For fun and money

The recent transplantation of an unusual allosteric effect from crocodile to human haemoglobin has implications for both molecular evolution and the engineering of artificial blood substitutes.

More than 30 years ago, Jeffrey Wyman [1] said that haemoglobin may be considered to be the "hydrogen atom of molecular biology" because, over and over again, it has proven to be the ideal molecular guinea pig for the development of new biophysical techniques, such as protein crystallography, and the testing of new ideas about the structural basis of biological regulation. All biology students are familiar with haemoglobin's sigmoidal O_2-binding curve [2], a reflection of the way O_2 binds cooperatively to this sophisticated molecular machine, which has been selected during evolution to optimize O_2 delivery to respiring tissues.

Cooperative O_2 binding to haemoglobin inspired Jacques Monod, the father of allostery. The classical Monod–Wyman–Changeux [3] model accounts for haemoglobin's cooperativity by postulating that the protein can exist in two conformational states — the R (relaxed) and T (tense) states — which differ in quaternary structures (haemoglobin is an $\alpha_2\beta_2$ tetramer) and O_2 affinity, and which are in equilibrium at every ligation state. Deoxyhaemoglobin is thought to exist largely in the T state, with a relatively low O_2 affinity; as the four oxygen-binding sites are progressively occupied, the equilibrium is increasingly shifted towards the R state, increasing the O_2 affinity of the still-unoccupied sites and thus accounting for the sigmoidal O_2-binding curve.

There are many subtle variations on this basic theme. Thus, a number of small effector molecules — such as H^+ (pH), diphosphoglycerate, CO_2 and Cl^- — are known to bind haemoglobin and modulate its O_2 affinity. These effector molecules generally lower haemoglobin's O_2 affinity, so that high concentrations of effector molecules at sites in the body where O_2 is required improve the efficiency of O_2 delivery. A wealth of sophisticated experimental data on haemoglobin has been gathered over the years, and convincing structural interpretations of these results have been proposed by Max Perutz [4,5], based on the X-ray crystallographic analyses that he pioneered.

The control of the O_2 affinity of haemoglobin by pH is known as the Bohr effect, after a paper published in 1904 by Christian Bohr and colleagues (quoted in [2]). It is less well known, however, that Christian Bohr (father of the famous physicist Niels Bohr) also discovered the effect of CO_2 on the O_2 affinity of haemoglobin. In a recent paper, Komiyama *et al.* [6] have reported some very interesting results on a related phenomenon, inspired by the unique physiological role of bicarbonate in crocodiles [7]. This work sheds light on the molecular evolution of haemoglobin, and points the way forward in attempts to design artificial blood substitutes.

Transplantation of an allosteric site

The ability to hold your breath for a long time would be very useful if you were a hunter living on the shoreline or river bank and had jaws powerful enough to grab prey and trail it under water. You would then not have to risk losing your meal when you released your grip to give it the killing bite: it would be enough to hold it and wait until the victim was drowned. In order to adopt this hunting strategy, crocodiles make use of an unique allosteric effect [7], which enables their haemoglobin drastically to lower its affinity for O_2 upon binding bicarbonate, the anion resulting from hydration of the CO_2 that accumulates as the final product of respiration.

Crocodile and man diverged over 300 million years ago, and only 68 % of their haemoglobin α-chain residues, and 51 % of their β-chain residues, are identical. Starting from the premise that a limited number of substitutions may account for the bicarbonate effect (the others being due to evolutionary drift), the work of Komiyama *et al.* [6] was initially directed to the identification of the residues involved in bicarbonate binding. Their strategy was an alternative to the usual approach of disrupting a specific function by making point mutations in the original haemoglobin: instead, they attempted to identify a discrete region of crocodile haemoglobin that could be grafted into the homologous human protein and confer the bicarbonate-dependent allosteric effect on the resulting hybrid protein.

How could the residues that confer the bicarbonate-dependent allosteric effect be identified? A systematic search of all possible substitutions in all possible combinations would not have been practicable in the absence of a way of generating and then selecting among a huge number of variants, by some procedure analogous to screening bacteria for antibiotic resistance or phage libraries for expression of some sequence or epitope. The first step was to identify the contribution of the two types of chain — α and β — to the bicarbonate-binding site. To this end, the hybrids $\alpha(croco)_2\beta(human)_2$ and $\alpha(human)_2\beta(croco)_2$ were constructed and expressed in *Escherichia coli*; only the latter retained a measurable bicarbonate effect, implying that the β chain is particularly important in this allosteric effect.

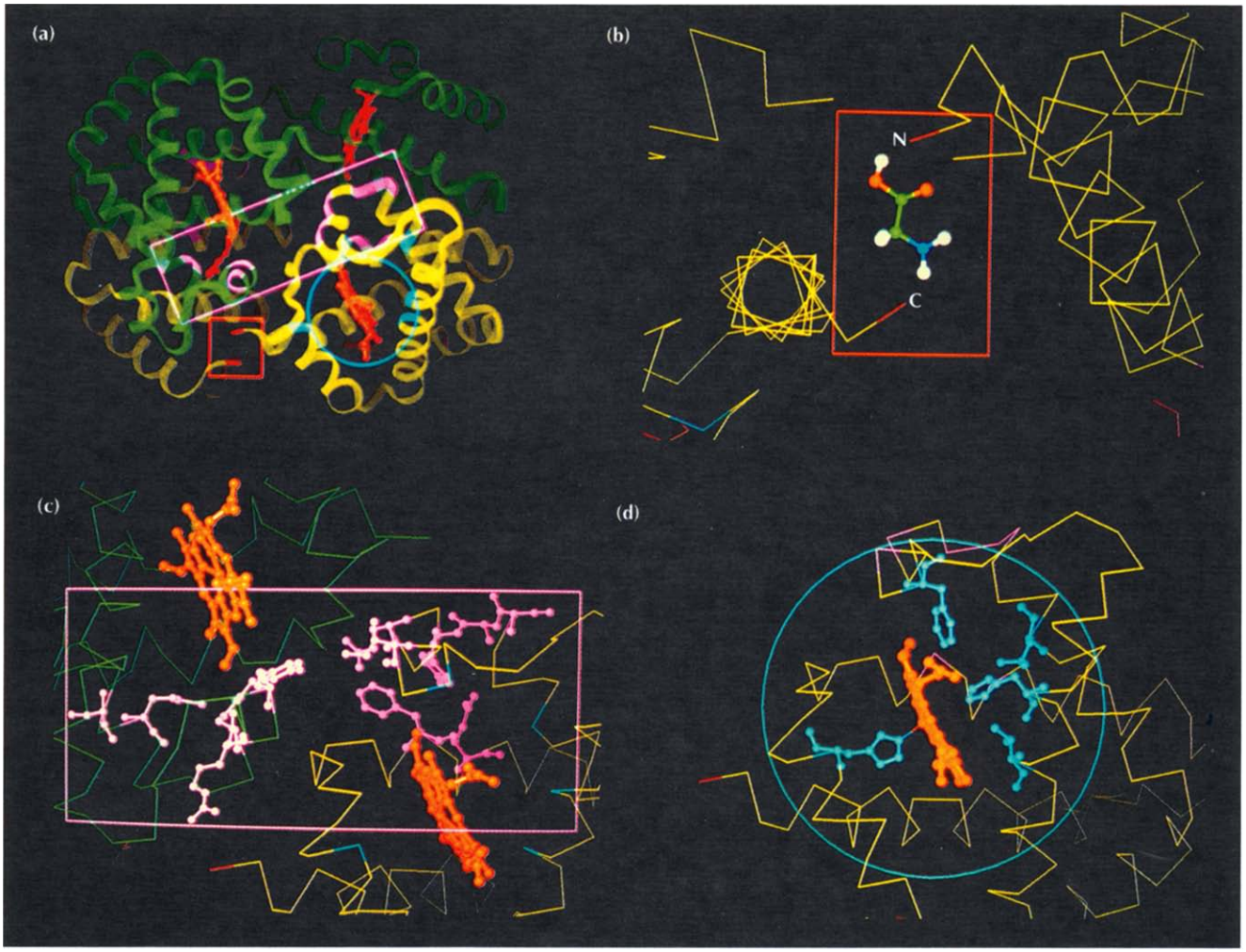

Fig. 1. **(a)** A model of T-state human deoxyhaemoglobin. The α chains are yellow, the β chains green and the haems red/orange. Detailed views of the regions highlighted in the coloured boxes are shown in the other panels. **(b)** The region within the red box. The carboxyl and amino termini of the two α chains are sufficiently close to allow fusion, linked by a glycine residue (shown in a ball-and-stick representation), to create one long 'double' α chain. As explained in the text, this may be useful in the design of a blood substitute. **(c)** The region within the pink box, showing the twelve residues substituted to create Scuba haemoglobin, superimposed on the human deoxyhaemoglobin structure. The side chains of these residues (shades of pink) are clustered in the 'sliding region' at the $\alpha_1\beta_2$ interface; bicarbonate is thought to bind at this interface, acting as a clamp that stabilizes the molecule in the T state. **(d)** The region within the blue circle, showing the α-chain O_2-binding site. The side chains of some key residues — His(F8), His(E7), Val(E11), Phe(CD1) and Leu(B10) — are shown in blue; mutations at these sites change the O_2 affinity and autoxidation rate.

The next step was to identify the important residues in the β chain, and here the approach taken was to compare the sequences of haemoglobin β chains from three crocidilian species — *Caiman crocodylus*, *Alligator mississippiensis* and *Crocodylus niloticus* — the assumption being that sites important in the allosteric effect would be conserved. These comparisons identified a cluster of five conserved residues that, in the three-dimensional haemoglobin structure, are present at the $\alpha_1\beta_2$ interface (Fig. 1a). This interface is of crucial importance in the transition between the high (R) and the low (T) affinity states of haemoglobin [4,5]. These five residues were transplanted into human β chain, creating a hybrid chain termed β(SC4).

The hybrid $\alpha(croco)_2\beta(SC4)_2$ displayed a full bicarbonate effect, whereas the hybrid $\alpha(human)_2\beta(SC4)_2$ proved to possess only a small (but non-zero) bicarbonate effect. Incidentally, this indicated — consistent with the molecular interpretation of the Bohr effect [5] — that the control by bicarbonate is not an all-or-none phenomenon. The last step in the construction of 'scuba haemoglobin' was to 'humanize' crocodile α chains in order to conserve only those crocodile residues required for a full bicarbonate effect. The resulting hybrid α chain, containing seven crocodile residues, was assembled with the β(SC4) chains, generating scuba haemoglobin with the bicarbonate-binding site shown in Figure 1c.

The twelve mutations that confer on scuba haemoglobin the bicarbonate effect typical of crocodile haemoglobin [7] are in two neighbouring regions across the $\alpha_1\beta_2$ interface: the β chain 'C helix' and the α chain 'FG corner' (see [4]). Bicarbonate bound at this site is thought

to act as a molecular clamp, stabilizing haemoglobin in its low affinity (T) state. The amino acid residues proposed by Perutz and Fermi to interact directly with the bicarbonate anion are Tyr 41_β, Lys 38_β and Tyr 42_α (also a tyrosine in human haemoglobin); but substituting crocodile residues 38 and 41 alone in the human β chain does not introduce the bicarbonate effect. The price that had to be paid for grafting this new allosteric site into human haemoglobin is a large (and still mysterious) increase in O_2 affinity; this indicates that not all of the other differences between human and crocodile haemoglobin are neutral, but that more engineering may be needed to create a hybrid molecule that fully reproduces the O_2-binding properties of crocodile haemoglobin.

Molecular evolution

When gene and protein sequences started to become available in the 1970s, they stimulated fresh debate about the classical Darwinian view that evolution occurs by the natural selection of adaptive variations. In particular, the view was put forward [8] that most sequence changes are actually functionally neutral, the result of random genetic drift. This 'neutralist' view was opposed by the 'selectionists', who argued that the fixation of mutations implies that they have some selective advantage. These two views are not, of course, mutually exclusive: most neutralists admit adaptive changes are possible, and *vice versa*. The debate turns on the relative importance of the two processes to the observed sequence variation.

In support of the neutralist viewpoint, the rate of evolutionary sequence change for a given protein — the 'molecular clock' — was found, curiously enough, to be relatively constant, and not dependent on species-specific parameters such as generation time and environment. The global structure and function of each particular protein seemed to be the main determinants of its evolutionary rate. Both these constraints play a relevant role; interestingly enough, from a recent comparison [10] of ~2000 proteins of known three-dimensional structure, only 150 unique types of fold have been identified, and some of these are exemplified by proteins that have no obvious similarities in sequence or function. In the globin family as a whole, although sequence conservation can be as low as 16 %, the typical myoglobin fold observed by Kendrew and Perutz is highly conserved. Kendrew and Perutz have suggested that the exclusion of polar residues from 32 specific sites is sufficient for a protein to have the globin fold.

From the wealth of information available on globins, the molecular clock was estimated to tick with an average rate of one substitution per α or β chain every 2–3 million years [11]. A closer look, however, shows that the clock is not at all regular. For example, analysis of the evolutionary rate since a tetrameric haemoglobin was first assembled from the ancestral monomeric globin has highlighted an acceleration–deceleration pattern between ancestor and descendant, arguing for positive selection rather than neutral fixation [11,12]. From analysis of the

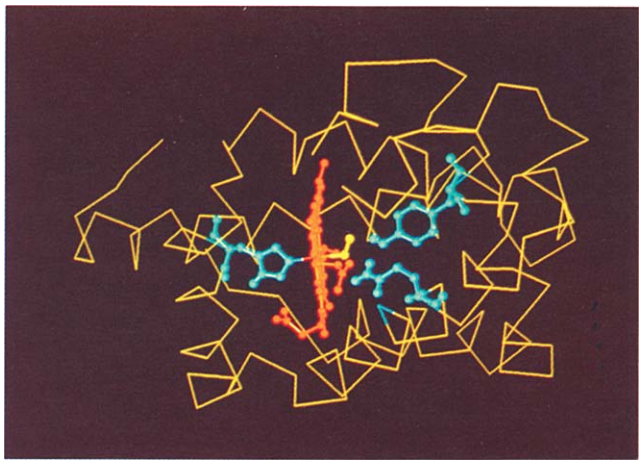

Fig. 2. The distal haem pocket of mutant sperm whale myglobin YQR, as determined by X-ray crystallography (our unpublished data). The side chains of residues Tyr(B10) and Gln(E7) (highlighted in blue) are at the appropriate distance and orientation to interact with bound O_2 (shown in yellow).

genealogical trees, therefore, neither positive selection nor neutral drift alone are the sole force in globin evolution, but both seem to have had different relevance over different time spans.

The work of Komiyama *et al.* [6] shows that transplantation of a unique function with a strong selective advantage is possible by grafting a relatively small number of residues at key positions. Thus, among all the amino-acid differences between human and crocodile haemoglobins — 110 out of 287 residues — twelve and possibly fewer residues are responsible for the bicarbonate effect. This finding strongly argues that adaptive changes can be brought about by few substitutions of key residues, rather than by the gradual accumulation of mutations that each produce a very small shift in chemical affinity [13].

Engineering a blood substitute

To be able to synthesize wholly artificial blood in the laboratory is a desirable, but not a realistic, task for the near future. However, encouraging attempts have been made to engineer a 'blood substitute' — or an oxygen-carrying 'plasma expander' — by suitable modifications of human haemoglobin. A number of potential advantages for such a blood substitute can be envisaged (see [14]). These include, first, the availability of the substitute for immediate use in emergencies, without the need for group typing; second, the ability to store the substitute in a ready-for-use formulation for years; and third, the ability to eliminate the risk of viral transmission by heat treatment. The driving force behind this biotechnological endeavour are projected sales worth $10 billion per year, even with applications limited to emergency cases, organ perfusion and the prevention of cardiac ischemia (using balloon angioplasty, for example).

A number of problems have to be overcome in the design of a form of haemoglobin suitable for use as a blood substitute. One problem comes from the dissociation of

oxygenated haemoglobin tetramers into $\alpha\beta$ dimers [2,12], which are rapidly eliminated through the kidney, causing renal damage in the process. Several approaches have been taken to solving this problem, all involving modifications to stabilize the tetrameric state of haemoglobin. This has been done, first, by chemically crosslinking the two α chains across their interface; second, by genetically engineering fusion between the carboxyl and amino termini of the two α chains [15] (Fig. 1b); and third, by glutaraldehyde polymerization of haemoglobin tetramers. In all cases, dimerization has been practically abolished, and as a consequence the life-time of injected haemoglobin after modification is considerably extended [14].

The O_2 ligand of haemoglobin is coordinated by the Fe^{2+} atom at the centre of the haem cofactor. The stability of the Fe^{2+}-O_2 complex is the fundamental property of O_2 carriers [2,12], and differentiates haemoglobins and myoglobins from O_2 adducts of free haem, which very rapidly oxidizes into the physiologically inactive Fe^{3+} form in the presence of O_2. The stability of the Fe^{2+}-O_2 complex depends on the amino-acid side chains lining the haem pocket on the distal side of the Fe^{2+} atom (Fig. 1d). A hydrogen bond between bound O_2 and the distal histidine at position E7 is a key interaction in stabilizing the Fe^{2+}-O_2 complex [5]. In the past few years, detailed structural and functional data on myoglobin and haemoglobin mutants have provided a clue to a detailed quantitative understanding of these phenomena [16]. In a nutshell, it was shown that there is a correlation between three key parameters: the absolute values of the velocity constant for O_2 dissociation; the hydrogen-bonding capability and overall hydrophobicity of the side chains on the distal side of the haem Fe^{2+} atom; and the rate of autoxidation of the haem iron.

We recently decided to engineer onto the sperm whale myoglobin framework a binding site with an O_2 dissociation velocity constant high enough for physiological O_2 delivery, but low enough for the Fe^{2+}-O_2 complex to be reasonably stable [17]. We transplanted into sperm whale myoglobin a distal-site architecture inspired by *Ascaris* haemoglobin, which has an extremely low O_2 dissociation velocity constant as a result of two hydrogen bonds between bound O_2 and distal residues Gln(E7) and Tyr(B10) [18]. This was achieved with a triple myoglobin mutant (called YQR) whose crystallographic structure (Fig. 2) shows that Gln(E7) and Tyr(B10) on the distal side are conveniently located to interact with bound O_2. Remarkably, while the O_2 dissociation velocity constant of YQR is much slower than that of wild-type myoglobin, its oxygen affinity is very similar and its autoxidation rate is two-fold lower, implying that this may be a suitable strategy for engineering a stable blood substitute.

Making sure that haemoglobin's allosteric control mechanisms are conserved is the most complex requirement in engineering a blood substitute. The role of intracellular diphosphoglycerate in efficient O_2 delivery by human blood [12] is crucial for acclimatization when we go to the mountains; infused haemoglobin that is devoid of this important allosteric control because of the absence of diphosphoglycerate in plasma has a high O_2 affinity, and is totally unsuitable for use as an O_2 carrier. The elegant synthesis of scuba haemoglobin [6] is indeed very promising, as transfused haemoglobin may be allosterically regulated by the bicarbonate produced as a catabolite of tissue respiration; a smart idea has yielded a new molecule thanks to a combination of ingenuity, sophisticated engineering strategy and all-round incentive!

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