Enzymic conversion of α -oxyprotohaem IX into biliverdin IX α by haem oxygenase

Takeo YOSHINAGA,* Yukari SUDO* and Seiyo SANO†

*Department of Public Health, Faculty of Medicine, Kyoto University, Kyoto 606, Japan, and †Shiga University of Medical Science, Ohtsu, Shiga 520–21, Japan

Conversion of four isomers of *meso*-oxyprotohaem IX into the corresponding biliverdin IX was attempted with a reconstituted haem oxygenase system in the presence of NADPH-cytochrome *c* reductase and NADPH. Only the α -isomer of *meso*-oxyprotohaem IX was converted effectively into biliverdin IX α , which was further reduced to bilirubin IX α by biliverdin reductase. Only trace amounts of biliverdins IX β , IX γ and IX δ were respectively formed from the incubation mixture of the corresponding oxyprotohaemin IX isomers with the complete haem oxygenase system under the same conditions. In a kinetic study, the K_m for α -*meso*-oxyprotohaem IX was 3.6 μ M, which was 2-fold higher than that for protohaem IX. The maximum velocity (V_{max}) of the conversion of α -*meso*-oxyprotohaem IX into biliverdin IX α was twice as fast as that of protohaem IX. These results demonstrate that α -*meso*-oxyprotohaem IX is an intermediate of haem degradation and it was converted stereospecifically into biliverdin IX α via verdohaem IX α .

INTRODUCTION

Haem catabolism is an important biological process, which is catalysed by haem oxygenase (EC 1.14.99.3) and coupled NADPH-cytochrome *c* reductase (EC 1.6.2.4). The enzymic reaction has been studied in several laboratories during the past 20 years, and its main reaction pathway has been elucidated [1–14]. The first step is assumed to be hydroxylation at the porphyrin *meso* position to form an α -oxy derivative [5], which is further converted into biliverdin IX α via verdohaem IX α by two molecules of O₂.

Sano & Sugiura reported in 1982 that Fe(II)-oxy-*meso*-porphyrin-2-methylimidazole complex, a model for the first stable intermediate in the haem oxygenase reaction, showed porphyrin π -radical characteristics and that this compound could be oxidized stepwise to Fe·*meso*-biliverdin by two molecules of O₂ [6]. An Fe(II)-oxy-*meso*-porphyrin derivative was also converted into *meso*-biliverdin IX α in the reconstituted haem oxygenase system [7]. The oxidoreductive cleavage of verdohaem IX α -2-picolin complex to Fe·biliverdin *in vitro* was reported by Itano's group, but the step from α -oxyprotohaemin IX to verdohaem IX was not investigated [8]. Sano and his co-workers succeeded in the synthesis of α -oxyprotohaemin and showed that α -oxyprotohaemin IX was converted into biliverdin IX α with a clear cleavage reaction mechanism using apo-myoglobin and reducing agents [4]. However, the natural enzyme system was not used in that study.

In the present paper we report that the conversion of synthetic α -oxyprotohaemin IX into biliverdin IX α is efficiently catalysed by a reconstituted haem oxygenase system, which is composed of homogeneously purified haem oxygenase, NADPH-cytochrome *c* reductase and NADPH.

MATERIALS AND METHODS

Materials

Protohaemin IX chloride was purchased from Sigma Chemical Co. Four benzoyloxyprotohaemin isomers were synthesized according to the method described by Sano *et al.* [4,9]. For purification, alumina 90 and silica gel 60 precoated plates from Merck (Darmstadt, Germany), silica gel C-300 for chromatography from Wako and an h.p.l.c. column (Cosmosil SSL) from Nacalaitesque (Kyoto, Japan) were used. Biliverdin

IX α , biliverdin IX β , biliverdin IX γ and biliverdin IX δ were synthesized according to the method of Bonnett & McDonagh [10], followed by t.l.c. as described previously [11,12] after esterification with trimethyloxonium tetrafluoroborate [13]. Trimethyloxonium tetrafluoroborate was purchased from Fluka A.G., Buchs, Switzerland, and di-isopropylethanolamine was from Eastman Organic Chemicals.

Preparation of haem-degradation enzymes

Purification of haem oxygenase was performed from 2 kg of fresh bovine spleen according to a previously described method [14]. The purified enzyme was homogeneous and showed the same specific activity as reported previously [14].

NADPH-cytochrome *c* reductase was also purified from bovine liver microsomes according to the method of Yasukochi & Masters [15], with some modifications [14], and showed a specific activity of 120 units/mg of protein.

Biliverdin reductase was prepared from the supernatant fraction of ultracentrifuged bovine spleen homogenate and was partially purified by (NH₄)₂SO₄ fractionation followed by column chromatography through DEAE-cellulose [14,16].

Assay of haem-degradation enzymes

Enzymic activities of haem-degradation enzymes were determined by the method described previously [14] using a Hitachi automatic spectrophotometer (200–20) equipped with a thermal control system. Haem oxygenase activity with α -oxyprotohaem IX as substrate was determined similarly to protohaemin IX. An assay for kinetic analysis was carried out at substrate concentrations of 2, 4, 8, 16 and 32 μ M. The substrates were incubated with 12 units of haem oxygenase, 0.7 unit of NADPH-cytochrome *c* reductase and 9.5 units of biliverdin reductase in the presence or in the absence of 0.8 mM-NADPH in 1.2 ml of 0.1 M-potassium phosphate buffer, pH 7.5, for 5 min at 37 °C, with shaking in the dark. The amount of derived bilirubin was measured by the difference in spectra between the reaction mixtures in the presence and in the absence of NADPH at 468 nm, and then the velocity as enzymic activity was determined.

Difference-spectral experiment

The difference spectra of the conversion of α -oxyprotohaemin IX into biliverdin IX α or bilirubin IX α were measured with a

Shimadzu UV-356 computer-controlled spectrophotometer at 37 °C. At first, the baseline was calibrated with two cuvettes containing 30 μ M- α -oxyprotohaem IX, 44 units of haem oxygenase and 0.7 unit of NADPH-cytochrome *c* reductase in 400 μ l of 0.1 M-potassium phosphate buffer, pH 7.5. Measurement of spectral change was started by the addition of 10 μ l of 0.1 M-NADPH into the sample cuvette and 10 μ l of water into the reference side, followed by the recording of spectra at 2 min intervals. The reduction of biliverdin IX α to bilirubin IX α was started by the addition of 10 μ l of biliverdin reductase (9.5 units) to both cuvettes.

Extraction and esterification of reaction product for h.p.l.c. analysis

Oxyprotohaem isomers (50 nmol) were incubated with 50 units of haem oxygenase, 1.0 unit of NADPH-cytochrome *c* reductase and 2 mM-NADPH in 1.0 ml of 50 mM-potassium phosphate buffer, pH 7.5, for 30 min at 37 °C in the dark with shaking. After incubation, the reaction products were chilled on ice and mixed well with 5 ml of methanol by vigorous bubbling with N₂ gas, then extracted twice with 2 ml of chloroform. Subsequent procedures were performed under N₂ gas to avoid oxidation by air. The chloroform layers were combined and dried with solid anhydrous Na₂SO₄ and evaporated under reduced pressure. The dried residue was dissolved in 1.0 ml of ethanol containing 0.1 ml of water and 15 μ l of di-isopropyl-ethanolamine, and then 5 mg of solid trimethylxonium tetra-fluoroborate was added twice at 5 min intervals. After stirring the mixture for 10 min, 3 ml of water and 3 ml of chloroform was added. The esterified compound was extracted into the chloroform layer, which was washed with deoxygenated distilled water, dried with solid Na₂SO₄ and then the solvent was evaporated. Just before the analysis, samples were dissolved in 30 μ l of methanol/chloroform (1:1, v/v).

H.p.l.c. analysis of products from reaction of α -oxyprotohaem

Analysis of products derived from the incubation of α -oxyprotohaem IX with haem-degradation enzymes was performed with a Hitachi h.p.l.c. apparatus (type 655) equipped with a Shimadzu spectrophotometric detector (SPD-1). The column used was composed of silica (Cosmosil 5SL) (4.6 mm diam. $\times$ 150 mm), and an isocratic solvent system with hexane/methanol/ethyl acetate (902:33:65, by vol.) at a flow rate of 1.0 ml/min was used. Biliverdin IX α as the reaction product was monitored at 375 nm [17].

RESULTS

Spectral change in the conversion of α -oxyprotohaemin IX into biliverdin IX α by complete haem oxygenase system

The spectra of α -oxyprotohaem IX with haem oxygenase and NADPH-cytochrome *c* reductase in reference and sample cuvettes showed an almost straight baseline from 420 nm to 950 nm (Fig. 1, spectra A). Upon addition of NADPH to the sample cuvette, the spectra showed a significant increase in absorption at λ_{max} 650 nm with small shoulders at 630 nm and 688 nm within 4 min (Fig. 1, spectra B). The absorption around 420–520 nm gradually decreased with time. After 15 min, the absorption spectra showed only λ_{max} 650 nm accompanied by a small shoulder at 688 nm. This product with an absorption at λ_{max} 650 nm was tentatively identified as biliverdin IX α and was converted into bilirubin IX α (λ_{max} 468 nm) by biliverdin reductase [18] (Fig. 1, spectra C). The absorption shoulder at 688 nm was assumed to be a reaction intermediate, i.e. α -verdohaem-haem oxygenase complex. Bilirubin IX α in the reaction mixture was extracted into chloroform and identified

Fig. 1. Difference spectra from the conversion of α -oxyprotohaem IX into bilirubin IX α

Spectra A: baseline was obtained between the cuvettes containing 30 μ M- α -oxyprotohaem IX, 44 units of haem oxygenase and 0.7 unit of NADPH-cytochrome *c* reductase in 400 μ l of 0.1 M-potassium phosphate buffer, pH 7.5. Spectra B: measurements of spectra from the conversion of α -oxyprotohaem IX into biliverdin IX α were started by the addition of 10 μ l of 0.1 M-NADPH to the sample cuvette and 10 μ l of water to the reference cuvette, followed by scanning at 2 min intervals. Spectra C: finally the spectral changes from biliverdin to bilirubin were measured by the addition of 10 μ l of biliverdin reductase (9.5 units) and scanned at 2 min intervals. Arrows show the time course.

by comparison with an authentic sample on the basis of its absorption spectrum (λ_{max} 454 nm).

H.p.l.c. analysis of the product of α -oxyprotohaemin IX incubated with the complete haem oxygenase system

The reaction product derived from α -oxyprotohaemin IX incubated with the haem-degradation enzymes (without biliverdin reductase) was extracted with chloroform under N₂, followed by methyl esterification (see the Materials and methods section). H.p.l.c. analysis showed that the product was found to be exclusively biliverdin IX α dimethyl ester (Fig. 2, profile B). It was shown that more than 12 nmol of biliverdin IX α was formed in the complete haem oxygenase system from 30 nmol of α -oxyprotohaem IX after a 30 min incubation, but less than 1 nmol of biliverdin was formed in the absence of NADPH (Fig. 2, profile D). No biliverdin IX α was formed during the incubation without haem-degradation enzymes (Fig. 2, profile, C).

Comparative study of α -oxyprotohaem degradation by each haem-degradation enzyme and by the complete haem oxygenase system

When α -oxyprotohaem was incubated with haem oxygenase alone, i.e. omission of NADPH-cytochrome *c* reductase from the complete system, the addition of NADPH did not induce characteristic spectral changes (Fig. 3, spectra A). After the addition of biliverdin reductase, a minor peak located at λ_{max} 450 nm appeared, but did not coincide with that of bilirubin IX α (λ_{max} 488 nm). This result showed that biliverdin IX α was not formed by haem oxygenase alone even in the presence of a reducing agent.

When α -oxyprotohaem was incubated with NADPH-cytochrome *c* reductase alone, the spectra maintained a straight baseline. The addition of NADPH to this system resulted in a gradual increase of absorption around 500–540 nm, but no absorption peak appeared at 650 nm as observed in the case of the complete haem oxygenase system (Fig. 3, spectra B). Upon

Fig. 2. H.p.l.c. profiles of biliverdin IX dimethyl ester isomers and reaction products derived from the incubation of α -, β -, γ - and δ -oxyprotohaem IX with the complete haem oxygenase system

Measurement was carried out at 375 nm of profiles obtained with a silica column and an isocratic solvent system with hexane/methanol/ethyl acetate (902:33:65, by vol.). Samples were prepared as described as in the Materials and methods section. Profile A: authentic samples of biliverdin IX dimethyl ester (α , β , γ and δ). Profile B: biliverdin IX α dimethyl ester derived from α -oxyprotohaem IX incubated with the complete haem oxygenase system. Profile C: reaction product derived from α -oxyprotohaem IX incubated without the complete haem oxygenase system. Profile D: reaction product derived from α -oxyprotohaem IX incubated with the complete haem oxygenase system minus NADPH. Profile E: reaction product derived from β -oxyprotohaem IX incubated with the complete haem oxygenase system. Profile F: reaction product derived from γ -oxyprotohaem IX incubated as for profile E. Profile G: reaction product derived from δ -oxyprotohaem IX incubated as for profile E.

the addition of biliverdin reductase, the low broad peak around 500–540 nm was diminished and a new peak appeared ($\lambda_{\max}$ 690 nm), which was assumed to be the absorption peak of α -verdohaem–NADPH–cytochrome *c* reductase complex. However, no sign of bilirubin IX α formation was observed. Therefore neither biliverdin IX α nor bilirubin IX α was formed with NADPH–cytochrome *c* reductase alone, which is in contrast with the case with the complete haem oxygenase system followed by the addition of biliverdin reductase.

Kinetic analysis of conversion of α -oxyprotohaem into bilirubin IX α

Kinetic analysis of the conversion of α -oxyprotohaem into bilirubin IX α catalysed by the complete haem oxygenase system plus biliverdin reductase was carried out and the values were compared with those obtained from similar experiments with protohaem IX as a natural substrate. The enzymic activity was assayed with substrate concentrations of 2, 4, 8, 16 and 32 μ M and the concentrations of haem oxygenase, NADPH–

Fig. 3. Difference spectra obtained from the incubation of α -oxyprotohaem IX with incomplete haem oxygenase system

Spectra A: spectra obtained from the reaction system in which NADPH–cytochrome *c* reductase was omitted from the complete system. Spectra B: spectra obtained from the reaction system in which haem oxygenase was omitted from the complete system. Baseline was obtained between the cuvettes containing 30 μ M- α -oxyprotohaem IX and 44 units of haem oxygenase or 0.7 unit of NADPH–cytochrome *c* reductase in 400 μ l of 0.1 M-potassium phosphate buffer, pH 7.5. Measurements of spectra were started by the addition of 10 μ l of 0.1 μ M-NADPH into the sample cuvette and 10 μ l of water into the reference cuvette respectively, followed by scanning at 2 min intervals. Finally spectral changes were measured by the addition of 10 μ l of biliverdin reductase (9.5 units) and scanning at 2 min intervals. Arrows show the time course.

Fig. 4. Lineweaver–Burk plot of reaction velocity of incubation with protohaem IX and α -oxyprotohaem IX with haem-degradation enzymes

Indicated amounts of protohaem IX or α -oxyprotohaem IX were incubated with 12 units of haem oxygenase, 0.5 unit of NADPH–cytochrome *c* reductase and 9.5 units of biliverdin reductase in the presence or in the absence of 0.8 mM-NADPH in 1.2 ml of 0.1 M-potassium phosphate buffer, pH 7.5, for 5 min at 37 °C. The amounts of bilirubin formed were determined by difference spectra between the reaction mixture in the presence and in the absence of NADPH at 468 nm. The details are described in the text.

cytochrome *c* reductase and biliverdin reductase were 12, 0.5 and 9.5 units respectively. The results are presented in Fig. 4 as Lineweaver–Burk plots. Extrapolation of the two plotted lines for α -oxyprotohaem IX and protohaem IX yielded K_m values of 3.6 and 1.8 μ M respectively. The K_m value of α -oxyprotohaem IX was approximately twice that of protohaem IX, and is slightly

Fig. 5. Difference spectra from the reaction of the three isomers (β , γ and δ) of α -oxypyrrohaem IX with haem-degradation enzymes

The spectra were measured in the presence of β -oxypyrrohaem IX (A), γ -oxypyrrohaem IX (B) and δ -oxypyrrohaem IX (C) as described in the legend to Fig. 1. After the baseline had been recorded (—), NADPH was added (---), followed by the addition of biliverdin reductase (—). To simplify the spectrum obtained from each step, the first scanning and that after 14 min of scanning are shown. Finally CO gas was bubbled into the cuvette (·····). Details of the experiments are presented in the legend for Fig. 1 and in the text.

larger than that reported previously [14,18,19]. The $V_{\max}$ value of α -oxypyrrohaem IX was about twice that of pyrrohaem IX.

Experiments with β -, γ - and δ -oxypyrrohaemin IX and the complete haem oxygenase system

β -, γ - and δ -Oxypyrrohaemins IX were each incubated with the complete haem oxygenase system. In the case of β -oxypyrrohaem IX being used as substrate in place of the α -isomer in the reaction system, the difference spectra showed a slight increase in absorption intensity in the regions from 500 to 540 nm and from 600 to 700 nm with an indistinct shoulder at 413 nm (Fig. 5, spectra A, continuous line). Owing to the small change in the spectrum, it is not clear whether or not biliverdin IX β is formed from β -oxypyrrohaem IX by the enzyme system. Upon the addition of biliverdin reductase to the reaction mixture, a new peak appeared at 678 nm but the small shoulder at 413 nm was diminished. It is still difficult to judge whether biliverdin IX β was enzymically formed or not, because of the small spectral change.

When γ -oxypyrrohaem IX was used as a substrate, the absorption spectrum showed a slight increase around 520 nm and 410 nm with a shoulder (Fig. 5, spectra B, continuous line). Addition of biliverdin reductase to the system gave an increase of absorption around 660 nm with two shoulders at 520 nm and 410 nm. However, the spectrum was essentially unchanged. These spectral observations did not provide evidence for the formation of biliverdin IX γ via biliverdin IX γ .

In the case of δ -oxypyrrohaem IX the spectral change was the smallest of the three tested isomers. A slight increase of absorption from 500 to 540 nm was also observed (Fig. 5, spectra C, continuous line). Upon addition of biliverdin reductase to the system, a small peak appeared at 663 nm. This was not due to addition of the haem oxygenase system, because a similar increase in the absorption at the same wavelength was observed even when haem oxygenase was omitted from the complete enzyme system (see Fig. 3, spectra B).

Throughout these spectral investigations with isomers of oxypyrrohaem IX, peaks located at wavelengths from 660 to 678 nm (β , 678 nm; γ , 660 nm; δ , 663 nm) were always observed after the addition of biliverdin reductase and these peaks shifted to shorter wavelengths (β , 624 nm; γ , 618 nm; δ , 620 nm) with CO bubbling, indicating that the reaction proceeded partially to the verdohaem stage and then stopped. The CO bubbling in each reaction mixture resulted in the possible formation of verdohaem-CO compound.

Each chloroform extract from the reaction mixture of the haem oxygenase system (without biliverdin reductase) was esterified and then h.p.l.c. analysis was carried out (Fig. 2, profiles E, F and G). The results strongly suggest that only trace amounts of biliverdins β , γ and δ were formed. These results demonstrate that β , γ and δ isomers of biliverdin IX are not essentially formed in the complete haem oxygenase system, and that only the α -isomer is exclusively converted into biliverdin IX α .

Inhibition of α -oxypyrrohaem degradation to biliverdin IX α by the other isomers (β , γ and δ) of oxypyrrohaem

α -Oxypyrrohaem IX can bind to the active site of the haem oxygenase system as in the case of pyrrohaem IX. If the other isomers of oxypyrrohaem, i.e. β , γ and δ isomers, could bind to the same active site on the enzyme as in the case of α -oxypyrrohaem IX, the yield of biliverdin IX α converted from α -oxypyrrohaem should be decreased. As shown in Table 1, all the isomers (β , γ and δ) decreased haem oxygenase activity by 42–64%. The decrease was nearly two-thirds for α -oxypyrrohaem IX and nearly half for pyrrohaem IX.

Considering the concentrations of substrate and inhibitor and

Table 1. Inhibition of haem oxygenase activity for conversion of pyrrohaem IX or α -oxypyrrohaem IX into biliverdin IX α by β , γ and δ isomers of oxypyrrohaem

The reaction mixture contained 10 μ M-pyrrohaem IX or α -oxypyrrohaem IX as the substrate, 50 units of haem oxygenase, 0.7 unit of NADPH-cytochrome *c* reductase and 9.4 units of biliverdin reductase in the presence or in the absence of 0.8 mM-NADPH in 1.2 ml of 0.1 M-potassium phosphate buffer, pH 7.5. Each of the three isomers of oxypyrrohaem IX was used at an inhibitor concentration of 20 μ M. Incubation was carried out for 5 min at 37 °C with shaking. The other assay conditions are described in the Materials and methods section.

Substrate...	Pyrrohaem IX		α -Oxypyrrohaem IX	
	Bilirubin formed (nmol)	Inhibition (%)	Bilirubin formed (nmol)	Inhibition (%)
None (control)	3.1	0	5.8	0
β -Oxypyrrohaem IX	1.6	48	2.1	64
γ -Oxypyrrohaem IX	1.5	52	2.1	64
δ -Oxypyrrohaem IX	1.8	42	2.5	57

K_m values of the two substrates, the extents of decreases in enzymic activity by the three isomers against the two types of substrate suggested that inhibition took place in a competitive manner. These results indicate that four isomers of oxyprotohaem can bind to the active site of the haem oxygenase system with almost the same affinity, and that the reaction is isomer-specific.

DISCUSSION

Sano and his co-workers presented the reaction mechanism for the conversion of α -oxyprotohaem–apomyoglobin complex to Fe(III)–biliverdin IX α with two equimolar amounts of O₂ via an intermediate, verdohaem IX α · π radical [4]. However, apomyoglobin and haem oxygenase have different protein environments even in the haem-binding site, and it is not yet known to what extent the reactions in the apo-myoglobin model system mimic those that occur in the haem oxygenase system. The present study shows that α -oxyprotohaem IX is involved as the first intermediate in the degradation sequence of protohaem catalysed by the complete haem oxygenase system, since the features of degradation of α -oxyprotohaem to biliverdin or bilirubin, especially in terms of the kinetics, were shown to be essentially the same as that of protohaem degradation.

The second intermediate corresponding to verdohaem IX α [4] appeared in the spectral analysis at the early stage of incubation of α -oxyprotohaem with the haem oxygenase system, and was also observed in the incubation of the other type of isomers with haem-degradation enzymes. The intermediate compound seems to accumulate in small amounts when the enzymic formation of biliverdin is strongly inhibited, as in the cases of β -, γ - and δ -oxyprotohaem IX. Indeed, the intermediate compound accumulated when α -protohaem IX was incubated with NADPH and NADPH–cytochrome *c* reductase, in which enzyme system haem oxygenase was depleted from the complete haem oxygenase system. Moreover, the appearance of the intermediate was distinctive when biliverdin reductase was added to the enzyme system. These observations concord with the proposal made by Yoshinaga *et al.* that haem-degradation enzymes, including biliverdin reductase, make a ternary complex centred around the haem-binding site [20]. Such a complex may stabilize the second intermediate in the cases of the three oxyprotohaem isomers.

It is interesting to note that only trace amounts of biliverdins IX β , IX γ and IX δ were respectively formed from the incubation mixture of the corresponding oxyprotohaem IX isomer with the complete haem oxygenase system. These findings can explain the reaction mechanism that involves α -type bile pigment as the major component *in vivo*, whereas β , γ and δ types are only trace components in the bile. Our data can also account for the results obtained by Yoshida *et al.* [7] that α -type *meso*-bilirubin IX was obtained from the incubation products of a mixture of four isomers of oxymesohaem IX with the complete haem oxygenase system.

Previous reports on product analysis clearly demonstrated specificity, where the cleavage position of the methene bridge is highly restricted in the α -type [12,18,21]. Frydman *et al.* also found structural specificity of the α -methene position and an effect of the side chains of synthetic haem IX analogues on the haem oxygenase reaction [22]. The binding of protohaem IX is fixed by the two propionyl groups in the haem oxygenase system,

and this fixation is important for the first addition of molecular O₂ to the α -methene bridge to form α -oxyprotohaem IX, followed by further oxidation to biliverdin IX α via verdohaem IX α . It is very reasonable to consider that the α -oxyprotohaem used in this experiment was converted into bilirubin IX α .

The reason why only trace amounts of β -, γ - and δ -oxyprotohaems were converted into the corresponding biliverdin isomers may be as follows. The question is whether β , γ - and δ -oxyprotohaem isomers are incorporated into the correct position in haem oxygenase as in the case of α -oxyprotohaem. Competitive inhibition of α -biliverdin formation from α -oxyprotohaem in the haem oxygenase system by β -, γ - or δ -oxyprotohaem indicates that oxyprotohaem isomers (β , γ and δ) can bind to the active site of the enzyme at the same position as in the case of α -oxyprotohaem, but further oxygenation by either molecular O₂ or activated oxygen does not help the process to proceed smoothly as in the case of α -oxyprotohaem. The determining factor for the selectivity of the α -methene bridge is obviously its location in the iron porphyrin ring opposite the propionic acid chains. The reason why further oxygenation does not proceed sufficiently in the cases of β -, γ - and δ -oxyprotohaem IX in the complete haem oxygenase system remains to be interpreted, but perhaps it is due to the tertiary structure of the haem oxygenase. Different electron delocalization at a pyrrole carbon atom adjacent to the *meso*-oxy bond of β , γ and δ isomers compared with that in α isomers seems to be unlikely.

Under the conditions described in this paper, the homogeneous haem oxygenase preparation contained 0.3 mol of protohaem/mol of the enzyme as an impurity [14]. The amount of biliverdin converted from contaminated protohaem was less than 3% of that derived from α -oxyprotohaem IX. Spectral change of the flavin moiety in the pure NADPH–cytochrome *c* reductase [23,24] was negligible compared with the spectral change that occurred in the conversion of α -oxyprotohaem IX into biliverdin IX α via biliverdin IX α .

Lastly, there is a slight difference in the reaction between apomyoglobin and haem oxygenase systems. The sequence of α -methene-bridge-cleavage reaction (Scheme 1) is indistinguishable in the two systems. In the apo-myoglobin system, reductant was not required for the second oxygenation step from α -oxyprotohaem IX to verdohaem IX α , i.e. spontaneous reduction of Fe(III) to Fe(II) occurred in the presence of O₂ without any reducing agent, followed by verdohaem formation [4]. On the other hand, NADPH and NADPH–cytochrome *c* reductase are required in the complete haem oxygenase system. From α -oxyprotohaem IX, only a small amount of biliverdin IX α was formed in the haem oxygenase system without NADPH. It is not clear why trace amounts of biliverdin are formed in this system. The reaction pathway from verdohaem IX α to biliverdin IX α , however, seems to be similar in two enzyme systems.

Sano *et al.* reported slight discrepancies between the spectral properties of the intermediate ($\lambda_{\max}$ 660 and 704 nm) of the apomyoglobin system and the intermediate ($\lambda_{\max}$ 638 and 688 nm) of the haem oxygenase system [7]. The present study confirms the latter spectra.

Preparation of equimolar amounts of α -oxyprotohaem IX and haem oxygenase at high concentrations (e.g. 0.1–0.5 mM) and stoichiometric titration with O₂ was found to be difficult compared with the case of the apo-myoglobin system. However,

Scheme 1. Sequence of the haem-cleavage reaction

there was no discrepancy between the two systems concerning the proposed reaction mechanism, which was strongly supported by the difference in spectral analysis, product analysis by h.p.l.c. and kinetic analysis of the present investigation.

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