge. We have previously shown (Frydman et al., 1979) that the enzymatic oxidation of hemin XIII (**4**) and hemin III (**5**) gave only the corresponding α-biliverdins. This demonstration was carried out by reduction of the α-biliverdins to the α-bilirubins and degradation of the latter to the corresponding azo pigments. To further confirm the validity of these results, we prepared the [α-^{14}C]hemin XIII (**4**) and oxidized it enzymatically to bilirubin XIII-α (**29**). The latter was isolated by TLC and was found to be devoid of radioactivity (Table VI). These results confirm the previous data that the enzymatic oxidation of the synthetic hemins and iron porphyrins [iron porphyrin (**12**) was also oxidized at the α-methine bridge (Frydman et al., 1979)] is α selective.

Optimum pH. An optimum pH of 7.4 was found for hemin IX by using Tris-HCl buffer and sodium phosphate buffer. When barbital buffer was used; the heme oxygenase had optimum activity at pH 8.5–9.0. This last buffer as well as Tris-HCl buffer increased heme oxygenase activity by 50% as compared to phosphate buffer. When the synthetic iron

porphyrins were assayed as substrates in the different buffers, it was found that Tris-HCl was the best.

Kinetics. An apparent K_m = 91 μM was found for hemin IX (**1**) in sodium phosphate buffer. A K_m of 125 μM was determined for hemins XIII (**4**) and III (**5**), and for the iron 1,4-bis(β-hydroxyethyl)porphyrin (**12**). The V_{max}'s were 30, 36, 50 and 67 nmol of bilirubin (mg of protein)$^{-1}$ (15 min)$^{-1}$ for hemin IX (**1**), hemin XIII (**4**), hemin III (**5**), and the iron porphyrin **12**, respectively. An apparent K_m of 400 μM and a V_{max} of 100 nmol of bilirubin (mg of protein)$^{-1}$ (15 min)$^{-1}$ were found for iron 2,4-dilauroylporphyrin (**11**).

Effect of Sulfhydryl Compounds. Certain sulfhydryl compounds exerted an interesting effect on the activity of the microsomal-bound heme oxygenase when the different iron porphyrins were used as substrates. Reduced glutathione (5–10 mM) exerted an activating effect on the oxidation of heme IX (**1**) (the activation varied between 20% and 80%), and of deuterohemin **13**. It had no effect when hematohemin **18**, mesohemin **25**, iron 2,4-dilauroylporphyrin (**11**), and the isopentylhemin derivatives (5 mM) were used as substrates. Dithiothreitol and mercaptoethanol (5 mM) exerted a strong inhibitory effect on the enzymatic oxidation of hemin IX and of all the synthetic iron porphyrins used.

Discussion

The results summarized in Tables I, II, and III indicate that dicarboxylic porphyrins which have in their rings C and D the array of substituents of hemin IX (**1**) are substrates of heme oxygenase. Synthetic hemins like hemin XIII (**4**) and hemin III (**5**) are even better substrates than the natural hemin IX. We think that the essential structural feature that makes an iron porphyrin a substrate of heme oxygenase is the presence of the vicinal propionic acid side chains at C_6 and C_7. The presence of strong electron-withdrawing substituents in rings A and B affects only to a lesser degree the substrate capacity of the hemins. The decrease in the formation of the corresponding bilirubins is less than what could be expected of the strong deactivating effect of the acyl side chains (Table II).

There is a kind of balanced hydrophobic effect in the interaction between the substrate and the membrane-bound enzyme. The 2,4-dialkenylhemins are the best substrates of the enzyme, while their meso derivatives are enzymatically oxidized to a lesser degree (Table III). The introduction in the hemin nucleus of strongly hydrophobic chains (2,4-dilaurylhemins) does not necessarily make it a better substrate, although this can be due to steric factors.

The enzymatic oxidation of the hemins is α specific. The determining factor of the selectivity for the α bridge is obviously its location in the iron porphyrin structure opposite to the propionic acid side chains. The electronic contributions of the substituents on the α selectivity can be discarded in view of the formation of bilirubin XIII-α (**29**) and bilirubin III-α (Frydman et al., 1979) during the enzymatic oxidation of hemin XIII (**4**) and hemin III (**5**).

The specific inhibition of heme oxygenase by porphyrins, which have the same sequence of substituents as the hemins derived from them which are substrates of the enzyme (Table V), throws an interesting light on the mechanism of heme oxygenase. It suggests that these porphyrins bind to the active site of the enzyme in which they fit due to their "substrate-like" structure. In the absence of iron or in the presence of another chelating metal, they cannot be oxidized, but they are displaced neither by hemin IX nor by any other iron porphyrins.

Biliverdin reductase was induced together with heme oxygenase when cobaltous chloride was used to induce the latter. (R. B. Frydman, M. L. Tomaro, and B. Frydman, unpublished results). It appears as an enzyme of broad specificity which reduces all the biliverdins formed during the oxidation of the iron porphyrins. We found that it also reduces biliverdins isomeric with the α-biliverdins, although a recent report (Noguchi et al., 1979) informed us that a purified biliverdin reductase was highly specific for biliverdin IX-α.

It can be concluded that the microsomal heme oxygenase system is prepared to dispose of exogenous iron porphyrins by oxidation followed by reduction of the α-biliverdins to the α-bilirubins. Not unlike the other microsomal mixed-function oxidases, it has a specificity defined by certain substrate features, which is nevertheless broad enough to oxidize a large number of closely related compounds which are not part of the normal metabolism.

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