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Haem Degradation in Abnormal Haemoglobins

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The coupled oxidation of certain abnormal haemoglobins leads to different bile-pigment isomer distributions from that of normal haemoglobin. The isomer pattern may be correlated with the structure of the abnormal haemoglobin in the neighbourhood of the haem pocket. This is support for haem degradation by an intramolecular reaction.

Because of the asymmetry in the arrangement of the side chains around the protohaem IX molecule (Fig. 1*a*) the methine-bridge carbon atoms, designated α, β, γ and δ (Fig. 1*a*), are not equivalent. Four isomeric bilirubin IX molecules are therefore possible following haem degradation, but the bilirubin found in mammalian bile consists almost exclusively of the α-isomer, designated bilirubin IXα (Fig. 1*d*). Several intermediates must necessarily be formed during haem breakdown and the first is probably the oxyhaem species (iron–oxophlorin complex) shown in Fig. 1(*b*). The next stage involves ring opening to produce biliverdin (Fig. 1*c*), which is enzymically reduced to bilirubin (for reviews see Jackson, 1974; Schmid & McDonagh, 1975). It is likely (but not proven) that, during catabolism, the haem of haemoglobin dissociates from its apoprotein to act as a substrate for haem oxygenase (Tenhunen *et al.*, 1968), a haem-cleaving enzyme specific for degradation at the α-methine bridge. However, treatment of protein-free haem or haemoglobin with ascorbate and molecular O_2 *in vitro* readily results in biliverdin formation by a process of so-called coupled oxidation. The coupled oxidation of protein-free haem yields an almost random mixture of biliverdin isomers, whereas the coupled oxidation of normal human haemoglobin (Table 1) yields approx. 65% α-isomer and 35% β-isomer (O'Carra, 1975). The haemoglobin apoprotein therefore has the ability to confer a high degree of isomer selectivity even in the absence of haem oxygenase. The molecular mechanism by which the apoprotein controls isomer selectivity in the coupled oxidation of haemoglobin may be similar to that which occurs in the haem oxygenase reaction. Thus, although haem probably does not normally under degradation while associated with globin, the coupled reaction may be considered as a model for the reaction *in vivo*.

Although the structure of human haemoglobin is well known (Perutz, 1976), an explanation for the way in which apoprotein directs selectivity of ring cleavage is not immediately apparent, since the α-methine bridge is buried in a hydrophobic region of the protein, whereas the γ-methine bridge, which is exposed to external reagents, is not attacked. A recent approach to this problem suggests that degradation may occur via intramolecular attack by an activated O_2 molecule (bound to the haem iron atom), at a methine-bridge carbon atom, to produce the oxyhaem (Brown, 1976). In protein-free degradation, reaction is possible at any of the methine bridges since there is no restriction of rotation about the Fe–O bond. However, such free rotation may not be possible for an activated O_2 molecule bound to haemoglobin because of the steric effect of amino acid residues close to the distal side of the haem plane. Examination of the X-ray structure of haemoglobins reveals that access of an iron-bound O_2 molecule to the γ- and δ-bridges would be hindered by histidine residue E7 and valine residue E11 for both the α- and the β-subunits. The β-bridges are also partially blocked by phenylalanine residue CD1. By contrast, in both types of subunit the α-positions appear to be completely accessible and it is clear, on this model, that these would be the preferred points of attack as observed experimentally.

It is possible to test this hypothesis by determining whether variation in the blocking amino acid residues, and others in the neighbourhood of the haem pocket, influences the isomer pattern obtained on coupled oxidation. Fortuitously, haemoglobin is the one protein where an abundance of such amino acid substitutions have been found and documented. In this report, we present results from the degradation of several abnormal haemoglobins containing such substitutions.

Experimental

Samples of abnormal haemoglobins were given by Professor E. R. Huehns (Hb Bristol), Professor K. H. Winterhalter (Hb Zurich), Professor D. Labie (Hb Djelfa), Professor H. Lehmann and Dr. R. Carrell (Hb Koln and Hb Saskatoon). HbS and HbA were obtained from the Department of Haemotology, University of Leeds. HbS and Hb Zurich were pure abnormal samples and all other samples were

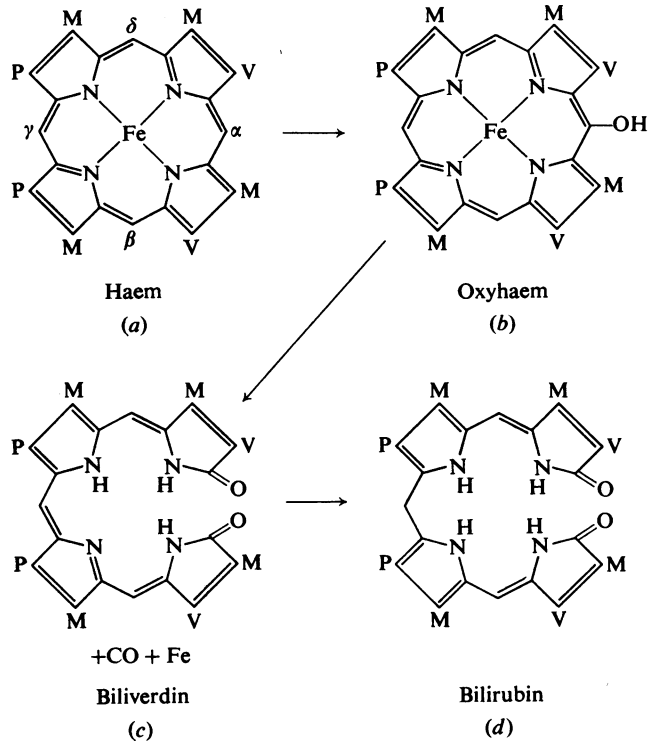

Fig. 1. *Degradation of haem to bile pigments*
Abbreviations: M, $-CH_3$; V, $-CH{=}CH_2$; P, $-CH_2CH_2CO_2H$.

Table 1. *Biliverdin isomers obtained from degradation of abnormal haemoglobins*
Coupled oxidation and t.l.c. was carried out as described in the text. In general the values refer to the means of three determinations where sufficient material was available (otherwise of two determinations). For Hb Bristol, the amount of material available was very small and the accuracy of the data is less than for other haemoglobins.

Haemoglobin	Substitution	Residue number	Abnormal haemoglobin in samples (% of total)	Biliverdin (%)			
				α	β	γ	δ
Hb A	—		—	65	35	—	—
Hb Bristol	β-E11 Val → Asp	67	30	54	37	—	9
Hb Djelfa	β-FG5 Val → Ala	98	15	63	37	—	—
Hb Koln	β-FG5 Val → Met	98	15	54	40	—	6
Hb S	β-A3 Glu → Val	6	100	66	34	—	—
Hb Saskatoon	β-E7 His → Tyr	63	30–40	51	36	6	6
Hb Zurich	β-E7 His → Arg	63	100	23	62	13	2

haemoglobins containing only a proportion of the abnormal material as shown in Table 1. All other materials used were as previously described (Brown *et al.*, 1974).

Coupled oxidation of haemoglobin (50mg) was carried out by incubation with sodium ascorbate (10mg) in 10ml of 0.1M-phosphate buffer (Na_2HPO_4/NaH_2PO_4), pH7.4, at 37°C for 2h with shaking (O'Carra & Colleran, 1969). The reaction mixture was cooled on ice, and 3ml of glacial acetic acid and 8ml of 5M-HCl added. Unreacted haem was extracted immediately into ether (2×15ml). Biliverdin was extracted into chloroform (10ml) and the solution evaporated to dryness. Biliverdin dimethyl esters were prepared by treatment with methanol/sulphuric acid (19:1, v/v) (O'Carra & Colleran, 1969). For visual

estimation of small samples, t.l.c. was carried out on precoated aluminium-backed silica gel G plates irrigated with chloroform/acetone (97:3, v/v) (Bonnett & McDonagh, 1973). For quantitative analysis of isomers, silica gel G-coated glass plates (20cm × 20cm × 0.25mm) were used and irrigated with n-heptane/butan-2-one/acetic acid (10:5:1, by vol.) (O'Carra & Colleran, 1969). Successive elution of isomer bands with chloroform and methanol was followed by evaporation to dryness in a stream of N_2. After redissolution in 1ml of chloroform, relative proportions of isomers were calculated from values of A_{380} and also A_{650}. Mixed t.l.c., with authentic biliverdin dimethyl ester isomers, confirmed the identity and purity of each isomer eluted. Reference experiments by the above procedures on synthetic mixtures of isomers proved that the isolation technique itself did not selectively alter the isomer proportion observed.

Results and Discussion

Results from the coupled oxidation of several abnormal haemoglobins are shown in Table 1, from which it is clear that unusual biliverdin isomers are indeed formed. In assessing the data in Table 1, it should be noted that in any abnormal tetramer half the chains are normal and these might be expected to contribute no unusual isomers on degradation. For Hb Zurich, it appears that the substitution of the distal histidine residue by arginine results in significant formation of biliverdin IX-γ and a decrease in the amount of the α-isomer formed. Studies of the structure of Hb Zurich (Perutz & Lehmann, 1968; Winterhalter & Wuthrich, 1972; Winterhalter *et al.*, 1972) suggest that arginine residue E7 is too large to occupy the position corresponding to histidine residue E7 in HbA and therefore lies further away from the haem group. In terms of the intramolecular degradation hypothesis outlined earlier, the blocking effect of the residue at position E7 would be consequently decreased. The observed formation of biliverdin IX-γ is therefore consistent with this hypothesis. Similarly Hb Saskatoon, also with a substitution of the histidine β-E7 residue, yields a significant amount of the γ-isomer (and, in this case, also the δ-isomer) on degradation.

The substitution of the valine β-E11 residue by aspartate (Hb Bristol) might be expected to perturb the normal protecting influence of this residue for the δ-methine bridge, since a charged residue has replaced a neutral residue. Accordingly Table 1 shows that a significant amount of δ-isomer is formed on degradation of this haemoglobin. Hb Koln and Hb Djelfa are of interest since both have a substitution at the same residue in a short interhelical segment on the proximal side of the haem. Hb Djelfa contains an alanine residue at position FG5, replacing the valine residue of HbA, whereas Hb Koln contains a methionine residue. Table 1 shows that Hb Djelfa showed an almost normal isomer pattern on degradation and no formation of γ- or δ-isomers. However, degradation of Hb Koln produced a significant amount of biliverdin IX-δ. This can be explained if the substitution of the valine residue by the smaller alanine caused no perturbation of the haem pocket, but the substitution by the larger methionine residue caused a significant change in the haem position relative to valine residue at position E11, which normally protects the δ-bridge. For HbS, with a substitution remote from the haem pocket, no significant perturbation of the haem environment would be expected and this is consistent with the observation (Table 1) of a normal isomer pattern on degradation.

These results appear to lend substantial support to the theory of intramolecular haem degradation outlined earlier. In addition, however, they have interesting implications for the study of the structure of abnormal haemoglobins. It is apparent that the isomer pattern obtained on degradation is very sensitive to the position of residues around the haem pocket. In this way the asymmetry of the haem group may be harnessed to act as an intrinsic probe for the determination of structural information for those abnormal haemoglobins whose X-ray structure has not yet been determined.

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