Formation of bile pigments by coupled oxidation of cobalt-substituted haemoglobin and myoglobin

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Treatment of cobalt-substituted haemoglobin and myoglobin with ascorbate and molecular O_2 (coupled oxidation) resulted in biliverdin formation from the cobalt(II) derivatives but not from the cobalt(III) derivatives. This was apparently due to the inability of ascorbate to reduce cobalt(III) haemoproteins. Isomer analysis of the biliverdins produced from coupled oxidation of cobalt(II) oxyhaemoglobin suggested that the orientation of the cobalt protoporphyrin IX in the haem pocket differed slightly from that of the haem in native haemoglobin.

Haem degradation *in vivo* is catalysed by the microsomal enzyme haem oxygenase (EC 1.14.99.3), which requires molecular O_2 and reducing capacity, supplied from NADPH via the enzyme NADPH:cytochrome *P*-450 oxidoreductase (Brown & Troxler, 1982). The reactions between molecular O_2 , a reductant (for example ascorbate) and haem derivatives have proved useful model systems for the study of the mechanism of haem catabolism in living systems. These so-called coupled oxidation reactions result in haem degradation to the bile pigment biliverdin, elimination of a methene bridge carbon atom as CO and loss of the iron atom of haem (Scheme 1), all features in common with biological haem degradation (for detailed reviews see Jackson, 1974; Schmid & McDonagh, 1979; Brown & Troxler, 1982).

Although some details of the mechanism of the haem-degradation process remain uncertain, the

main features are now reasonably clear and appear to be common to both biological and non-biological systems (Brown & Troxler, 1982). It appears that the reaction must proceed via iron(II), presumably to facilitate the binding and activation of molecular O_2 . It is probable that, during this activation process, an electron is transferred from iron(II) to molecular O_2 . The metal atom must therefore be able to exist in both the +2 and +3 oxidation states.

In principle, any one of the four methene bridge carbon atoms (Scheme 1) may be attacked and, because of the asymmetry of the arrangement of side-chain groups around the periphery of the haem molecule, four biliverdin isomers are possible. Degradation of protein-free haem (in pyridine) does indeed produce all four isomers (Bonnett & McDonagh, 1973; O'Carra, 1975), but, after coupled oxidation of haemoproteins, the actual isomer pattern obtained appears to reflect

Scheme 1. Formation of biliverdin by coupled oxidation of haem

M = $-CH_3$; V = $-CH=CH_2$; P = $-CH_2CH_2CO_2H$.

the conformation of the protein in the neighbourhood of the haem pocket (Brown, 1976; Brown & Docherty, 1978; Brown *et al.*, 1981) or, alternatively, the orientation of the haem within the haem pocket (Docherty & Brown, 1982). Thus coupled oxidation of myoglobin yields exclusively biliverdin IX- α , whereas degradation of normal human haemoglobin yields 65% α -isomer and 35% β -isomer.

Iron is not unique in its ability to bind molecular O₂ and to exist in both +2 and +3 oxidation states. For example cobalt(II) protoporphyrin IX can be substituted for haem in haemoglobin and myoglobin with retention of O₂-binding ability (Hoffman & Petering, 1970; Hoffman *et al.*, 1971; Dickinson & Chien, 1973; Yonetani *et al.*, 1974), and the complex may also exist in the cobalt(III) state. In principle, therefore, it would seem possible that cobalt protoporphyrin IX could replace haem as a substrate for microsomal haem oxygenase and in coupled oxidation reactions. However, attempts to produce bile pigment from coupled oxidation of cobalt protoporphyrin IX in pyridine appear to have been unsuccessful, although treatment of cobalt octaethyl porphyrin with H₂O₂ produces the corresponding oxophlorin (Bonnett & Dimsdale, 1972). Similarly, although one report that cobalt protoporphyrin IX can act as a substrate for haem oxygenase has appeared (Maines & Kappas, 1977), several other studies have concluded that cobalt protoporphyrin IX is not transformed to biliverdin by haem oxygenase (Schacter & Waterman, 1974; Yoshida & Kikuchi, 1978; King & Brown, 1978).

Since it is not clear why cobalt protoporphyrin IX should not be converted into bile pigment, we have pursued the system further. In the present work, we report studies on the attempted coupled oxidation of cobalt protoporphyrin IX-substituted haemoproteins and, in the light of knowledge gained from these experiments, we have further attempted to define conditions for the use of cobalt protoporphyrin IX as a substrate for haem oxygenase.

Experimental

Materials

All commercially obtained materials were of analytical-reagent grade and all solvents were redistilled before use. Cobalt(III) protoporphyrin IX was prepared by the method of Yonetani *et al.* (1974) from protoporphyrin IX disodium salt (Sigma Chemical Co.). Manganese(III) protoporphyrin IX was prepared from manganese(II) acetate by the same method. Protohaemin chloride was obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K.

Purification of haemoglobin

Fresh haemolysates were prepared by mixing 1 vol. of adult human erythrocytes with 2 vol. of cold distilled water and allowing lysis to occur (30 min). Cell debris was removed by centrifugation (15000g for 60 min), and the haemoglobin-rich supernatant was dialysed for 16 h against 50 mM-Tris/HCl buffer, pH 8.4, containing 0.1 mM-EDTA. The haemoglobin was purified by column chromatography on DEAE-cellulose (Pharmacia) as previously described (Huisman & Dozy, 1962). Haemoglobin solutions were concentrated in an Amicon ultrafiltration cell containing a PM-10 membrane.

For preparation of globin (apohaemoglobin), purified haemoglobin was dialysed against distilled water to render it salt-free. Apohaemoglobin was prepared from this material by the method of Winterhalter & Huehns (1964) and stored as a freeze-dried powder. Apomyoglobin was prepared from sperm-whale myoglobin type III (Sigma Chemical Co.) by using the same method.

Preparation of reconstituted haemoproteins

Cobalt(II) haemoproteins (deoxy and oxy forms). These were prepared broadly by following the method of Yonetani *et al.* (1974). Freeze-dried globin was dissolved in distilled water to a concentration of 8 mg/ml, and the solution was dialysed against 0.1 M-sodium phosphate buffer, pH 7.0. This was deoxygenated by passing N₂ over the solution surface for 10 min at 0–5°C. A 1.1–1.3 molar equivalent of cobalt(III) protoporphyrin IX (per globin subunit) was dissolved in aq. 50% (v/v) pyridine that had been saturated with Na₂S₂O₄ and deoxygenated. This solution was added dropwise to 10 vol. of a deoxygenated globin solution under a stream of N₂ at 0–5°C. The mixture, which was constantly stirred throughout, was immediately applied to a Sephadex G-25 column (2.5 cm × 15 cm) equilibrated with 10 mM-sodium phosphate buffer, pH 6.0, and eluted with the same buffer. This gel-filtration step removed pyridine, dithionite and unbound porphyrin. The eluted solution was adsorbed on a CM-cellulose column equilibrated with 10 mM-phosphate buffer, pH 6.0, and washed with several volumes of the same buffer. The cobalt protoporphyrin IX-substituted haemoprotein was eluted with 50–100 mM-phosphate buffer, pH 7.0, and concentrated by ultrafiltration. The material was stored under an atmosphere of O₂ at 0–5°C and used within 24 h. Because of the relatively low affinity of cobalt-substituted haemoglobin for O₂, significant quantities of cobalt(II) deoxyhaemoglobin are formed if the material is stored under air (Yonetani *et al.*, 1974).

Cobalt(III) haemoproteins (met form). Apohaemoglobin and apomyoglobin were reconstituted with cobalt(III) protoporphyrin IX by using the above procedure except that dithionite was omitted from the aqueous pyridine solution before the mixing with globin. After gel filtration and ion-exchange chromatography the cobalt(III) protoporphyrin IX derivatives of haemoglobin and myoglobin were stored aerobically at 0–5°C. This form of globin was unable to bind O₂ (Yonetani *et al.*, 1974).

Manganese haemoglobin. This was prepared by the method of Yonetani & Asakura (1969), which was developed for reconstitution of natural (iron) myoglobin.

Haemoglobin. The method of Winterhalter & Huehns (1964) was used for reconstitution of haemoglobin from haem and apohaemoglobin.

Atomic absorption spectrometry

Determination of iron and cobalt concentrations was performed with an International Laboratories IL-157 atomic absorption spectrometer, with the use of commercial standards to calibrate for analysis of iron and cobalt.

Coupled oxidations with ascorbate

Reconstituted haemoglobin derivatives (20–60 mg) were incubated in 10 ml of 0.1 M-phosphate buffer, pH 7.0, with 100 mg of sodium ascorbate at 37°C. After 2 h the reaction was stopped by cooling in ice and acidifying with 8 ml of 3 M-HCl and 4 ml of acetic acid. The mixture was extracted with peroxide-free diethyl ether (2 × 25 ml) to remove any free haem present and chloroform (2 × 10 ml) to isolate biliverdin. The chloroform extract was evaporated to dryness and the residue methylated with methanol/conc. H₂SO₄ (19:1, v/v) (O'Carra &

Colleran, 1969). Quantitative and qualitative isomer analyses were performed by using t.l.c. and spectrophotometry as described previously (Brown & Docherty, 1978).

Results and discussion

Attempted coupled oxidation of cobalt(III) derivatives

When excess of ascorbate is added to iron(III) haemoglobin or iron(III) myoglobin, rapid reduction to the iron(II) oxidation state occurs, as indicated by changes in the absorption spectrum. Coupled oxidation then proceeds via the iron(II) forms (Brown & Troxler, 1982). When ascorbate was added in a similar way to the cobalt(III) analogues of haemoglobin and myoglobin, no corresponding spectral changes were observed. This finding suggests that ascorbate is unable to reduce cobalt(III) haemoproteins rapidly, a result that is consistent with studies by Yonetani *et al.* (1974), which highlighted the difficulty in reduction of cobalt(III) haemoproteins to the oxygenated cobalt(II) forms.

Prolonged exposure of cobalt(III) haemoproteins to ascorbate and O₂ under coupled oxidation conditions (see the Experimental section) resulted in no appreciable spectral changes and formation of only very small traces of biliverdin (Table 1), much less than would be expected from coupled oxidation of iron(III) haemoproteins (O'Carra, 1975). The traces of biliverdin could be estimated spectroscopically (Table 1), but there was insufficient for quantitative isomer analysis by t.l.c.

Attempts were made to produce biliverdin by coupled oxidation of cobalt(III) protoporphyrin IX in pyridine under the established conditions for coupled oxidation of pyridine haemochrome

Table 1. Formation of biliverdin by coupled oxidation of various globin derivatives

Various amounts (20–60 mg) of protein were treated with ascorbate and O₂ as described in the text. Biliverdin was determined spectrophotometrically after isolation by t.l.c. (Brown & Docherty, 1978).

Globin derivative	Biliverdin isolated (nmol)	Ratio of biliverdin yield to protein used (nmol/mg)
Native oxyhaemoglobin	194	3.29
	173	4.22
Cobalt(III) haemoglobin	1.4	0.02
Cobalt(III) myoglobin	4.4	0.09
Reconstituted oxyhaemoglobin	99	2.30
	60	1.40
Cobalt(II) oxyhaemoglobin	46	1.64
	52	1.93
Cobalt(II) oxymyoglobin	66	1.61
	45	2.25
Manganese(III) haemoglobin	1.2	0.04
Apohaemoglobin	0	0
Apohaemoglobin containing 4.4 mol% haem	1.2	0.04

Table 2. *Determination of iron and cobalt during preparative procedures for reconstitution of haemoproteins*
Iron and cobalt were determined by atomic absorption spectroscopy, as described in the text. Atomic absorption spectroscopy showed that the cobalt acetate used in the preparation of cobalt protoporphyrin IX contained only 0.006% (w/w) iron.

Sample	Iron content (nmol/ml)	Cobalt content (nmol/ml)	Molar ratio iron/protein
Native haemoglobin solution (0.95mg/ml of water)	61	0	1.03
Apohaemoglobin solution (1.5mg/ml of water)	0.8	0	0.0087
Apomyoglobin solution (2.5mg/ml of water)	1.4	0	0.0087
Protoporphyrin IX	Not detected	Not detected	—

(Bonnett & McDonagh, 1973; O'Carra, 1975). However, although a slow reduction of cobalt(III) appeared to take place, no biliverdin was formed.

Coupled oxidation of cobalt(II) oxyhaemoproteins

Coupled oxidation of the cobalt(II) oxy derivatives of haemoglobin and myoglobin resulted in the incubation mixture becoming brown-green and the appearance of an absorption band around 630nm. After extraction, substantial quantities of blue-green pigment were observed, and this was shown to be biliverdin by t.l.c. analysis and comparison with authentic standards (Brown & Docherty, 1978). The yields obtained (expressed in terms of protein used) are shown in Table 1, along with corresponding values for reconstituted (iron) oxyhaemoglobin. It is clear from these data that coupled oxidation of cobalt(II) oxyhaemoproteins proceeds with a yield of biliverdin comparable with that of coupled oxidation of the reconstituted iron protein.

The overall yield of coupled oxidation reactions in terms of the ratio of biliverdin recovered to haem used is small (approx. 5%), and this has been fully discussed elsewhere (Docherty & Brown, 1982). Because of this small yield, it is necessary to examine the possibility that the apparent coupled oxidation of cobalt(II) oxyhaemoproteins to biliverdin might, in fact, be due to contamination either by free haem or by the normal iron haemoproteins. Table 1 shows that, as expected, coupled oxidation of apoproteins does not occur, and, even in the presence of 4.4mol% of added haem, only small traces of biliverdin are formed. The possibility that some iron haemoprotein may have been reconstituted during the preparation of the cobalt haemoproteins was examined by determination (by atomic absorption spectroscopy) of the iron and cobalt contents of the various components used in the preparative procedures. From these results (shown in Table 2) it is clear that no appreciable quantities of haem could have been incorporated during the reconstitution of apoproteins with cobalt(II) protoporphyrin IX. This was confirmed by spectral measurement of

Table 3. *Biliverdin isomer distribution after coupled oxidation of various globin derivatives*

The isomer distribution was determined spectrophotometrically after methylation of biliverdin produced by coupled oxidation and separation by t.l.c. (Brown & Docherty, 1978).

Globin derivative	Isomer distribution(%)			
	α	β	γ	δ
Native oxyhaemoglobin	65	35	—	—
Reconstituted oxyhaemoglobin	68	26	—	6
Cobalt oxyhaemoglobin	—	94	—	6
Cobalt oxymyoglobin	95	5	—	—

the cobalt haemoproteins, which would have detected contamination above approx. 5% by the normal iron haemoproteins. Final confirmation that the biliverdin formed in these experiments is due to direct reaction of the cobalt haemoproteins and not to contaminant iron haemoproteins was provided by the biliverdin isomer patterns (see below).

Attempted coupled oxidation of manganese(III) protoporphyrin IX

Manganese(III) substituted haemoglobin behaved very similarly to the cobalt(III) haemoproteins in that only traces of biliverdin were recovered after coupled oxidation (Table 1).

Biliverdin isomer patterns after coupled oxidation

The distributions of biliverdin isomers in the various experiments, determined by t.l.c. analysis (Brown & Docherty, 1978), are shown in Table 3. For native haemoglobin, the biliverdin consisted of 65% α -isomer, 35% β -isomer but none of the γ - or δ -isomers, in agreement with previous results (O'Carra, 1975; Brown & Docherty, 1978). Coupled oxidation of reconstituted haemoglobin produced 6% δ -isomer at the expense of β -isomer. This is due to haem disorder, i.e. a small proportion of the reconstituted protein containing haem groups at an orientation 180° different from that of native

haemoglobin, by rotation about the α - γ axis. This phenomenon has been well documented (La Mar *et al.*, 1980; Docherty & Brown, 1982).

For cobalt(II) oxyhaemoglobin, the isomer pattern also revealed 6% δ -isomer, suggesting a similar level of haem disorder, but, surprisingly, no α -isomer. Assuming that some haem disorder has occurred on reconstitution, this implies that all of the biliverdin produced arises from the region of the haem pocket normally occupied by the β -methene bridge. However, for cobalt(II) oxymyoglobin, almost 100% α -isomer was obtained, as is the case for coupled oxidation of native myoglobin (O'Carra, 1975).

General discussion

The present study represents the first report of the formation of bile pigments from coupled oxidation of cobalt protoporphyrin IX. It is clear, however, that degradation requires the participation of the cobalt(II) oxidation state, since the cobalt(III) haemoproteins, which are not readily reduced, did not react significantly. This resistance of cobalt(III) derivatives towards reduction by ascorbate probably also explains the lack of reactivity of cobalt(III) protoporphyrin IX in pyridine and also its failure to act as a substrate for haem oxygenase (King & Brown, 1978). Presumably, if a complex between haem oxygenase and cobalt(II) protoporphyrin IX could be prepared, degradation to bile pigment might well be possible.

Previous work has shown that for many haemoprotein derivatives the relative orientation between haem and apoprotein can be expressed in terms of the isomer pattern obtained after coupled oxidation, i.e. this pattern is a sensitive probe of the environment in which the haem finds itself (Brown, 1976; Brown *et al.*, 1981). For cobalt(II) oxyhaemoglobin, the present results suggest that the β -methene bridge is more accessible, and the α -bridge less so, than in oxyhaemoglobin itself. Previous work, based on calculation of interaction energies, has shown that this can be accomplished with relatively little change in the haem-protein relationship, whereas formation of γ - or δ -isomer requires more substantial changes (Brown *et al.*, 1981). However, similar calculations for myoglobin (normal pattern, 100% α -isomer) shows that rather more significant changes would be required for formation of β -isomer. The present work is also consistent with these predictions. It may therefore be concluded that the binding of cobalt(II) protoporphyrin IX in the haem pockets of both

haemoglobin and myoglobin apoproteins is similar to, but not identical with, that of haem in the native proteins.

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