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Theoretical prediction and experimental measurement of the bile-pigment isomer pattern obtained from degradation of catalase haem

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Degradation *in vitro* of the haem in catalase by a 'coupled oxidation' reaction yields products in which approx. 45% of the haem groups have been cleaved at the α-methene bridge, 55% at the β-bridge and a trace at the δ-bridge. Molecular-mechanics calculations with the three-dimensional structural co-ordinates of catalase shows that these proportions of products can be accounted for by the relative accessibility of the four methene bridges to a haem-linked oxygen molecule, thus further confirming Brown's [(1976) Biochem. J. **159**, 23–27] hypothesis that the first stage of haem catabolism *in vivo* is selective attack by haem-bound oxygen, with selectivity conferred by the surrounding protein moiety.

INTRODUCTION

Haem is degraded to bile pigments *in vivo* during the catabolism of haemoproteins in mammals and in the formation of photosynthetic pigments in algae. The first stage of degradation involves cleavage of the protoporphyrin ring at one of the four methene bridges as a result of attack by oxygen on the methene carbon atom, which is ultimately eliminated as CO. The four methene bridges are sterically non-equivalent, and both the bilirubin in mammalian bile and the algal bile pigments consist almost entirely of the α-isomer. Little is known yet of the structures of the enzymes responsible for ring cleavage *in vivo*, but the oxygen complex of microsomal haem oxygenase (EC 1.14.99.3), which catalyses the breakdown of haem in mammals, has spectroscopic properties similar to those of oxyhaemoglobin and oxymyoglobin (Yoshida *et al.* 1980).

The degradation process has been simulated *in vitro* by a 'coupled oxidation' method in which haem, while in a protein complex in the form of myoglobin or haemoglobin, is allowed to react with a reducing agent and molecular oxygen. This appears to be a good model for biological haem cleavage, in which also there is a need for a reducing agent (NADPH *in vivo*) and molecular oxygen, and the products, biliverdin, CO and free iron, are identical.

The experiments *in vitro* also give selective degradation products, but the proportions of the four isomers depend upon the nature of the globin moiety (O'Carra, 1975; Brown & Docherty, 1978; Brown *et al.*, 1981; Docherty & Brown, 1984). The observation that, when free haem is degraded by the 'coupled oxidation' reaction, cleavage takes place almost randomly at all four bridges, shows that the selectivity is not due just to the intrinsic asymmetry of the haem group, but must arise in some way from the environment imposed by the surrounding protein. This is confirmed by the different proportions of the isomers arising from different globin moieties.

From an inspection of a three-dimensional model of haemoglobin, Brown (1976) suggested that the attack on the methene bridge was brought about by an oxygen molecule that was already bound to the haem iron atom and that the selectivity was determined by the relative accessibility of the four bridges. This proposal was reinforced by precise calculations by use of computer graphics and molecular mechanics (Brown *et al.*, 1981), which showed that the relative proportions observed for the four isomers were directly related to the interaction energies experienced by an oxygen molecule aligned *cis* to the relevant bridge carbon. For the globin chains, access to the γ-bridge is prevented by His-E7, to the δ-bridge it is prevented by Val-E11 and to the β-bridge it is impeded by Phe-CD1. Although these three residues are identical in sperm-whale myoglobin and the two human haemoglobin chains that were studied, subtle differences in molecular geometry vary the extent to which Phe-CD1 occludes the β-bridge. The only example of substantial production of a γ-isomer of biliverdin of which we are aware from a 'coupled oxidation' reaction occurs with the abnormal haemoglobin Zürich, in which His-E7 is replaced by arginine, the side chain of which is known to point out of the haem pocket. In order to examine further the hypothesis that selectivity of haem degradation arises through constraints on the accessibility of the bridges to haem-bound oxygen, we have now studied, both by experiment and by theoretical calculations, the breakdown of the haem of catalase, a protein with a structure and function very different from that of the globins and one in which the haem groups are buried deep within the tetrameric molecule, with their propionate groups pointing inwards and co-ordinate to internal basic side chains. We must emphasize that we are not endeavouring to throw any direct light on the enzymic function of catalase, but are using the enzyme in this work in order to provide a protein cage of known structure around a haem group.

EXPERIMENTAL

Degradation of the haem of catalase

Ox liver catalase (EC 1.11.1.6) obtained as an aqueous suspension (Sigma, Poole, Dorset, U.K.) was centrifuged

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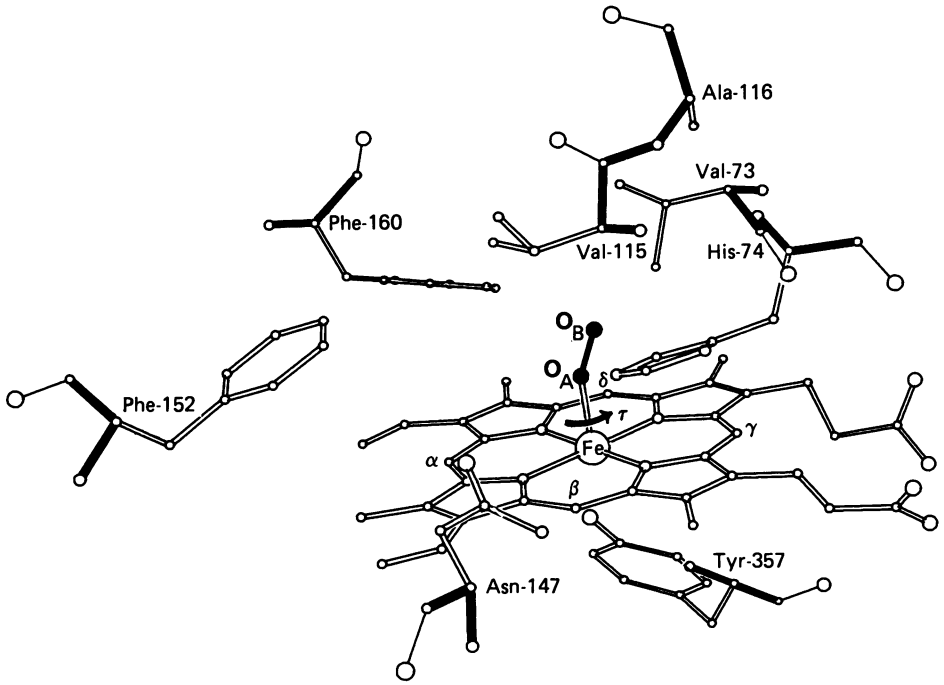

Fig. 1. Haem group of catalase and the neighbouring parts of the polypeptide chain

The four methene bridges are labelled α, β, γ, δ. O_AO_B is the haem-linked oxygen molecule, τ the torsion angle defining its orientation about the bond to the iron atom (Fe).

for 10 min in a bench centrifuge (3000 *g*); the supernatant was extracted and the pellet re-suspended in 10 ml of distilled water and stored overnight at 4 °C. This solution was combined with the supernatant and the concentration of catalase found to be 25.5 mg·ml^{-1} by measurement of its A_{405} ($\epsilon_{mM} = 130$ mM^{-1}·cm^{-1} at 405 nm). For each coupled oxidation reaction, 7.84 ml (equivalent to 200 mg of catalase) was added to 4.0 ml of 0.1 M-phosphate buffer, pH 7.4, and adjusted to 20.0 ml with distilled water. A 100 mg portion of sodium ascorbate was added and the mixture incubated for 2 h at 37 °C with continuous shaking in the dark. The solution was cooled in ice before the addition of 3 ml of acetic acid and 8 ml of 5 M-HCl. Peroxide-free diethyl ether (2 × 15 ml) was used to extract any remaining free haem. Biliverdin was then extracted into chloroform (5 ml) and evaporated to dryness with N_2. Methylation was carried out overnight at 4 °C by dissolution in approx. 1 ml of 5% (v/v) H_2SO_4 in methanol.

The methanolic biliverdin solution was diluted four-fold with water and its dimethyl ester extracted into 1 ml of chloroform. The chloroform extract was washed with 4 vol. of water until the washings were no longer acid (six to eight times) and evaporated to dryness under N_2 as described above.

Thin-layer chromatography was carried out on silica-gel plates. Two systems were used: (1) n-heptane/butanone/acetic acid (0:5:1, by vol.) and (2) 4% (v/v) acetone in chloroform.

It had previously been established (O'Carra & Colleran, 1969; Bonnett & McDonagh, 1973) that system (1) separates the α-isomer well, but does not distinguish easily between the β-, γ- and δ-isomers of biliverdin, and that system (2) will separate the β-, γ- and δ-isomers from each other, but does not readily separate the α- from the γ- or δ-isomers. System (1) was therefore used first to separate the α-isomer, followed by system (2).

Chromatography was carried out in the dark because of the light-sensitivity of biliverdin. On completion of chromatography, isomers were removed from the plates and extracted in chloroform. They were evaporated to dryness under N_2 and then re-dissolved in a known volume of chloroform. The concentrations were then determined by measurement of A_{650} by using known absorption coefficients (biliverdin IX-α, $\epsilon = 14300$ M^{-1}·cm^{-1}; biliverdin IX-β, $\epsilon = 15400$ M^{-1}·cm^{-1}; biliverdin IX-γ, $\epsilon = 17700$ M^{-1}·cm^{-1}; biliverdin IX-δ, $\epsilon = 15600$ M^{-1}·cm^{-1}; Vernon, 1982).

Theoretical calculations

The three-dimensional co-ordinates of ox liver catalase, as determined at 0.25 nm resolution by Rossmann and colleagues (Murthy *et al.*, 1981), were obtained from the Protein Data Bank (Bernstein *et al.*, 1977) file 3CAT. This structure determination was of a non-liganded form of the enzyme, so that we had to deduce the position that would be occupied by a haem-bound oxygen molecule. We placed the proximal, O_A, atom at a covalent bond distance of 0.18 nm from the Fe atom on a line perpendicular to the best plane through the four methene-bridge C atoms. Fig. 1 shows the haem group of catalase and the amino acid residues that are adjacent to it. There is some doubt as to the exact co-ordination of the O_2 molecule to the haem Fe in haem proteins, with both covalent and ionic contributions being present. The crystal structures of oxyhaemoproteins reveal a variation of geometry, particularly in the FeO_AO_B bond angle. The distal, O_B, atom was therefore placed at a series of distances and inter-bond angles with respect to the Fe and O_A atoms (see the Results and discussion section). As is

Table 1. Comparison of experimental results on ox liver catalase with calculated accessibilities of methene-bridge carbon atoms for attack by haem-bound oxygen

The observed proportion of isomers was determined by the coupled-oxidation method described in the Experimental section. The reproducibility of the method has been assessed in previous work, where the S.D. for determination of proportions of isomers was ±2.6% (Docherty & Brown, 1982). The data for relative probability at the various positions are averages of those shown in Table 2 for calculations in which hydrogen atoms were omitted and the iron atom was assumed to lie in the plane of the haem.

Carbon atom ...	α	β	γ	δ
Observed proportion of isomers (%)	45	55	—	Trace
Relative probability of attack (%) (iron atom in plane, hydrogens omitted)	45	51	1	3

usual with X-ray-diffraction studies of proteins, no co-ordinates were available for hydrogen atoms. A computer program was therefore used to generate the co-ordinates of hydrogen atoms according to standard molecular geometry. The hydrogen atoms of methyl groups were placed in staggered positions. Lennard-Jones potential functions, $E = -a_{ij}r^{-6} + b_{ij}r_{ij}^{12}$, where r_{ij} is the distance between atoms i and j and a_{ij} and b_{ij} are constants characteristic of the types of atoms concerned, were used to calculate the variation of energies due to van der Waals interactions between atom O_B and all neighbouring atoms as the oxygen molecule was rotated at 10° intervals around the Fe–O_A bond. We considered only those atoms less than 0.75 nm from the O_A atom as van der Waals interactions between more distant atoms are negligible. For a_{ij} and b_{ij}, we used the constant of Scott & Scheraga (1966).

We made no attempt to calculate electrostatic interactions due to partial charges, as it was clear that the effect of non-bonded steric interactions would predominate in defining the relative accessibility of the bridges, electrostatic energies varying only slowly with distance.

Catalase is a tetrameric enzyme with identical subunits. The non-hydrogen atoms of a neighbouring subunit come within 0.75 nm of the O_A atom on one subunit, but we found that they were insufficiently close to have a significant effect on O_B as it was rotated.

RESULTS AND DISCUSSION

Our experimental results showed that the δ-isomer was present as a trace, but in insufficient quantity for accurate determination of its concentration. No γ-isomer was detected. The major components were α-isomer (45%) and β-isomer (55%) (Table 1). These results are consistent with those quoted by O'Carra (1975).

We calculated the variation of van der Waals energy with rotation angle, τ, for three values of O_AO_B bond length (0.118, 0.120 and 0.123 nm) combined with six values of FeO_AO_B inter-bond angle (120°, 125°, 130°, 140°, 150°, 160°). Fig. 2 shows the curves for bond length 0.120 nm and two values of inter-bond angle (120° and 160°) with hydrogen atoms omitted. The effect of varying the bond length was very small compared with varying the angle, and all of the curves showed a maximum (unfavourable) energy between the δ- and γ-positions and a broad minimum energy covering both the α- and β-positions. The expression

$$\frac{n_1}{n_2} = \exp\left(-\frac{E_1 - E_2}{RT}\right)$$

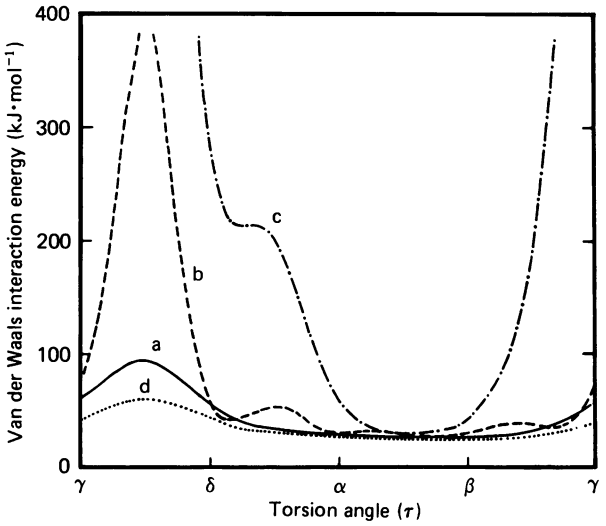

Fig. 2. Variation of van der Waals interaction energies as the oxygen molecule is rotated through 360° about the Fe–O_A bond

a, Hydrogen atoms omitted, $O_AO_B = 0.120$ nm, $FeO_AO_B = 160°$; b, hydrogen atoms omitted, $O_AO_B = 0.120$ nm, $FeO_AO_B = 120°$; c, hydrogen atoms included, $O_AO_B = 0.123$ nm, $FeO_AO_B = 160°$; d, iron atom displaced 0.033 nm towards the proximal ligand (Tyr-357), hydrogen atoms omitted, $O_AO_B = 0.123$ nm, $FeO_AO_B = 160°$. Interaction energies are calculated relative to an arbitrary baseline torsion angle, τ, indicated with respect to the positions of the four methene bridges.

where n_1 and n_2 represent the numbers of molecules having conformations of energies E_1 and E_2 respectively, was used to evaluate the relative probability of the O_B atom taking a position *cis* to each of the methene bridges, giving the values shown in Table 2.

The addition of hydrogen atoms (Fig. 2, curve c) restricted the accessible range, but without changing qualitatively the *relative* accessibilities of the four bridges. We note that a number of the hydrogen atoms belong to the methyl groups of alanine, valine and isoleucine residues and that such groups are able to rotate from their staggered conformations at relatively little cost in energy; this, together with the comparative 'softness' of the hydrogen potential functions, is often used as a justification for omitting separate hydrogen atoms from molecular-mechanics calculations. We therefore consider our calculations with fixed hydrogen positions to be

Table 2. Effects of varying Fe–O_A bond length, FeO_AO_B inter-bond angle, displacement of Fe from haem plane and inclusion of hydrogen atoms on O_B energy

The data were obtained by calculating the van der Waals energies corresponding to each of the four methene-bridge positions in the catalase molecule and using these energies to calculate the relative probability of the O_B atom taking up a position *cis* to the appropriate methene bridge as described in the text.

Fe–O_A bond length (nm)	FeO_AO_B inter-bond angle (°)	Fe	H atoms	Probability of attack (%)			
				α	β	γ	δ
0.123	160	In plane	Omitted	45.0	51.5	0.9	2.6
0.123	125	In plane	Omitted	45.0	52.0	0.2	2.8
0.118	160	In plane	Omitted	43.0	49.0	3.0	5.0
0.118	120	In plane	Omitted	45.0	51.0	0.7	2.6
0.123	160	Displaced	Included	35.0	65.0	—	—
0.123	160	Displaced	Omitted	38.0	43.0	7.7	11.3

extreme and unsuitable fof quantitative deductions from the calculated energies.

The Fe atoms and haem groups of myoglobin and haemoglobin are not exactly co-planar, movement of the Fe atom from the plane and puckering of the porphyrin ring varying according to the co-ordination state of the iron (see, e.g., Phillips, 1978). We therefore examined the effect on our potential curves of displacing the Fe atom from the plane of the ring by 0.033 nm (the largest displacement yet seen in a haemoprotein) towards the proximal ligand, Tyr-357. Again, although there is a significant change in the interaction energy curve (Fig. 2, curve d), the *relative* accessibilities of the four bridges are much the same.

For those calculations in which hydrogen atoms were omitted and the iron atom was assumed to lie in the plane of the haem, the relative probabilities of O_B being *cis* to a methene bridge are shown in Table 1 to be quite remarkably close to the proportions we observed experimentally. The other variations that we tried (including hydrogen atoms explicitly and displacing the Fe atom) would still lead to the general conclusions (i) that the α- and β-positions are both favourable, with the β-position being marginally of lower energy, and (ii) that the γ- and δ-positions are much less favourable, with the δ-position being the more probable of the two. It should be noted that, as the haem group is nearly symmetrical about the line joining the α- and γ-methene-bridge carbon atoms, there might well be a rather low energy penalty incurred by inserting the haem group the wrong way round within the protein. Although we have no evidence that this actually occurs in catalase *in vivo*, the crystallographic data would be unlikely to detect such disorder if 5 or even 10% of the groups were the opposite way round to the rest. The effect would be to interchange the environments of the β- and δ-bridges and this would give an alternative explanation for the occurrence of traces of the δ-isomer.

In describing our work on haemoglobin and myoglobin (Brown *et al.*, 1981) we acknowledged some shortcomings which apply equally to the present work. Attack by O_B on a methene bridge would not necessarily require O_AO_B to be exactly co-planar with the bond from Fe to the bridge carbon, although the probability of a productive reaction would be expected to fall off with increasing non-co-planarity. Moreover, the atoms of a protein are far from being stationary. Both of these effects would be expected to tend to equalize the relative probabilities of attack at the four bridges. As with the previous work, the very satisfactory agreement between experiment and calculation encourages us to believe that the deficiencies of our model are of relatively small effect and that our present observations give further support for Brown's (1976) proposal that the first stage of haem degradation, both *in vivo* and *in vitro*, comprises attack on a methene bridge by a haem-bound oxygen molecule and that the stereoselectivity arises from steric hindrance of the oxygen by the surrounding protein. It is of particular interest that the haem groups of catalase are inserted with their propionate side chains towards the interior of the protein, whereas in the globins they lie on the surface. The relative accessibility of the α- and β-methene bridges in both catalase and the globins is not therefore correlated with their disposition within the protein environment, but again we wonder whether any biological advantage attaches to the α- and β-bridges, the two flanked by the most hydrophobic porphyrin side chains, being also the most accessible to oxygen.

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