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Haem degradation in human haemoglobin *in vitro*

Separation of the contribution of the α- and β-subunits

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Human haemoglobin was prepared containing [^{14}C]haem in either the α- or the β-subunits. Coupled oxidation of such hybrid haemoglobins with ascorbate and O_2 showed that the biliverdin produced by the α-subunits contained approx. 55% α-isomer and 45% β-isomer, whereas that produced by the β-subunits contained approx. 75% α-isomer and 25% β-isomer. Coupled oxidation of isolated α- and β-subunits gave approx. 70% α-isomer, 30% β-isomer and 78% α-isomer, 22% β-isomer respectively. These results are consistent with calculations of differences in the haem environment in the two subunit types.

Haem degradation to biliverdin forms the major pathway for catabolism of haemoproteins in mammals and for the biosynthesis of certain photosynthetic pigments in algae. Because of the asymmetry in the arrangement of the haem side chains, four biliverdin isomers are possible, depending on which methene bridge (α, β, γ or δ) is the site of attack (see Fig. 1). Haem degradation *in vivo*, however, occurs almost exclusively at the α-bridge. The reaction utilizes molecular O_2 and NADPH and is catalysed by the microsomal enzyme haem oxygenase (Schmid & McDonagh, 1975). The way in which the haem oxygenase protein controls the selectivity of haem cleavage has not yet been studied directly.

The degradation has been simulated *in vitro* by a coupled oxidation method, in which the haem of haemoproteins is degraded to biliverdin while the haem remains associated with haemoprotein (Jackson, 1974; O'Carra, 1975). Coupled oxidation involves treatment of the haemoprotein with molecular O_2 and ascorbate in aqueous solution. In this system (and indirectly in the biological system) there is now strong evidence that the chief determinant of which bridge is attacked (and hence which biliverdin isomer is formed) is the accessibility of an iron-bound O_2 molecule to the various bridge carbon atoms (Brown, 1976). This accessibility may be restricted by the various amino acid residues on the protein. The isomeric composition of the biliverdin produced can there-

Abbreviations used: Hb, haemoglobin; IC II, intermediary compound II.

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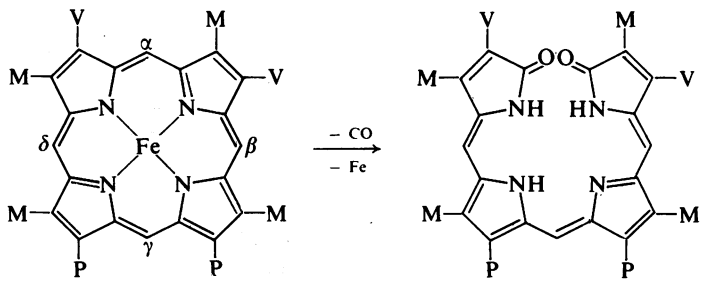

Haem Biliverdin IXα

Fig. 1. *Haem degradation to biliverdin*
M, $-CH_3$; V, $-CH{=}CH_2$; P, $-CH_2CH_2CO_2H$.

fore be used as a chemical probe of the haem environment within the haemoprotein. For native human haemoglobin degradation leads to 65% biliverdin IXα and 35% biliverdin IXβ, whereas coupled oxidation of myoglobin yields only biliverdin IXα (O'Carra, 1975; Docherty & Brown, 1982). These experimental findings correlated well with predictions made by examination of molecular models (Brown, 1976) and with calculations using an interactive computer display system to explore the relative acessibilities of the four methene bridges to a haem-bound O_2 molecule in myoglobin and haemoglobin (Brown *et al.*, 1981*a*).

The use of degradation as a chemical probe of the structure of the haem environment has been reported in studies of abnormal human haemoglobin (Brown & Docherty, 1978) and also more recently in a study of haem disorder in reconstituted haemoglobin (Docherty & Brown, 1982). This latter phenomenon results from an insertion of haem into globin with an orientation 180° different from the natural orientation (rotation about the α–γ axis; see Fig. 1). This phenomenon was readily detected as an appearance of biliverdin IX-δ after coupled oxidation of reconstituted haemoglobin, the δ-bridge occupying the environment previously occupied by the β-bridge.

In previous studies of coupled oxidation of haemoglobin, no attempt has been made to assess the contributions of each type of subunit within the tetramer to the biliverdin isomer pattern produced. Such information would be useful, not only for correlation with calculations (Brown *et al.*, 1981*a*), but also in terms of understanding differences in the haem environment within each type of subunit. We now report such studies with hybrid haemoglobins in which either the α-subunits or the β-subunits were selectively labelled with [^{14}C]haem.

Experimental

Materials

Tris, sodium ascorbate and *p*-hydroxymercuribenzoic acid were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Before use, *p*-hydroxymercuribenzoic acid was purified by the method of Boyer (1954). 5-Amino[4-^{14}C]laevulinic acid hydrochloride (sp. radioactivity 58mCi/mmol) was obtained from Amersham International (Amersham, Bucks., U.K.). Dodecane-1-thiol was from Fluka (Buchs, Switzerland). BDH Chemicals (Poole, Dorset, U.K.) supplied most of the laboratory reagents, which were of analytical-reagent grade.

Purification of haemoglobin

Haemolysates were prepared by addition of distilled water to washed erythrocytes (from outdated blood for transfusion), followed by centrifugation to remove cell debris. Haemoglobin A (HbA) was purified by ion-exchange chromatography on DEAE-cellulose (Huisman & Dozy, 1962).

Preparation of [^{14}C]haem-hybrid haemoglobins

[^{14}C]Haem (sp. radioactivity 20×10^{10} d.p.m./mol) was prepared biosynthetically by addition of 5-amino[4-^{14}C]laevulinic acid to reticulocyte-rich rabbit blood as previously described (Brown *et al.*, 1981*b*). Three techniques were utilized for the preparation of hybrid haemoglobin tetramers containing [^{14}C]haem in either the α- or the β-subunit.

Subunit exchange. This method utilizes the phenomenon whereby a mixture of two haemoglobins taken to mildly acid pH forms monomers (Huehns *et al.*, 1964), which on return to neutrality will recombine to give hybrid tetramers. A mixture of ^{14}C-labelled foetal haemoglobin (HbF) and [^{12}C]HbA yields HbA carrying [^{14}C]haem in only the α-subunits (Bunn & Jandl, 1968). [^{14}C]HbF was prepared by incubating fresh umbilical-cord blood with 5-amino[4-^{14}C]laevulinic acid, followed by ion-exchange chromatography on DEAE-cellulose. An equimolar mixture of [^{14}C]HbF and [^{12}C]-HbA was dialysed against 0.1M-sodium acetate buffer, pH 4.5, for 48h. Dialysis against 10mM-Tris/HCl buffer, pH 7.0, allowed monomer recombination and hence hybrid formation to occur. Haemoglobins were subsequently separated by ion-exchange chromatography (Bunn & Jandl, 1968).

Subunit recombination. Globin was prepared by precipitation in acidified acetone at −20°C (Winterhalter & Huehns, 1964) and recombined with [^{14}C]haem to give haemoglobin in the Fe^{3+} CN^- form as described by Winterhalter & Huehns (1964). Subunit separation was achieved by reaction with *p*-hydroxymercuribenzoate. The subunits were isolated by ion-exchange chromatography with the use of the two-column technique described by Geraci *et al.* (1969). *p*-Hydroxymercuribenzoate was removed from the isolated subunits by treatment with dodecanethiol (De Renzo *et al.*, 1967), and this process was monitored by determination of free thiol groups in the isolated subunits (referred to below as αSH and βSH) (Benesch & Benesch, 1974). The hybrid tetramer [^{14}C]α_2[^{12}C]β_2 was obtained by mixing equimolar quantities of [^{14}C]αSH and [^{12}C]βSH subunits. [^{12}C]α_2[^{14}C]β_2 hybrid tetramer was obtained in a similar manner with the appropriate ^{14}C- and ^{12}C-containing subunits. Recombination was confirmed by chromatography on Sephadex G-100.

Reconstitution via half-filled intermediates. The addition of non-saturating amounts of haem to

globin results in the formation of a series of intermediates containing 0, 1, 2, 3 and 4 molecules of haem per globin tetramer (Winterhalter, 1966; Winterhalter & Deranleau, 1967). When the molar ratio of haem/globin subunits is 1:2 the major product is a tetramer containing two haem groups located solely in the α-subunit (intermediary compound II, IC II; Winterhalter & Deranleau, 1967). IC II prepared with [^{12}C]haem may be converted into haemoglobin by addition of [^{14}C]-haem to give the [^{12}C]α_2[^{14}C]β_2 hybrid. The [^{14}C]α_2[^{12}C]β_2 species may likewise be prepared by addition of [^{12}C]haem to [^{14}C]IC II.

Preparation of IC II species and conversion into haemoglobin were performed exactly as described by Winterhalter & Deranleau (1967). The resultant hybrids were converted from the Fe^{3+} CN^- form into the oxy form via the carbonmonoxy derivative. Formation of HbCO was achieved by reduction with dithionite in the presence of CO, excess dithionite being removed by passage through a column of Sephadex G-25. Repeated evacuation and equilibration with O_2 yielded HbO_2. All conversions were monitored by spectrophotometric means in the range 500–650nm.

The distribution of the [^{14}C]haem between the two types of subunit within the tetramer after hybrid formation was assessed by chain separation by reaction with *p*-hydroxymercuribenzoate and chromatography by the single-column technique of Geraci *et al.* (1969), followed by scintillation counting of the radioactivity of the isolated subunits. Similar determinations were also performed on residual haemoglobin after coupled oxidation, to ensure that no redistribution of the labelled haem had occurred during the course of the reaction.

Coupled oxidation of haemoglobins

The chemical degradation of the haem of haemoglobin, followed by the purification by t.l.c., extraction and spectrophotometric quantification of the resultant biliverdin isomers, were performed as previously described (Brown & Docherty, 1978). Although the biliverdin recovered corresponds to only about 5% of the total initial haem, it has been clearly shown that losses of biliverdin during extraction are not isomer-selective (Docherty & Brown, 1982). After determination of the relative proportion of each biliverdin isomer by absorption spectroscopy, biliverdin samples were evaporated to dryness under a stream of N_2 in glass scintillation vials and redissolved in 0.2ml of methanol. Then 5ml of scintillation cocktail (0.4% 2,5-diphenyloxazole in toluene) was added. Counting efficiency was determined by the internal-standard method with n-[^{14}C]hexadecane.

Results

In all procedures used to prepare [^{14}C]haem-hybrid haemoglobin tetramers, care was taken to prevent formation of methaemoglobin to minimize redistribution of the [^{14}C]haem between subunits as a result of haem exchange (Bunn & Jandl, 1968). In preliminary experiments, where significant methaemoglobin formation occurred, up to 20% of the labelled haem was located in the wrong subunit type, as assessed after chain separation and scintillation counting. Such preparations were not used for haem-degradation studies. Fig. 2 shows the distribution of [^{14}C]haem between the subunit types in a [^{14}C]α_2[^{12}C]β_2 hybrid prepared by the IC II method. Over 95% of the labelled haem is present in the α-chains. Similar analysis of a portion of the same hybrid that had not reacted, recovered after coupled oxidation, showed no significant redistribution of the labelled haem between the subunit types. Haem exchange therefore does not occur under the conditions employed in the coupled oxidation reaction.

Spectrophotometric determination of the isomer distribution of biliverdin recovered from the coupled oxidation of hybrid haemoglobins (Tables

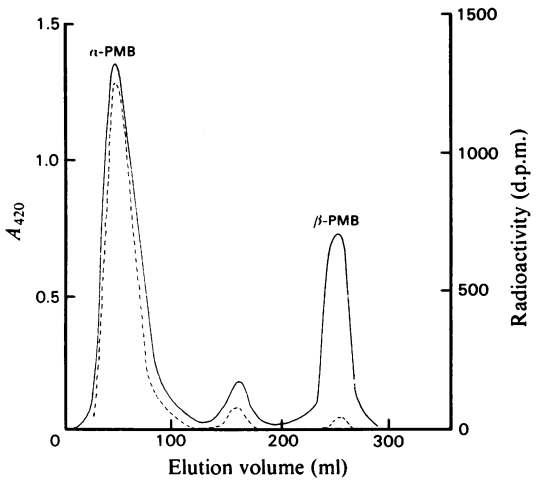

Fig. 2. *Distribution of* [^{14}C]*haem between the subunit types in a* [^{14}C]α_2[^{12}C]β_2 *hybrid prepared by the IC II method*

The selectively labelled tetramer was prepared by addition of [^{12}C]haem to [^{14}C]IC II as described in the text. After reaction with *p*-hydroxymercuribenzoate, the mixture of subunits was resolved on DEAE-cellulose as indicated in the Experimental section (Geraci *et al.*, 1969). ——, A_{420}; ----, radioactivity. α-PMB and β-PMB indicate subunits with thiol groups blocked by reaction with *p*-hydroxymercuribenzoate.

Table 1. *Biliverdin isomers recovered from coupled oxidation of hybrid haemoglobins containing [^{14}C]haem in α-subunits*

	Biliverdin isomer (% of total biliverdin recovered)				^{14}C in biliverdin isomers (% of total d.p.m. recovered in biliverdin)			
Method of preparation	α	β	γ	δ	α	β	γ	δ
Subunit exchange	63	37	0	0	55	45	0	0
Subunit recombination	64	33	0	3	52	46	0	2
IC II	65	31	0	4	56	41	0	3

Table 2. *Biliverdin isomers recovered from coupled oxidation of hybrid haemoglobins containing [^{14}C]haem in β-subunits*

	Biliverdin isomer (% of total biliverdin recovered)				^{14}C in biliverdin isomers (% of total d.p.m. recovered in biliverdin)			
Method of preparation	α	β	γ	δ	α	β	γ	δ
Subunit recombination	67	30	0	3	76	21	0	3
IC II	64	32	0	4	75	23	0	2

1 and 2) revealed close agreement with the 65% α/35% β pattern observed in native HbA. The occurrence of small amounts of biliverdin IX-δ formed from hybrid haemoglobin prepared by methods involving reconstitution of globin with haem are in agreement with recent studies on haem disorder in reconstituted haemoglobins (Docherty & Brown, 1982).

Table 1 also shows the distribution of radioactivity in the biliverdin isomer obtained by coupled oxidation of hybrid haemoglobin containing [^{14}C]haem in the α-subunits. Similar data are shown in Table 2 for the complementary experiments involving labelling of the β-subunits. In both cases there is good agreement between the results for the hybrids obtained by various methods. Clearly, substantially less of the biliverdin IX-β isomer is formed from degradation of the haemoglobin β-subunit than from the α-subunit.

Significantly, the average of the biliverdin isomer distributions from the two subunits within the hybrid tetramer closely agrees with the overall isomer pattern obtained from the native haemoglobin tetramer. This strongly suggests an equal contribution of the two subunits to the overall recovery of biliverdin, showing that the haem of one subunit is not preferentially degraded.

The biliverdin isomer patterns obtained from coupled oxidation of isolated haemoglobin subunits are shown in Table 3. The results show that, as in the intact tetramers, only α- and β-isomers are formed. Also shown in Table 3 are results for coupled oxidation of the IC II species, which is a globin tetramer containing haem only in the α-subunit. This species also yields predominantly α- and β-isomers of biliverdin with the expected occurrence of δ-isomer due to haem disorder in reconstituted haemoglobin.

Table 3. *Biliverdin isomers recovered from coupled oxidation of isolated α- and β-subunits of human haemoglobin*

Results are expressed as average of two determinations on the same preparation of each subunit derivative. α-SH and β-SH indicate subunits with free thiol groups, α-PMB and β-PMB indicate subunits with thiol groups blocked by reaction with *p*-hydroxymercuribenzoate, and IC II contains haem only in the α-subunits.

	Biliverdin isomer (% of total biliverdin recovered)			
Subunit derivative	α	β	γ	δ
α-SH	65	35	0	0
α-PMB	70	30	0	0
β-SH	80	20	0	0
β-PMB	76	24	0	0
IC II	67	30	0	3

Discussion

Of the three methods used for preparation of hybrid haemoglobins, two involved reconstitution of globin with [^{14}C]haem *in vitro*. By contrast, in the third method, subunit exchange, ^{14}C-labelled α-subunits were formed directly by biosynthesis. Despite these differences in methods of preparation, all of the hybrid tetramers yielded a biliverdin isomer pattern essentially the same as that observed in native haemoglobin (with the exception of δ-isomer formation due to haem disorder). On

this basis, the hybrid tetramers appear to have been prepared with a native protein conformation in the vicinity of the haem-binding pocket. As predicted from studies on haem disorder in reconstituted haemoglobins (Docherty & Brown, 1982), small amounts of biliverdin IX-δ were observed on coupled oxidation of hybrids prepared by methods involving reconstitution of globin with haem. It is especially noteworthy that [^{14}C]-biliverdin IX-δ was formed irrespective of which subunit type contained [^{14}C]haem, indicating that the haem disorder phenomenon is not confined to one subunit type.

From the results, variations in the haem environment in the two subunit types both within the haemoglobin tetramer and as isolated subunits may be explored. The quantitative differences in the distribution of radiolabel in the biliverdin recovered from hybrids containing [^{14}C]haem in the α- or the β-subunit suggest significant differences in the accessibility of the α- and β-methene bridges of haem in the two subunit types. Approx. 45% of the IX-β isomer is recovered from biliverdin formed from the α-subunit, whereas only 25% of the β-isomer is formed from the β-subunit. Previous studies (O'Carra, 1975) have shown that none of the β-isomer is formed by degradation of myoglobin haem. These findings are in good agreement with predictions made with the use of an interactive computer display system to examine the relative accessibilities of the four methene bridges to a haem-bound oxygen in the three haem-polypeptide chain complexes (Brown *et al.*, 1981*a*). These calculations revealed a decreasing accessibility of the β-methene bridge in the order Hb α-chains, Hb β-chains, myoglobin and suggested that this altered accessibility to the β-methene bridge appeared to be due to minor variations in the orientation of a haem-pocket phenylalanine residue relative to the haem. The results of the present study are compatible with a positioning of the phenylalanine residue closer to the haem β-methene bridge in the β-subunits as compared with the situation of this residue in the α-subunits.

On dissociation of the native tetramers to isolated subunits only a slight alteration in the biliverdin isomer pattern is observed in the case of β-subunits, whereas a greater difference is observed with α-subunits, with less of the biliverdin IXα isomer being formed. The apparently greater conformational change in the α-subunits may be related to the observations that α-subunits exist as dimers whereas the β-subunits are tetrameric (Rosemeyer & Huehns, 1967). The tetrameric arrangement of β-subunits may result in a haem-pocket conformation similar to that in the native tetramer. It should be noted, however, that the *p*-hydroxymercuribenzoate-blocked β-subunits, which exist as monomeric rather than tetrameric species, yielded biliverdin isomer patterns differing only slightly from the distributions obtained from β-SH subunits. The slightly larger differences observed in the α-subunits on dissociation of the tetramer agrees with e.s.r. studies (Jan & Asakura, 1979) on hybrid haemoglobins containing spin-labelled haem in the α- or β-subunits, where dissociation of the tetramer into isolated subunits caused larger perturbations in the α-chains than in the β-chains.

The degradation of IC II tetrameric globin containing haem only in the α-subunits resulted in a biliverdin isomer pattern similar to that of isolated α-subunits, but different from that obtained from degradation of the haem of α-subunits within fully reconstituted haemoglobin. Thus the influence of the β-subunits on the haem environment of the α-subunits in the intact tetramers is not merely a protein–protein interaction but requires the presence of haem in the subunits. Whether this is due to conformational differences brought about by the insertion of haem itself or is a result of the classical concepts of haem–haem interactions during haemoglobin oxygenation cannot be distinguished in the present study.

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