Stereospecific Haem Cleavage

A MODEL FOR THE FORMATION OF BILE-PIGMENT ISOMERS IN VIVO AND IN VITRO

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A new approach is suggested for an explanation of stereospecific haem degradation to biliverdin and bilirubin. A model is proposed in which an oxygen molecule, bound to the haem iron atom, attacks a methene-bridge carbon atom in an intramolecular reaction. Specificity of macrocyclic ring cleavage is explained on the basis of the different accessibilities of the bound oxygen molecule to the four methene bridges. The consequences of these ideas are assessed in relation to coupled oxidation in model systems and to haem catabolism.

The degradation of haem to bile pigment is a much-studied process in living animals, in tissue extracts and in chemical model systems [for reviews, see Lathe (1972), Jackson (1974), O'Carra (1975) and Schmid & McDonagh (1975)]. In mammals, bilirubin is produced by the action of biliverdin reductase on the intermediate biliverdin (Fig. 1) and is excreted in the bile. Bilirubin is also formed in the microsomal 'haem oxygenase' system (Schmid & McDonagh, 1975), but from haem or haemoprotein degradation *in vitro*, produced by coupled oxidation with ascorbate, biliverdin is the final product.

Because of the asymmetry of the side chains in the protoporphyrin IX ligand, the methene bridges α , β , γ and δ (shown in Fig. 1) are not equivalent and cleavage of the macrocyclic system at these positions can give rise to four isomeric biliverdins or bilirubins. However, the bilirubin found in bile consists exclusively of the α isomer, designated bilirubin IX α . The explanation of this specificity in molecular terms has eluded workers in this area for many years. Early work

by Lemberg (1956) suggested that specificity was a natural consequence of the asymmetry of the haem, i.e. that the α bridge was intrinsically more reactive than the others. Alternatively it was possible that cleavage occurred at all four positions, but that the products formed from β , γ and δ cleavage were catabolized by alternative routes.

The development of more sophisticated chromatographic techniques (Rudiger, 1968; O'Carra & Colleran, 1969; Bonnett & McDonagh, 1973) for the separation and analysis of bile-pigment isomers has shed new light on the problem, and shown that neither of these views is correct. The observations by Petryka *et al.* (1962) and O'Carra & Colleran (1969) that chemical degradation of haem in aqueous pyridine gave an almost random mixture of isomers revealed that there were no large differences in the intrinsic reactivity of the four methene carbon atoms. O'Carra & Colleran (1969) carried out further work on the coupled oxidation (with ascorbate) of haemoproteins and found that from myoglobin, biliverdin IX α was

Fig. 1. Formation of bile pigments by haem degradation

M = $-\text{CH}_3$, V = $-\text{CH}=\text{CH}_2$, P = $-\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$. Because of the asymmetry of the side-chain groups, the methene bridges α , β , γ and δ are not equivalent and four isomers of biliverdin and bilirubin are possible. Only the α isomers are shown.

obtained exclusively, whereas from haemoglobin the product contained about 60% of the α isomer and 40% of the β isomer. These workers were therefore able to demonstrate the imposition of specificity of cleavage by apoproteins even under conditions *in vitro*. With denatured myoglobin or with haem-albumin a random mixture of isomers was obtained.

Although it was clear that apoproteins were somehow directing specificity, the molecular factors involved were obscure in spite of the known structure of both haemoglobin and myoglobin. As O'Carra (1975) has pointed out, on the basis of accessibility to external reagents the α -methene bridge might be expected to be the least susceptible to attack, since it lies towards the interior of the proteins in a hydrophobic region. It was therefore suggested that degradation might be faster in a hydrophobic rather than a hydrophilic environment and that acceleration of the rate of cleavage at the α position might be responsible for the observed α specificity. In support of this theory, it was shown that coupled oxidation proceeds faster in a hydrophobic solvent (benzene) than in aqueous systems (O'Carra & Colleran, 1970). Brown *et al.* (1974), however, have argued that a kinetic specificity introduced by virtue of an acceleration of reaction rate at the α position should result in a consequent increase in total degradation rate compared with denatured myoglobin or free haem. Such an increase is not observed experimentally. Further, the apparent influence of the hydrophobicity of the haem environment on degradation rate in model systems may be accounted for in other ways, such as increased oxygen solubility or prevention of haem aggregation (Brown *et al.*, 1974; Brown & King, 1976).

Previous theories for the origin of protein-induced specificity of haem cleavage have therefore considered the problem in terms of the accessibility of the methene bridges to agents outside the protein or in terms of the hydrophobicity of the environment of the various bridges. An explanation is now suggested on a somewhat different basis, i.e. the accessibility of the various methene bridges to an oxygen molecule already bound to the iron atom.

Oxygen Activation

Essential to any theory of haem-cleaving specificity is an understanding of the way in which molecular oxygen becomes activated before bridge oxidation. Evidence is now accumulating that oxygen is activated by the very haem molecule that it will ultimately destroy (see below). It seems likely that the oxygen molecule becomes bound to ferrohaem and that an intramolecular reaction ensues, which results in transfer of an oxygen atom to the methene bridge to form the oxyhaem species, generally accepted as the first intermediate in the degradative pathway (Jackson, 1974; O'Carra, 1975; Schmid & McDonagh, 1975). In

support of this hypothesis are the following points. (a) Free porphyrins do not undergo cleavage in any of the usual haem-degrading systems. Work in our laboratory (S. B. Brown & S. Grundy, unpublished work) has shown that this is not due to porphyrin aggregation, which, by analogy with haem systems, might be expected to prevent degradation. Iron is therefore required for macrocyclic ring cleavage. (b) Haem degradation could be envisaged as an intermolecular process in which a haem molecule is able to complex with and activate an oxygen molecule and then cause the degradation of a second haem molecule. This would be consistent with (a), since iron would almost certainly be required for oxygen activation. However, in such a process it would be expected that the second haem molecule, which is acting solely as a substrate rather than an oxygen activator, could be replaced by a free porphyrin molecule. In this case, exposure of mixtures of haem and free porphyrins to the coupled oxidation system would lead to degradation of the iron-free porphyrin as well as the haem. Experiments carried out on the coupled oxidation of such mixtures (S. B. Brown & S. Grundy, unpublished work) have shown that although the haem is degraded, the porphyrin remains unchanged. These results suggest that haem degradation cannot be an intermolecular process, i.e. the degradation of one haem molecule, catalysed by another. (c) Degradation appears to require iron in the $\text{Fe}^{(II)}$ oxidation state and ferrihaem species are completely unreactive (Brown & King, 1976). Since it is well known that only ferrohaems are able to bind molecular oxygen, this is further evidence that such oxygen binding is required for degradation. In the case of the haemoproteins, the ability to bind molecular oxygen is an intrinsic property of the protein. For free haem, the presence of excess of reducing agent is required to maintain the iron atom in the $\text{Fe}^{(II)}$ oxidation state. This is accomplished in coupled-oxidation systems by ascorbate. (d) Ligands which bind very strongly to haem, for example imidazole, completely inhibit haem cleavage in model systems (S. B. Brown & P. O'Carra, unpublished work). Presumably this inhibition arises because of the inability of molecular oxygen to compete with imidazole for binding at the sixth position on the iron atom. (e) It has been well established that dimeric haem species are not readily degraded (Brown *et al.*, 1974; Brown & King, 1976), probably because no oxygen binding is possible in the dimer.

Fig. 2 shows, in cross section, how a bound oxygen molecule may interact with a methene bridge. Such an orientation of the oxygen atoms is consistent with X-ray data (e.g. the picket-fence model of Collman *et al.*, 1974) and has been suggested by Antonini & Brunori (1971), partly by analogy with the well-defined azide-haem complex. Antonini & Brunori (1971) further suggest that the angle between the

Fig. 2. Possible scheme by which oxygen bound to haem iron may interact with a methene-bridge carbon atom

The Figure is a cross-section perpendicular to the haem plane along a line joining the γ and α methene-bridge carbon atoms. The bound oxygen molecule O(A)–O(B) is shown with the O(A)–O(B) bond at an angle to the haem plane that is consistent with X-ray data (Antonini & Brunori, 1971). Oxidation is envisaged as an attack by the oxygen atom O(B) on a methene-bridge carbon atom (in this case the α bridge).

Fe–O(A) bond and the porphyrin plane may be less than 90° . Clearly this would facilitate attack by the O(B) atom at a methene-bridge carbon atom.

The nature of the electronic rearrangements during oxidation of the bridge carbon atom are unknown. However, assuming the initial species to be the complex Fe(II)–O₂, the most likely ways in which molecular oxygen may be activated are either by transfer of one electron to oxygen to form Fe–O₂^{•−} (a superoxo species) or by a two-electron transfer to produce Fe–O₂^{2−} (a peroxo species). These electrons are supplied either by NADPH in biological systems or by ascorbate in coupled oxidation systems. Whether electron transfer occurs directly from reducing agent to iron-bound O₂ or whether this process is mediated by the formation of higher oxidation states of iron, such as have been postulated for peroxidase and catalase action (Schonbaum & Lo, 1972; Jones & Middlemiss, 1974), is not known. Similar mechanisms have also been suggested to account for the activity of cytochrome P-450 (Estabrook *et al.*, 1975). Indeed, the analogy between the postulated haem-cleaving reaction and hydroxylations involving cytochrome P-450 is a striking one, the only difference being that in the former case the 'substrate' carbon atom is part of the haem molecule, whereas in the latter case the substrate is presumably bound to cytochrome P-450 apoprotein in juxtaposition to haem.

It is well known that H₂O₂ causes cleavage of the haem macrocycle in the absence of molecular oxygen (Gray, 1953) and this reaction has been studied in some detail (Brown & Jones, 1968; Jones *et al.*, 1973). This may be taken as evidence in support of the Fe–O₂^{2−} species, although it appears that any possible reaction between externally generated superoxide ion and haem has not yet been investigated.

Specificity of Cleavage

Whatever the precise electronic rearrangements during methene-bridge oxidation, the proposition that

the oxygen molecule bound to the iron atom is itself the agent of haem oxidation leads to rather different criteria for the imposition of specificity than those previously considered. Now, it is not necessary to consider the accessibility of the methene bridges to external reagents, but to the oxygen molecule bound to their parent iron atom. For free haem derivatives, the O(A)–O(B) bond shown in Fig. 2 would be allowed completely free rotation about the Fe–O(A) bond. All four methene bridges would be equally available to the attacking oxygen atom, O(B), leading to a random mixture of isomers. The same argument would apply to haem bound to proteins, where the haem is not buried within the protein. This situation occurs with the haem adduct of serum albumin (Beaven *et al.*, 1974) and presumably also with myoglobin denatured in 8M-urea. In both these systems, a random isomer mixture is obtained (O'Carra, 1975). However, in the haemoproteins, where haem is tightly bound within the protein, a different situation would apply. Because of interactions of the oxygen atom O(B) with amino acid residues on the protein, rotation about the Fe–O(A) bond would be restricted. The O(A)–O(B) bond would therefore point in a direction dictated, not by hydrophobic–hydrophilic factors as suggested by O'Carra (1975), but by the positive exclusion of the O(B) atom from certain positions because of steric hindrance. Oxidation would then occur only at the bridge carbon atom(s) that were accessible to the oxygen atom O(B).

Fig. 3. Oxygen-binding site in sperm-whale myoglobin

M = –CH₃, V = –CH₂=CH₂, P = –CH₂CH₂CO₂H. The bonds of all amino acids within 0.6nm of the haem plane (distal side) are shown in projection on to the haem plane. An oxygen molecule would bind above the plane of the paper in the manner shown in Fig. 2. Van der Waals radii are not shown, to aid clarity, but clearly should be taken into account in assessing the accessibility of the various methene-bridge carbon atoms to the iron-bound oxygen molecule. It is evident that only the α bridge is likely to be accessible. Data were obtained from Antonini & Brunori (1971).

These ideas have been tested by examining the known structures of haemoglobin and myoglobin. Fig. 3 shows the orientation of the haem in myoglobin (sperm whale) relative to amino acid residues on the distal side of the iron atom, i.e. the side at which oxygen is bound. The positions of all residues with atoms within 0.6 nm of the haem plane are shown in projection on to the haem plane. Examination of Fig. 3 shows at once that access of oxygen atom O(B) to some of the methene bridges is restricted. For the γ -methene bridge, histidine-64 completely blocks any possibility of oxidation. Similarly the atoms of valine-68 are blocking the δ bridge. The β -methene bridge appears to be more accessible, but allowing for the Van der Waals radius on the benzene-ring atoms of phenylalanine-43, it is likely that this position also would be blocked. By contrast, the α position is completely accessible and it is clear that this would be the preferred point of attack for the O(B) atom. These observations accord exactly with experiment, since native myoglobin in coupled oxidation yields 100% of the α isomer.

Parallel considerations for haemoglobin (human) reveal a similar but not identical situation. Here again the δ and γ bridges are completely blocked by the corresponding residues, valine-62 and histidine-58 for the α subunits and valine-67 and histidine-63 for the β subunits. Again the α -methene bridge is relatively accessible, but in this case, leucine-101 (α subunit) and leucine-106 (β subunit) appear to be sufficiently close to the α -carbon atom so that some hindrance may occur. On the other hand, the β -methene carbon atom appears to be more accessible than in myoglobin, since the corresponding phenylalanine residue (phenylalanine-43 for the α subunit, phenylalanine-42 for the β subunit) is further away from the haem plane. These observations for haemoglobin are also in agreement with experiment, where the proportions of α and β isomers are 60 and 40% respectively.

Discussion

It appears from these considerations that cleavage specificity is determined by accessibility of the methene bridges to oxygen from within the haemoprotein molecule rather than to external reagents. In the degradation of haem *in vivo*, it seems likely that a membrane-bound protein, appearing in the microsomal fraction, binds the haem released from haemoglobin. It is also probable that for efficiency of haem removal, very strong haem binding is required whether the haem is scavenged directly from haemoglobin or taken up as free haem from the external medium. Such tight binding would be consistent with the high degree of specificity observed in the bile pigments produced after cleavage. This haem-binding

Fig. 4. Postulated structure of the ligand-binding site in catalase

M = $-\text{CH}_3$, V = $-\text{CH}=\text{CH}_2$, P = $-\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$. Since catalase gives 50% each of biliverdin IX α and biliverdin IX β on coupled oxidation, it is likely that the γ and δ bridges are completely inaccessible to the iron-bound oxygen molecule. On this basis, the shaded zone would be inaccessible to ligands bound to iron, including peroxide substrate. The ligands would be diverted towards either the α or β position.

protein is identifiable with the haem oxygenase identified and characterized by Schmid and co-workers (Schmid & McDonagh, 1975). On the basis of the above model, it may therefore be predicted that, in the haem-haem oxygenase enzyme-substrate complex, access of a bound oxygen molecule to the β , γ and δ bridges of haem is restricted by amino acid residues of the enzyme. A necessary property of this protein is the ability, at least partially, to stabilize the haem in the iron(II) form during oxygen binding before oxygen activation. In this respect it behaves in an identical fashion with cytochrome P-450. However, the work of Yoshida *et al.* (1974) and Maines & Kappas (1974) suggests that cytochrome P-450 and haem oxygenase are distinct proteins, although it has been suggested (O'Carra & Colleran, 1976; Schmid, 1976) that haem oxygenase may be a modified form of cytochrome P-450.

These ideas can be extended to other, more complex, haemoproteins such as catalase and peroxidase, whose complete three-dimensional structure has not yet been elucidated. For example, on coupled oxidation catalase yields about 50% each of the α and β isomers (O'Carra, 1975). It is reasonable to infer that the γ and δ bridges are inaccessible to ligands bound to the central iron atom and that such ligands, including peroxide substrate, would be preferentially orientated towards the α and β positions. On this basis, Fig. 4 represents a postulated structure of the catalase active site shown in projection on to the haem plane. These ideas may be helpful in considering the mechanism of action of catalase and other haemoproteins. In this way the cleavage specificity may be

used as a probe of the active-site structure of haemo-proteins that are too large to readily permit X-ray analysis.

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