Haem disorder in reconstituted human haemoglobin

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Degradation of the haem of haemoglobin (used as a chemical probe of the haem–protein relationship), suggests that reconstituted human haemoglobin contains significant haem disorder. This results from the insertion of haem into globin with an orientation 180° different from the natural orientation. Haem disorder also slowly occurs in methaemoglobin solutions.

The X-ray structures of oxygen-binding haemoproteins have clearly shown that the haem group is orientated within the protein such that its hydrophilic propionic acid side-chains are directed toward the exterior of the molecule, whilst the opposite hydrophobic edge of the haem plane is buried deep within the protein (Kendrew & Watson, 1969; Perutz, 1976). However, because of the asymmetry in the arrangement of side-chains around the haem molecule, for a fixed position of the porphyrin plane there are two orientations (H_1 and H_2) which satisfy this condition. These are shown schematically in Fig. 1 for haemoglobin, relative to selected residues in close contact with the distal side of the haem (Brown, 1976). For haemoglobin and myoglobin, X-ray studies have concluded that the native proteins exist exclusively in the H_1 form, although the X-ray data probably would not detect very small amounts of the H_2 form. Recently, n.m.r. studies on a number of porphyrin-substituted haemoproteins have shown that, under certain circumstances in solution, both H_1 and H_2 forms can exist simultaneously.

La Mar *et al.* (1978) showed that myoglobin reconstituted as the cyanmet derivative with deuterohaem rather than with the natural protohaem contains both H_1 and H_2 forms. Moreover, the two forms were slowly interconvertible in solution. Further n.m.r. experiments by La Mar *et al.* (1980a) showed that deuterohaem-reconstituted horseradish peroxidase exists exclusively in the H_2 form, whilst the native protein is in the H_1 form. It was also found (La Mar *et al.*, 1980b) that, for the haemoglobins from the insect *Chironomus thummi thummi*, a mixture of H_1 and H_2 forms exists for the Fe(III) derivatives in solution, whereas an X-ray structure showed only the H_2 form (Steigemann & Weber, 1979). Apparently, no corresponding n.m.r. studies on human haemoglobin have been reported.

Although X-ray work on native human haemo-

globin shows that significant haem disorder could not exist, the question of possible haem disorder following reconstitution of haem with apoprotein is an important one on which there is apparently no experimental evidence at present. There have been many studies of reconstituted haemoglobins involving preparation of various hybrids and haemoglobins with metals other than iron, or porphyrins other than protoporphyrin IX. Haem disorder in these situations seems a distinct possibility and molecular models show that there would be no spatial problem in insertion of haem in the H_2 configuration.

We have investigated the possibility of haem disorder in reconstituted human haemoglobin using a technique recently developed for studying the structure of certain abnormal haemoglobins (Brown & Docherty, 1978). Using a simple ‘coupled oxidation reaction’ in which haemoglobin reacts with ascorbate and molecular oxygen, the haem is degraded to the bile pigment biliverdin. This reaction actually takes place whilst the haem is associated with apoprotein. Because of the asymmetry of the arrangement of the haem sidechains, four biliverdin isomers are possible depending upon which methene bridge (α , β , γ or δ) is attacked. There is now substantial evidence suggesting that accessibility of an iron-bound O_2 molecule to the various methene bridge carbon atoms is the chief criterion determining which bridge is attacked and hence which biliverdin isomer is formed (Brown, 1976; Brown *et al.*, 1981). By analysis of the product biliverdins (Brown & Docherty, 1978) it is therefore possible to obtain information on the accessibility of the various methene bridges within the haem pocket of the protein.

From Fig. 1, it may be seen that haem disorder results in a reversal of the positions occupied by the β and δ methene bridges within the protein. Coupled oxidation of native human haemoglobin yields about

Fig. 1. Possible orientations of haem within haemoglobin

(a) The orientation of the haem plane is shown relative to selected residues in close contact with the haem. These residues are shown in projection onto the haem plane. This is the naturally occurring orientation, here designated H_1 . (b) Rotation of the haem plane by 180° about an axis through the α and γ bridge carbon atoms yields the H_2 form. The rotation results in identical environments for the α and γ bridges, but those of the β and δ bridges have been exchanged.

two-thirds biliverdin-IX α and one-third biliverdin-IX β (O'Carra, 1975). If haem disorder occurs upon reconstitution of haemoglobin, some δ -isomer might be expected to occur at the expense of β -isomer. Coupled oxidation of myoglobin yields almost 100% α -isomer. In this case, since no β -isomer is formed, haem disorder would not be detectable by the coupled oxidation approach.

In the present work, we report studies of haem disorder in human haemoglobin, reconstituted in various ways.

Experimental

Materials and measurements

A mixture of all four biliverdin-IX isomers was prepared and analysed as described by O'Carra (1975). All other materials and measurements were as previously described (Brown & Docherty, 1978; Brown *et al.*, 1974).

Coupled oxidation of haemoglobin and isolation of biliverdin

Coupled oxidation reactions were carried out at pH 7.4 for 2 h at 37°C by addition of 10 mg of sodium ascorbate to 50 mg of haemoglobin in 10 ml of 0.1 M-sodium phosphate buffer. The resultant biliverdins were extracted, methylated and purified by t.l.c. Full details of these procedures have been described elsewhere (Brown & Docherty, 1978; Brown *et al.*, 1974).

Preparation of haemoglobin derivatives

Native human haemoglobin was prepared from haemolysates of normal adult blood. Haemolysate was dialysed against 50 mM-Tris/HCl buffer, pH 8.5, containing 10^{-4} M-EDTA, and the haemoglobin was purified on a DEAE-cellulose column equilibrated with the same buffer. Elution was by means of a linear gradient from the starting buffer to 50 mM-Tris/HCl, pH 6.6, also containing 10^{-4} M-EDTA. Haem-free globin was prepared by the method of Winterhalter & Huehns (1964) which gave yields in the region of 65–70%.

For all reconstitution experiments, globin was adjusted to pH 7.5 by dialysis against 10 mM-potassium phosphate buffer, pH 7.5. Reaction was carried out at 4°C by dropwise addition of chilled haem solution to the globin solution as described below. In all cases, excess haem was removed by passage through a short DEAE-cellulose column, pH 7, the protein-free haem remaining firmly bound to the ion exchanger.

For reconstitution via the aquomet ($\text{FeIII}\cdot\text{OH}$) form, haemin (10–15 mg) dissolved in 0.5 ml of 0.1 M-NaOH was diluted to 10 ml with water, added to globin solution (1%, w/v) in 1.2-fold molar excess and allowed to stand overnight at 4°C (yield approx. 60%). For preparation via the carbonmonoxy form, a 1% (w/v) sodium dithionite solution was prepared under N_2 and added to haemin (10–15 mg), dissolved in dilute alkali as before and the mixture (10 ml) equilibrated with CO. After 30 min, the haem

solution was added to globin solution (1%, w/v) in 1.2-fold molar excess and the system was allowed to stand overnight at 4°C (yield approx. 75–80%). Reconstitution via the cyanmet form was achieved by addition of haem (10–15 mg of haemin in 0.5 ml of 0.1 M-NaOH, diluted to 10 ml with 0.1 M-potassium phosphate buffer, pH 7.5, containing 0.1 M-KCN) to globin, resulting in a yield of approx. 95% reconstituted haemoglobin. Following reconstitution, the various derivatives were converted to oxyhaemoglobin (conversions monitored spectrophotometrically) to prevent continuous haem exchange. The met forms were first converted to carbonmonoxyhaemoglobin by saturation of the solutions with CO and reduction with excess dithionite. Residual dithionite was removed by passage of the carbonmonoxyhaemoglobin through a column of Sephadex G-25. Repeated evacuation and exposure to O₂ yielded oxyhaemoglobin.

A solution of methaemoglobin for haem exchange studies was prepared by addition of excess potassium ferricyanide to oxyhaemoglobin. Excess oxidizing agent was removed on a short Sephadex G-25 column at 4°C. The protein was incubated at 37°C (Bunn & Jandl, 1966) in the presence of streptomycin and penicillin (0.5 mg/ml) to permit haem exchange to occur. Samples were withdrawn at intervals and coupled oxidation was carried out as described above.

Results and discussion

The almost random mixture of all four biliverdin isomers recovered following coupled oxidation of haem in pyridine accurately reflects the initial haem cleavage process, i.e. there is no selective filtering out of particular isomers during the purification procedures (O'Carra, 1975). In the presence of protein, however, it is important to be sure that selective protein binding of one or more biliverdin isomers does not distort the isomer distribution observed following coupled oxidation. This was checked by incubation of a known mixture of all four biliverdin IX isomers with globin, followed by purification and quantification of the isomers under identical conditions to those used for coupled oxidation, i.e. at 37°C. In addition a different temperature (0°C) was also used. Results are shown in Table 1. The total recovery of biliverdin was relatively low and showed some variation with temperature. This suggests that biliverdin does indeed bind to the globin and is not wholly removed by the extraction procedure. Nevertheless, the distribution of isomers recovered following this procedure is almost identical with that applied initially, indicating that the biliverdin losses are not selective. These results give confidence in the assumption that the biliverdin isomer distribution obtained following coupled oxidation of haemo-

Table 1. *Recovery of biliverdin isomers from globin/biliverdin mixtures*

A mixture of all four biliverdin isomers of known composition (1.92 mg) was incubated with globin (apohaemoglobin) (50 mg) for 2 h in 10 ml of sodium phosphate buffer, pH 7.4. Biliverdin isomers were then extracted, purified and estimated as described in the Experimental section.

Biliverdin isomer	Proportion (%) before incubation	Proportion (%) after incubation at:	
		0°C	37°C
α	28	30	27
β	25	26	23
γ	26	24	26
δ	21	20	24
Total biliverdin recovered (%)		36	20

globin is a true reflection of the bridge selectivity of the initial haem cleavage process.

The isomer distribution following coupled oxidation of native human haemoglobin is well established at approx. 65% α -isomer, 35% β -isomer and none of the γ or δ isomers, although from a large-scale reaction, Bonnett & McDonagh (1973) observed a trace of δ -isomer. In the present work, only α and β isomers were found following coupled oxidation of native human haemoglobin. The mean of eight determinations was α -isomer 64.8% (S.D. = 2.6%) β -isomer 35.2% (S.D. = 2.6%).

Coupled oxidation of human oxyhaemoglobin, reconstituted via the various derivatives described above, was carried out as indicated in the Experimental section. Isomer analysis of the resultant biliverdin dimethyl esters yielded the results shown in Table 2. Small but significant proportions of δ -isomer were observed in each case, but no trace of γ -isomer was ever observed. Moreover, the proportion of α -isomer from coupled oxidation of these reconstituted haemoglobins remained approximately the same as in native haemoglobin, i.e. the δ -isomer appears to be formed at the expense of the β -isomer. These results are therefore consistent with a small but significant amount of haem disorder occurring during haemoglobin reconstitution from haem and globin. This disorder apparently occurs whichever method of reconstitution is used, though it appears to be more extensive for reconstitution via the carbonmonoxy derivative. When similar experiments were carried out with reconstituted horse myoglobin (Wood, 1978), only biliverdin IX- α was observed, as in the native protein. This shows that the formation of δ -isomer in haemoglobin is not likely to be due to any protein denaturation or failure to reconstitute properly the protein.

Table 2. *Coupled oxidation of human haemoglobin reconstituted under various conditions*

Reconstitution from haem and globin was carried out as described in the Experimental section. In each case the reconstituted protein was converted to oxyhaemoglobin before being subjected to coupled oxidation; 50mg of haemoglobin was used in each reaction. Native haemoglobin yields 65% α -isomer and 35% β -isomer.

Derivative used in reconstitution	Total biliverdin recovered (nmol)	Proportion of biliverdin isomers formed (%)				
		α	β	γ	δ	($\beta + \delta$)
Cyanmet	151	64	32	0	4	36
Cyanmet	152	66	30	0	4	34
Cyanmet	117	65	31	0	4	35
Aquomet	117	66	30	0	4	34
Aquomet	95	70	26	0	4	30
Carbonmonoxy	30	66	28	0	6	34
Carbonmonoxy	57	64	29	0	7	36

Samples of human haemoglobin, in which the subunits had been separated and then recombined (prepared for a different study to that reported here; Docherty, 1980) were also subjected to coupled oxidation. When subunits were dissociated and recombined without haem exchange, coupled oxidation yielded 66% α -isomer, 34% β -isomer and none of the γ or δ isomers. However, when the haem from either α or β subunits was replaced before recombination to yield tetramers, δ -isomer was again observed. These findings confirm that the observation of δ -isomer is a function of haem exchange and also show that the phenomenon occurs with both types of subunit.

Further studies were carried out by following coupled oxidation of methaemoglobin which had been incubated at 37°C for various times. Under these conditions, haem exchange has been shown to occur (Bunn & Jandl, 1966, 1968). Results from these studies are shown in Table 3. Initially, the isomer distribution following coupled oxidation is identical with that of native oxyhaemoglobin. However, a gradual increase in formation of δ -isomer is observed. This reaches a constant value of 3–4% within 16h, again primarily at the expense of a reduction in the amount of β -isomer formed. These data are clearly consistent with the gradual introduction of haem disorder as haem exchange proceeds. The maximum amount of δ -isomer formed (3–4%) is consistent with that shown in Table 2 for reconstitution via cyanmet and aquomet derivatives, but less than that observed for reconstitution via carbonmonoxyhaemoglobin. The kinetics of this progressive formation of δ -isomer correlate well with the rate of haem exchange in methaemoglobin, measured by Bunn & Jandl (1966, 1968). Using ^{59}Fe -labelled haem, these authors found that exchange was almost complete after about 16h.

Results from this work clearly support the idea that haem disorder occurs during reconstitution of haemoglobin and that an equilibrium exists between the H_1 and H_2 forms when haem exchange is

Table 3. *Coupled oxidation of haemoglobin following haem exchange*

Methaemoglobin was incubated at 37°C for the times shown, during which slow haem exchange occurred (Bunn & Jandl, 1968). Coupled oxidation was then carried out for 2h in each case as described in the Experimental section. Where δ isomer was detectable but not accurately quantifiable, this is indicated by +, ++, or +++ indicating a visual estimate of the amount present.

Incubation time (37°C) as methaemoglobin (h)	Biliverdin isomers formed (%)			
	α	β	γ	δ
0	66	34	0	0
0.5	65	35	0	0
1.0	67	33	0	0
2.0	66	34	0	+
3.0	66	34	0	++
4.0	67	33	0	+++
16.0	67	30	0	3
24.0	68	28	0	4
48.0	69	28	0	3

possible as in methaemoglobin solutions. In the latter case, once the haem-exchangeable derivative is converted to the non-haem-exchangeable oxyhaemoglobin or deoxyhaemoglobin (as occurs under coupled oxidation conditions), the existing distribution between H_1 and H_2 forms is effectively fixed.

It is known that the H_1 form yields only α and β isomers and it therefore seems a reasonable assumption that the H_2 form yields only α and δ isomers. From the observed amounts of β and δ isomers, it is therefore possible to evaluate approximately the proportion of each form. For reconstitution via the met forms of haemoglobin (approx. 4% δ -isomer and 31% β -isomer), the data would imply 89% of reconstituted molecules in the natural H_1 form and 11% in the H_2 orientation. For reconstitution via carbonmonoxyhaemoglobin, (about 6.5% δ -isomer)

the data suggest approx. 81% of molecules in the H₁ form and 19% in the H₂ form.

The implied haem disorder in reconstituted human haemoglobin is not surprising in view of the n.m.r. studies of La Mar and coworkers. In studies of *C. thummi thummi* haemoglobins, these authors found approx. 60% H₂ form and 40% H₁ form. It seems possible that this distribution resulted from haem exchange via the methaemoglobin and that the naturally occurring species is the H₂ form as observed in the X-ray studies of the corresponding oxyhaemoglobin (Steigemann & Weber, 1979), where haem exchange would not be expected.

Two issues are raised by the present results. The first concerns the interpretation of the large number of studies using reconstituted haemoglobins. Although our data show the proportion of H₂ form to be less than 20%, this may nevertheless be a significant factor in the evaluation of experimental data which has not hitherto been taken into account. A more fundamental question concerns the insertion of haem during haemoglobin biosynthesis. Since native haemoglobin consists only of the H₁ form, but combination of haem with globin *in vitro* yields some H₂ form, it is implied that some control process must exist *in vivo* for ensuring that haem is inserted into apoprotein with the correct orientation. The nature of this control process is unknown.

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