Methine Bridge Selectivity in Haem-Cleavage Reactions: Relevance to the Mechanism of Haem Catabolism

PÁDRAIG O’CARRA and EMER COLLERAN

Department of Biochemistry, University College, Galway, Republic of Ireland

The predominant haem type in biological systems is protohaem IX, whose unsymmetrical arrangement of side chains renders the four methine bridge positions (α, β, γ and δ) non-equivalent (Scheme 1). As a result, cleavage at each bridge produces a different biliverdin isomer, the four isomers being named after the parent haem-type and the methine bridge cleaved, thus biliverdin IXα, IXβ, IXγ and IXδ (Scheme 1).

It has long been the general opinion that naturally occurring biliverdin, and bilins derived therefrom (e.g. biliary bilirubin), are of the IXα isomeric type, and the assumption has been that this reflects a very high degree of specificity for the α-methine bridge during biological haem-cleavage processes. With some seemingly minor exceptions and reservations (cf. Rüdiger et al., 1968; O’Carra & Colleran, 1970a; Morell & O’Carra, 1974) this belief has been largely confirmed by recent results (see e.g. O’Carra & Colleran, 1970a; Gray et al., 1972). This ‘α-specificity’ of biological haem-cleavage processes has attracted the attention of workers over the years. It seemed to provide a clue whose explanation would constitute a major advance towards the elucidation of the causative factor(s) and mechanism of biological haem cleavage.

Two rival theories regarding the causative factor(s) and the α-specificity were repeatedly advanced. The first, advanced by Lemberg (see Lemberg, 1956), proposed that haem catabolism was a non-enzymic process involving purely chemical coupled oxidation of haem, haemochromes or haemoproteins with reductants such as ascorbate; the α-specificity was supposed to be due to an intrinsic lability of the α-methine bridge resulting from the structure of protohaem IX itself. The alternative
theory proposed that the cleavage event was catalysed by a haem-cleaving enzyme, whose active site imposed the $\alpha$-specificity on the cleavage process.

Proponents of both theories cited experimental evidence in support of their opposing viewpoints, but work published in the late 1960's and early 1970's showed that this seemingly conflicting experimental evidence had resulted largely from the unreliability of the experimental techniques previously available for isomer analysis of the bilirubin products of haem cleavage. Improved techniques showed that the $\alpha$-specificity is not attributable to any intrinsic property of the haem molecule itself, which is cleaved in an essentially random fashion at the four methine bridges when subjected to coupled oxidation in the 'free' form or as simple haemochromes (Rüdiger, 1968; O’Carra & Colleran, 1969, 1970a; Bonnett & McDonagh, 1970, 1973). At the same time, much of
Table 1. Methine bridge selectivity in the coupled oxidation of various haem derivatives and haemoproteins

The coupled oxidations were carried out with ascorbate as the 'oxygen-activating' reductant, except in the case of the microsomal preparations where NADPH (with or without ascorbate) was the most effective reductant. [From O'Carra & Colleran (1969) and P. O'Carra & E. Colleran, unpublished work.]

<table>
<thead>
<tr>
<th>Haem derivative subjected to coupled oxidation</th>
<th>Isomer composition of biliverdin product (%)</th>
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<tbody>
<tr>
<td>Haem</td>
<td>IXα  31</td>
</tr>
<tr>
<td>Pyridine haemochrome</td>
<td>IXβ  24</td>
</tr>
<tr>
<td>Haem–albumin (human)*</td>
<td>IXγ  24</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>IXδ  21</td>
</tr>
<tr>
<td>'Microsomal haemprotein' (P-450 or derivative?)</td>
<td></td>
</tr>
<tr>
<td>Haemoglobin (human)*</td>
<td></td>
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<tr>
<td>Catalase</td>
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* Proportions of isomers produced vary somewhat with species variations in the structure of albumin and of apohaemoglobin.

The evidence that had been put forward in favour of an enzymic haem-cleavage system was shown to be spurious (Colleran & O'Carra, 1970).

In our laboratory we instituted a search for a true enzymic system, but lack of really convincing results in this direction caused us to change our approach. We began to consider whether the $\alpha$-specificity might be induced, not by binding of haem to the active site of a haem-cleaving enzyme, but rather by its interaction with the haem-binding sites of well-defined haemoproteins having no 'purposeful' haem-cleavage role.

Our results quickly encouraged us to accept this viewpoint. As Table 1 shows, free haem, simple haemochromes (e.g. pyridine haemochrome) or relatively non-specific haem–protein complexes, such as haem–albumin, produce an essentially random mixture of the four biliverdin isomers, when subjected to coupled oxidation. But, when certain well-defined haemoproteins are subjected to similar coupled oxidation, a striking degree of bridge-specificity is imposed on the cleavage process. That such selectivity is induced in a very specific way by the haem-binding sites of the haemoproteins is evidenced by the fact that the selectivity disappears if the apoproteins are denatured (O'Carra & Colleran, 1969), but so long as these apoproteins retain their native conformations the selective bridge-cleavage survives removal and reinsertion of the haem groups or replacement of the natural protohaem by mesohaem or deuterohaem (cf. O'Carra, 1975).

The $\alpha$-specificity induced by the haem-binding sites of myoglobin and the 'microsomal haemoprotein' (Table 1) accorded so well with the $\alpha$-specificity of catabolic haem cleavage that a connexion between the two phenomena seemed evident. But the results obtained with haemoglobin, although interesting in themselves, seemed perverse in terms of our efforts to develop a simple model for haem catabolism. The cleavage in this haemoprotein is also highly selective in that it is directed completely away from the $\gamma$ and $\delta$ bridges, but it is directed to both the $\alpha$ and $\beta$ bridges. (Table 1) Since haemoglobin is the haemoprotein whose catabolism accounts for over 80% of total haem catabolism in mammals, the dual bridge selectivity accords poorly with the overwhelming preponderance of the $\alpha$-isomer type among the natural bilin products. Although complexing of haemoglobin with haptoglobin produced a very significant change of the cleavage pattern in favour of greater $\alpha$-specificity (O'Carra & Colleran, 1970b), any explanations involving haptoglobin seem to be eliminated by the subsequent discovery that at least some mammals seem to lack this protein entirely (P. O'Carra & E. Colleran, unpublished work).

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The mechanisms of the haem-cleavage process seems to involve binding of $O_2$ to the haem group (which must be pre-reduced by the reductant), followed by injection of further reducing equivalent(s) from the reductant used for the coupled oxidation ascorbate in the case of the soluble haemoproteins, NADPH in the case of the microsomal haemoprotein [which was initially thought to be cytochrome $P-450$, but may be a variant form thereof (see below)], The added electrons are probably then transferred to the bound $O_2$ molecule, converting it into an activated form, possibly the superoxide radical, which then hydroxylates the methine bridge, leaving it susceptible to further oxidation (cf. O'Carra, 1975).

The reductive activation of the bound oxygen of myoglobin and haemoglobin presumably represents a rather artificial situation, and it seems unlikely that the concentrations of ascorbate encountered in vivo would be sufficient to achieve such a result. In any case the attack on the methine bridge is clearly unrelated to the true functions of myoglobin and haemoglobin and must be considered an 'accidental' process or 'suicide' reaction.

The process occurring in the microsomal preparations was much more difficult to investigate and define owing to the complex composition of this material. However, in view of the close similarities of the results to those observed with the well-defined myoglobin system it seemed a reasonable assumption that the cleavage process followed a similar pattern in the two systems. The formation of activated oxygen is a normal function of the haem group of a major microsomal haemoprotein, cytochrome $P-450$, into which reducing equivalents are fed from NADPH. This seemed to tally with other evidence suggesting that cytochrome $P-450$ was the microsomal haemoprotein undergoing haem cleavage. The activated oxygen is 'normally' transferred out of the haem-containing site of cytochrome $P-450$ for hydroxylation of external substrates. Therefore our view was that in this case also an attack on the methine bridge of the haem group should be regarded as an 'accidental' or 'suicide' reaction unrelated to the true function of the haemoprotein (O'Carra & Colleran, 1969, 1975).

The fact that the haem group of cytochrome $P-450$ is known to undergo much more rapid turnover in vivo than the apoprotein (cf. Levin et al., 1973) is consistent with the suggestion that such an accidental cleavage process may be metabolically significant (cf. O'Carra, 1975; O'Carra & Colleran, 1976). Such turnover probably accounts for a large proportion of 'early labelled bilirubin'. We also advanced the idea that such turnover of cytochrome $P-450$ haem might account 'at second hand' for the $\alpha$-specific cleavage of the haem of degrading haemoglobin (O'Carra & Colleran, 1969). The suggestion was that (uncleaved) haem released from the haemoglobin might find its way into a haem pool in hepatic cells from which haem could be drawn to fill haem-binding sites of cytochrome $P-450$ left vacant by 'accidental' cleavage of the previous haem complement. Repeated cycles of such cleavage and replacement were envisaged as possibly accounting for cleavage of a large proportion of the haem deriving from senescent erythrocytes.

Some support for such a model, or at least for its molecular feasibility, was provided by the demonstration of analogous experimental models in vitro (O'Carra & Colleran, 1969, 1976). These derived from the observation that the biliverdin product formed by cleavage of the haem groups of haemoproteins could be displaced from the haem-binding sites by 'new' haem molecules from an external haem pool (i.e. free haem added to the incubation mixtures). The results indicated that such displacement could take place continuously during the course of a coupled oxidation and that repeated cycles of replacement and cleavage could take place, resulting in the cleavage of a number of haem molecules, one after the other, in a single haem-binding site. The otherwise very slow and random cleavage of free haem could thus be greatly accelerated and made bridge-selective by the presence of catalytic quantities of haemoproteins. The $\alpha$-specificity imposed on haem cleavage by the haem-binding sites of myoglobin and the microsomal haemoprotein applied equally to 'extra' haem cleaved in the presence of catalytic quantities of these haemoproteins.

In operating thus these haemoproteins, or more correctly their apoproteins, were
seen to be acting with all the attributes expected of a haem-cleaving enzyme system, but with the distinction that the haem-cleaving activity appears as an incidental attribute rather than a 'purposeful' function. The suggestion that cytochrome P-450 might be responsible for a large proportion of in vivo haem cleavage envisaged this protein as a consumer of new and second-hand haem for purposes other than the destruction of the haem.

On the other hand, the work of Tenhunen, Schmid and co-workers has promoted the widely accepted view that the microsomal membrane contains a 'purposeful' haem-cleaving enzyme, haem oxygenase. This view is based on extensive observations of NADPH-dependent haem cleavage promoted by microsomal preparations (e.g. Tenhunen et al., 1968, 1969, 1972). We have expressed the view that this haem oxygenase activity and the microsomal haem-cleavage process studied by us are probably attributable to the same causative agent, but interpreted differently (O'Carra & Colleran, 1969; Colleran & O'Carra, 1970). We still feel this to be so, but in view of recent results indicating a lack of direct correspondence between microsomal haem cleavage activity and cytochrome P-450 content (Yoshida et al., 1974; Maines & Kappas, 1974), we have modified our original view that the haemoprotein involved is necessarily cytochrome P-450 in its 'native' form. Some of the results of Maines & Kappas (1974) indeed indicate that microsomal haem-cleaving activity may be greatly increased by treatment of microsomal preparations in vitro with agents such as 4M-urea that convert cytochrome P-450 to a form no longer spectrally recognisable as cytochrome P-450. This might be interpreted in terms of formation of a 'perturbed' form of cytochrome P-450 in which the haem is more labile or less protected against accidental attack by the activated oxygen it helps to produce.

We are reluctant to drop our argument that microsomal haem cleavage is basically due to a haemoprotein rather than a conventional enzyme system. The similarities between the myoglobin system and the microsomal system seem too striking to be coincidental. It is of interest in this regard that some other similarities have been observed between features of cytochrome P-450 and myoglobin related to their haem-binding sites (cf. Lipscomb et al., 1973). However, it is clearly conceivable from much of the foregoing, that a haemoprotein could be pressed into service as a functional haem-cleaving enzyme, and one could imagine a labilized haemoprotein serving a function as a scavenger of excess haem.

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Lipscomb, J. D. & Gunsalus, I. C. (1973) Drug Metab. Dispos. 1, 1–5

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Intermediates in Haem Degradation

ANTHONY H. JACKSON, DAVID E. GAMES and GEOFFREY COOPER

Department of Chemistry, University College, Cardiff CF1 1XL, Wales, U.K.

In normal metabolism haem undergoes oxidative ring-opening at the α-position to form biliverdin, which is then immediately reduced to bilirubin (I). The latter subsequently undergoes a further series of hydrogenations by bacterial organisms during the course of excretion, and the end product is the colourless stercobilinogen (IV). Intermediates in this pathway may undergo oxidation in air to form urobilins in which the two central pyrrole rings are conjugated as the orange dipyrrolylmethene chromophore.

Stercobilin (III), the dehydrogenation product of stercobilinogen, is readily isolated from normal faeces. It is strongly laevorotatory, and can be crystallized as the hydrochloride salt. Chromic acid degradation to the imides (V) and (VI) was a major piece of evidence leading to the now well-accepted structure (III) (Gray & Nicholson, 1958). However, the electron-impact mass spectrum obtained some years later (Jackson et al., 1966) unexpectedly showed weak molecular ions at m/e 592 and 596, instead of the expected 594; this was attributed to disproportionation in the mass spectrometer. Other urobilins also showed apparently anomalous behaviour on mass spectrometry (Jackson et al., 1966; Watson et al., 1967), and it was not clear whether similar processes were occurring or whether the compounds isolated were mixtures of pigments with varying degrees of unsaturation (see below).

Recently we have examined (Games et al., 1974) the mass spectra of a large number of bile pigments by both electron-impact and field-desorption mass spectrometry. The latter involves dipping a special carbon-fibre-coated tungsten filament into a solution of the compound under investigation; after drying, this emitter is inserted into the mass spectrometer via the vacuum lock and a powerful electric field (8-11kV) is applied while simultaneously a small current (10-20mA) is passed through the filament. Under these conditions positive ions are formed and desorbed directly from the solid state and analysed by the mass spectrometer in the normal manner. Because the ionization process is very mild and little thermal energy is required (in contrast with electron-impact spectroscopy for which the compound must be volatilized by heating in the ion source) the molecular ion or quasi-molecular ion is often greatly enhanced or is the only ion in the spectrum. For example, bilirubin gives only a molecular ion on field-desorption spectroscopy, whereas with electron-impact spectroscopy substantial cleavages occur at the central methine bridge, and the molecular ion is relatively weak (Jackson et al., 1967). The electron-impact and field-desorption mass spectra of over 30 natural and synthetic compounds have now been determined in Cardiff (Games et al., 1974); the field-desorption spectra of the esters invariably showed the molecular ion as the base peak with little fragmentation at low wire currents, whereas in many of the electron-impact spectra the molecular ions were very weak. Enhancement of fragmentation could be achieved in some of the field-desorption spectra by increasing the wire current, and this was of assistance in providing structural information.

The field-desorption spectrum of stercobilin shows only a quasi-molecular ion at m/e 596 (i.e. M+2), but the field-desorption spectrum of the dimethyl ester exhibits the expected molecular ion at m/e 622; the electron-impact spectrum of the ester also shows this molecular ion, and although the intensity was very low (4%) owing to extensive fragmentation no disproportionation was observed (Games et al., 1974). The formation of M+1 and M+2 ions in field-desorption spectroscopy is now well recognized, especially with basic or acidic compounds, and we have also observed them with