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Visions & Reflections

The honorary enzyme haemoglobin turns out to be a real enzyme

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Haemoglobin research has contributed more to building the foundations of biochemistry than studies of any other protein [1]. At the beginning it was the ease of its purification from many organisms and its stability that drove researchers to the field. Later, haemoglobin proved to be a very complex system with fascinating properties that still remains a challenge to its investigators. Many 'firsts' are associated with haemoglobin. Crystallographic investigations of haemoglobin and its smaller cousin, myoglobin, yielded the first three-dimensional images of proteins and led to the concept of protein-fold conservation during divergent evolution. Our understanding of cooperativity and allosteric modulation of ligand binding was born out of haemoglobin studies. The molecular basis of a genetic disease was first elucidated for sickle cell anaemia. These are just a few milestones in haemoglobin research that have been successfully applied in enzymology and have made a huge impact in the field of biochemistry. It is somewhat ironic that haemoglobin, this gem of our knowledge of structure-function relationships and a pivotal subject in many biochemistry courses, is not an enzyme but 'just a carrier'.

The haem, protoporphyrin IX, is not only the oxygen-binding prosthetic group in globins but also catalyses the oxygen chemistry of peroxidases and cytochromes P450. Whereas different properties of the haem in cytochromes P450 versus those in globins can be largely attributed to the different proximal ligands, cysteine and histidine, respectively, much more subtle differences exist between peroxidases and globins, which both have a histidine in the proximal position [2].

Proteins that appeared late in evolution usually arose by gene duplication and divergence. Until recently, it appeared that the oxygen carrier function was so specialized that globins were not recruited to new tasks. A few months ago, however, it was found that some marine worms, *Amphitrite ornata*, evolved their globin into a powerful peroxidase, more precisely dehaloperoxygenase (DHP) [3]. *A. ornata* usually cohabits estuarine mudflats with other polychaete worms such as *Notomastus lobatus*, and hemichordata such as *Saccoglossus kowalewski*, which secrete brominated aromatics and other halocompounds as repellents. DHP catalyses the oxidative dehalogenation of polyhalogenated phenols in the presence of hydrogen peroxide at a rate at least 10 times faster than all known halohydrolases of bacterial origin [4] and allows *A. ornata* to prosper in such a toxic environment. If the substrate is 4-bromophenol, the products are quinone, bromide ion and water. It is the only known enzyme that can catalyse the breakage of a C–F bond, most likely due to the utilization of the oxidative power of hydrogen peroxide. Polychaetes are well represented by the Middle Cambrian [5], about 530 million years ago, so DHP activity must be younger than that. Conversely, the oxygen carrier function of globins is universal and much older [6]. It is thus apparent that DHP evolved from globins and not vice versa.

The mechanism of peroxidases, which explains how their function differs from oxygen carriers (globins), is usually referred to as the 'push and pull' theory [2, 7]. The push relates to the electron-releasing property of the proximal ligand. In globins, the $N^{\delta 1}$ atom of the proximal histidine forms a weak bifurcated hydrogen

bond; in peroxidases, the corresponding hydrogen bond is directed to the carboxylate of a conserved aspartate and is strong. The resulting polarization of the $N^{\delta 1}$–H bond leads to increased electron density at the haem iron, which promotes catalysis. The pull component in peroxidases is effected by the distal histidine, which functions as a base/acid facilitating transfer of the proton from the haem iron-bound hydrogen peroxide oxygen atom to the other peroxide oxygen atom. A nearby arginine plays an auxiliary role by polarizing the hydrogen peroxide molecule. The proton transfer is followed by the heterolytic cleavage of the haem-bound hydrogen peroxide molecule and results in the formation of so-called compound I, which contains an oxygen atom bound to the haem iron and one oxidative equivalent of peroxide as Fe^{IV}, whereas the other is in the form of a porphyrin radical. Peroxidases do not have a binding pocket for organic substrates; rather, the reaction takes place at the solvent-exposed haem edge, which leads to relatively low substrate selectivity. The folds of peroxidases and globins are completely different, but the haem environments are quite similar. Indeed, myoglobin has some intrinsic peroxidase activity; Watanabe and coworkers used site-directed mutagenesis to engineer variant myoglobins with enhanced catalytic activity, but these attempts yielded only moderate successes [8].

The detailed crystallographic characterization of DHP, a globin functioning as a superb peroxidase, has offered a unique perspective on the structure-function relationship in haem proteins [9]. What does it take to convert an oxygen carrier into a real enzyme? The answer is not much. A comparison of DHP with myoglobin or haemoglobin shows that there are very few changes on the distal side of the haem. The distal cavity in DHP is as hydrophobic as it is in the globins—there is no auxiliary arginine. The main difference is the position of the distal histidine, which is further from the haem iron. This is in agreement with the trend observed for globins and classical peroxidases [8]. This position is closer to the entrance to the distal cavity, and in addition to being more suitable for the heterolytic cleavage of hydrogen peroxide, it facilitates the movement of the histidine out of the distal pocket to make room for organic substrates. In the crystal structure of the complex between DHP and a substrate, 4-iodophenol, the histidine is out while the substrate occupies the pocket [9]. Thus, the proposed mechanism of DHP is sequential: hydrogen peroxide binds at the haem iron and undergoes heterolytic cleavage to form compound I; the distal histidine moves out of the cavity to make room for an organic substrate molecule; the oxygen atom bound to the haem carries out direct nucleophilic attack on an adjacent carbon atom of the phenyl ring of the substrate, and the products dissociate.

The lack of an auxiliary arginine in the distal cavity of DHP must lead to a weaker pull than that in peroxidases and should be compensated by a stronger push from the proximal side. Indeed, the haem proximal side in DHP is different from those found in other globins. Remarkably, the proximal histidine is shifted in the sequence by two residues, and as a consequence the main chain loop position is different and the plane of the imidazole moiety is rotated by about 60° with respect to the situation in myoglobin. The hydrogen bond formed by the $N^{\delta 1}$ donor is stronger in DHP than those in globins and is not to a charged carboxylate as in peroxidases but to a main chain carbonyl [9]. This is in excellent agreement with vibrational spectroscopy studies, which have shown that the Fe–N bond, the strength of which reflects the electron push, is stronger in DHP than in globins but not as strong as in peroxidases [10]. Another apparent failure of the electronic push theory in histidine-ligated haem systems was encountered earlier. Variant cytochrome c peroxidase in which the proximal histidine was replaced with a glycine

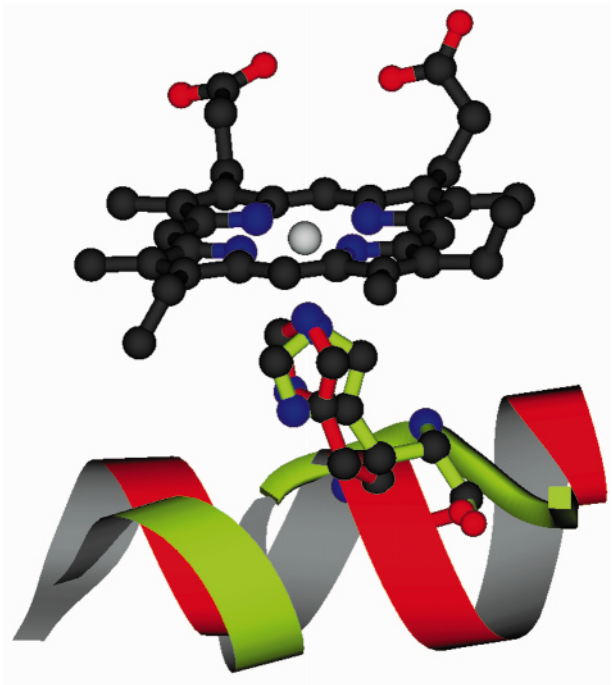

Figure 1. A Molscript [13] representation of the least-squares superposition optimizing haem overlap shows relative positions of the proximal histidine in DHP (yellow) and in myoglobin (red). The main chain with the histidine is in a coil conformation in DHP, whereas in the oxygen carrier it is in the helical conformation. To be approximately in the same spatial position the proximal histidine is shifted in the amino acid sequence by two residues, but the different conformation of the main chain results in the plane of the imidazole ring rotated by about 60°.

was crystallized in the presence of imidazole. Its crystal structure showed that imidazole from solution binds to the haem in essentially the same distance and orientation as the wild-type proximal histidine; thus, the difference is the absence of covalent tethering of the proximal ligand. Yet the variant enzyme had greatly reduced activity (5%) in the presence of imidazole [11]. This strongly suggested that the role of electron push in peroxidases is not as important as previously thought [12]. Rather, it appears that molecular dynamics plays an important role. In oxygen-carrying globins the proximal histidine is in a regular α helix, located close to its end. The corresponding helix in DHP is shorter, its last turn is unwound and the proximal histidine is not a part of the helix but is on a loop (fig. 1). However, in DHP the histidine $N^{\delta 1}$ is directly pointing into the terminal peptide carbonyl of the helix, and it appears that there should be a strong correlation between the helix motion and the electronic state of the histidine and, consequently, the haem iron. It is proposed that that this coupling of the proximal histidine to the molecular frame, different in DHP than in haemoglobin, plays an important role in defining globin function.

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