Species Adaptation in a Protein Molecule[1]

M. F. Perutz
MRC Laboratory of Molecular Biology

The allosteric properties of hemoglobins, especially their responses to ligands other than oxygen, vary widely in different classes of vertebrates. Knowing the stereochemistry of the cooperative effects in human hemoglobin, one can infer the stereochemical basis of these variations from the changes in amino acid sequence. The results indicate that the tertiary and quaternary structures of deoxy- and oxyhemoglobin have remained almost invariant during vertebrate evolution and that most of the amino acid replacements between species are functionally neutral. Adaptations leading to responses to new chemical stimuli have evolved by only a few (one to five) amino acid substitutions in key positions. Once such a response has become superfluous, it may be inactivated, not necessarily by a reversal of one of the original substitutions but by any other that happens to inhibit it.

Introduction

Species adaptation at the molecular level is a virgin field. Lewontin (1979) complained that "it has proved remarkably difficult to get compelling evidence for changes in enzymes brought about by selection, not to speak of adaptive changes. . . ." Such evidence has recently been gathered for hemoglobins whose response to different chemical stimuli varies widely in vertebrates living in different environments. How did this adaptation come about? Is it the result of changes in tertiary and quaternary structure? Has it been brought about by the gradual accumulation of minor mutations, each producing a small shift in chemical affinity, or by a few amino acid substitutions in key positions? Have similar changes in chemical affinity in different species been brought about by the same amino acid substitutions or by different ones? What selective advantage does possession of multiple hemoglobin genes offer to a species? Are most amino acid substitutions between species functionally significant, and have they evolved by Darwinian selection (Goodman 1981), or are they caused by random fixation of neutral or

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Address for correspondence and reprints: M. F. Perutz, MRC Laboratory of Molecular Biology, Cambridge CB2 2QH, England.

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nearly neutral mutations (King and Jukes 1969)? We can now at least begin to answer these questions.

The hemoglobins of two primitive vertebrates, the lamprey and the hagfish, are monomeric when oxygenated and aggregate into oligomers on deoxygenation. They contain only one type of chain. Hemoglobins of the higher vertebrates are tetramers made up of two α- and two β-chains, each containing between 141 and 147 amino acid residues. Each chain is linked to one heme. The α-chain contains seven and the β-chain eight helical segments, designated A–H; they are interrupted by nonhelical segments marked AB, BC, and so on. A short nonhelical segment, called NA, precedes the first helix, and another, called HC, follows the last helix (fig. 1). The four chains are arranged tetrahedrally around a twofold symmetry axis which runs along a water-filled cavity. Uptake and release of oxygen are accompanied by small changes in tertiary structure of the segments surrounding the hemes and by a large change in quaternary structure involving a rotation of one αβ-dimer relative to the other by 15°, together with a relative shift of 1 Å. The rotation that occurs on dissociation of oxygen widens the cavity between the two β-chains so that the cationic groups that form its lining can bind organic phosphates. The two quaternary structures will be referred to as the oxy or relaxed (R) and the deoxy or tense (T) structures (Fermi and Perutz 1981).

All vertebrate hemoglobins react cooperatively with molecular oxygen. Their oxygen equilibria are influenced by various chemical factors, known as hetero-

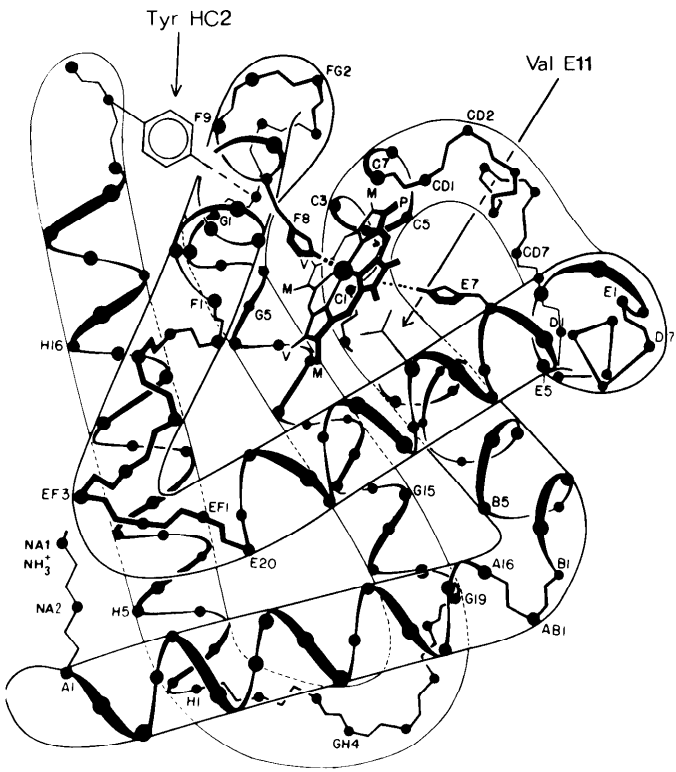

FIG. 1.—Tertiary structure of globin chain, showing helical and nonhelical segments and position of heme.

tropic ligands, and by temperature. In human erythrocytes these ligands are H^+, Cl^-, CO_2, and D-2,3-bisphosphoglycerate (DPG); they all reduce the oxygen affinity of hemoglobin in a physiologically advantageous manner by combining preferentially with the T structure. In addition, the uptake of protons on release of oxygen, known as the alkaline Bohr effect, facilitates the transport of CO_2 from the tissues to the lungs in the form of HCO_3^-. In 0.1 M chloride the uptake of protons vanishes at pH 6; below that pH protons are released on release of oxygen. This is known as the acid or reverse Bohr effect; it does not seem to have any physiological function in mammals but is dominant even at physiological pH in certain fish and amphibia. In humans a minor part of the CO_2 is carried from the tissues to the lungs in the form of carbamino ion bound to hemoglobin. DPG is an allosteric regulator which lowers the oxygen affinity and thereby facilitates the release of oxygen to the tissues. The reaction of human hemoglobin with oxygen is exothermic, so that the oxygen affinity decreases with rising temperature (Weatherall 1976; Bunn et al. 1977; Imai 1982).

The effects of heterotropic ligands arise because the T structure has a low affinity for oxygen and a high one for protons, chloride, organic phosphate, and CO_2. In the R structure these relative affinities are reversed. The transition between the two structures gives rise to the cooperativity of oxygen binding. Hill's coefficient n provides a measure of the cooperativity. In terms of allosteric theory, the equilibrium curve is described by the oxygen dissociation constants of the two alternative structures K_T and K_R and by the equilibrium constant $L = [T]/[R]$ in the absence of oxygen. Heterotropic ligands strongly influence K_T and L but exercise only a weak influence on K_R (Weatherall 1976; Imai 1982). (K_T and K_R are usually expressed in $[\text{mm Hg}]^{-1}$.)

Structural analysis of human hemoglobin has shown that all heterotropic ligands lower the oxygen affinity because they stabilize the T structure. They do so by forming salt bridges within and between the subunits (fig. 2). The binding sites of the heterotropic ligands are as follows: DPG binds in the cavity between the two β-chains to Val NA1(1)β, His NA2(2)β, Lys EF6(82)β, and His H21(143)β (fig. 3). (In this notation the first number gives the position of the residue along

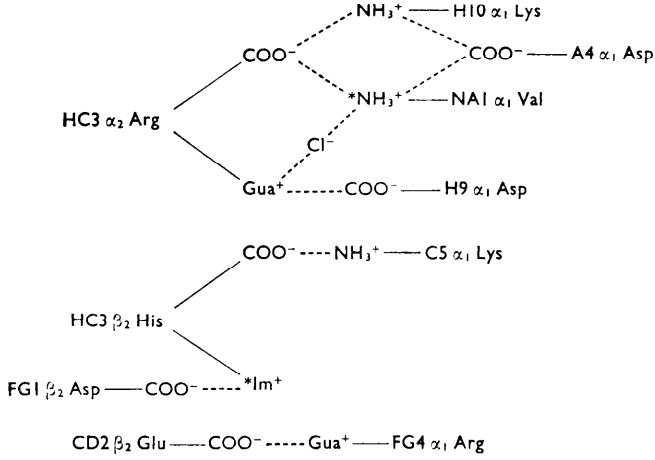

FIG. 2.—Salt bridges in human deoxyhemoglobin (Weatherall 1976). For details of the stereochemistry see Fermi and Perutz (1981).

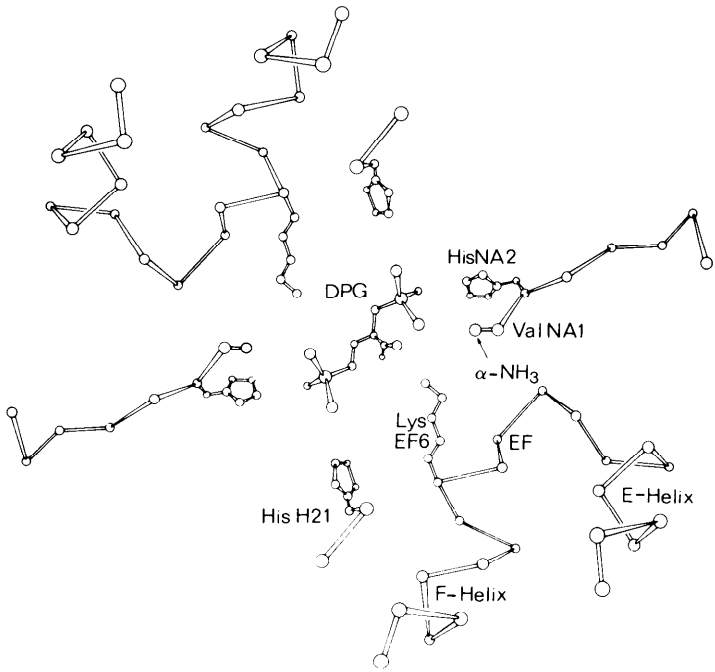

FIG. 3.—D-2,3-bisphosphoglycerate (DPG) binding site between the two β-chains in human deoxyhemoglobin. On transition to the oxy structure the α-amino groups move apart and the EF segments close up, so that the complementarity of the binding site to DPG is lost (Arnone 1972).

the helical or nonhelical segment, while the second number, in parentheses, gives its position in the amino acid sequence.) Bohr protons are bound by certain cationic groups that have their pK's raised in the R ⟶ T transition, because they are free in the R structure but form salt bridges with carboxylates, chloride, or phosphate in the T structure. In the absence of DPG, Bohr protons bind mainly to Val NA1(1)α, Lys EF6(82)β, and His HC3(146)β, making the hydrogen bonds shown in figure 2. In the presence of DPG, Bohr protons are also bound to His NA2(2)β and His H21(143)β. In vivo all these residues therefore contribute to the alkaline Bohr effect. CO_2 forms carbamino compounds with Val NA1(1)α and β. Chloride is bound in the internal cavity by Val NA1(1)α, Lys EF6(82)β, and probably at other minor sites. In the absence of DPG, His H21(143)β contributes to the acid Bohr effect (Weatherall 1976; Kilmartin et al. 1980; Perutz et al. 1980).

Fish Hemoglobins

Many teleost (bony) fish use hemoglobin for both respiration and secretion of oxygen into the swim bladder and the eye (Wittenberg and Haedrich 1974; Wittenberg and Wittenberg 1974; Steen 1979; Ingerman 1982). Each organ possesses an elaborate vascular network, the rete, which converts a gradient of increasing lactic acid concentration into a gradient of decreasing oxygen concentration by countercurrent circulation, thus causing oxygen to be discharged. This acid-activated discharge is known as the Root effect, but it is best considered as an enhanced version of the alkaline Bohr effect which causes so drastic a drop in the oxygen affinity at low pH that several hundred atmospheres of oxygen

pressure fail to saturate the hemoglobin with oxygen. The effect arises because the response of teleost fish hemoglobins to heterotropic ligands is markedly different from that of most mammalian hemoglobins. While in human hemoglobin Hill's coefficient n remains at or just below 3.0 independent of pH, in fish hemoglobins it drops to unity, or even below unity, near pH 6. This disappearance of cooperativity is due to inhibition of the allosteric transition from the T to the R structure at acid pH. Alkaline pH tends to inhibit the transition from the R to the T structure instead, so that n rises to a maximum of only just above 2.0 near neutral pH and in many, but not all fish, falls again at alkaline pH. Both the span of oxygen affinities covered by the pH range 6–9 and the magnitude of the alkaline Bohr effect ($\Delta \log p_{50}/\Delta$ pH) are larger in fish than in mammalian hemoglobins. In carp hemoglobin for instance, K_1, the association constant of the first oxygen molecule to be taken up, drops 100-fold between pH 9 and 6.5, compared with eightfold in human hemoglobin; over the same pH range K_4, the association constant of the last oxygen, drops 10-fold in carp compared with 1.2-fold in human. On oxygenation, human hemoglobin in deionized water releases no more than one proton per tetramer, while carp releases 3.6. However, the enhancement of the alkaline Bohr effect by chloride or phosphate is smaller in carp than in human hemoglobin. While the oxygen dissociation constants of the α- and β-subunits of human hemoglobin differ but slightly, those of fish hemoglobins at acid pH may differ by as much as 100-fold, which allows one pair of subunits to secrete oxygen into the swimbladder and the eyes against hydrostatic pressures as high as several hundred atmospheres (for details of the properties summarized here, see Noble et al. [1970]; Binotti et al. [1971]; Gillen and Riggs [1972]; Tan et al. [1972]; Brunori et al. [1973]; Brunori [1975]; Imai and Yonetani [1975]; Riggs [1979]; Chien and Mayo [1980]; Morris et al. [1981]; Morris and Gibson [1982]).

What makes the allosteric properties of these fish hemoglobins with Root effects so different from those of mammalian hemoglobins? Seeing that the amino acid replacements between, say, human and carp hemoglobin number over 140, this seems a forbidding problem, but systematic examination on my atomic model of human hemoglobin led me to the conclusion that the Root effect is caused mainly by a single amino acid replacement, that of cysteine F9β in mammals by serine in fish. My reasoning was as follows. In the T structure of all hemoglobins that exhibit an alkaline Bohr effect, the imidazole of the C-terminal histidine HC3β forms a salt bridge with Asp or Glu FG1β; this raises the pK of the histidine so that protons are bound. The C-terminal carboxyl of the same histidine forms a salt bridge with the invariant Lys C5α, whether or not the hemoglobin shows a Bohr effect. In mammals the sulfydryl of Cys F9β is in van der Waals contact with that oxygen of the C-terminus that is not bonded to Lys C5α, but it forms at most a very weak hydrogen bond with that oxygen. The OH of serine, however, forms strong hydrogen bonds. The atomic model shows that the OH of Ser F9β is so placed that it can donate a hydrogen bond to the free terminal oxygen atom of His HC3β and accept a hydrogen bond from the peptide NH of His HC3β (figs. 4, 5; table 1). These additional hydrogen bonds would stabilize the C-terminal salt bridges in the T structure, thereby raising the allosteric constant L and the pK of His HC3β and lowering the oxygen-binding constant K_T, especially of the β-chains (Perutz and Brunori 1982). Experimental tests of the theory are described below.

Fish hemoglobins exhibit at least one other feature that is likely to shift their allosteric equilibria toward the T structure. In the R structure of mammalian hemoglobins, the terminal carboxyl of His HC3β forms an external salt bridge with Lys HC1 of the same β-chain. In teleost fish hemoglobins this external lysine is replaced by a glutamine which would not bind the carboxyl in an aqueous

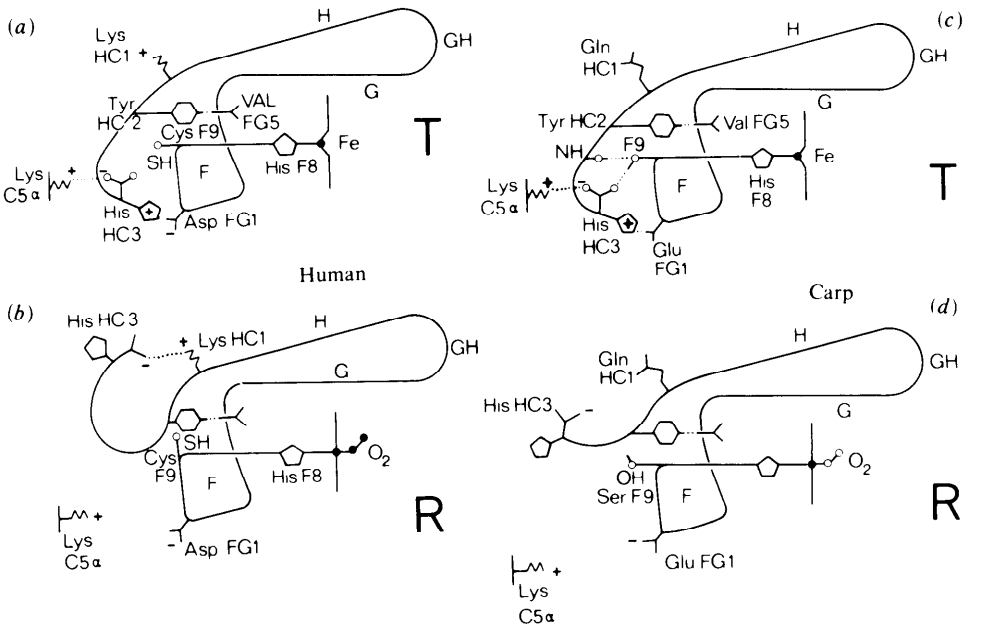

FIG. 4.—C-terminal salt bridges and the role of residue F9β in the T and R structures of mammalian and fish hemoglobins. *a*, Human T structure; *b*, human R structure; *c*, carp T structure; *d*, carp R structure. The letters F, G, and H denote helical segments; FG, GH, and HC denote nonhelical segments. The same notation is used in figs. 5–6 (Perutz and Brunori 1982).

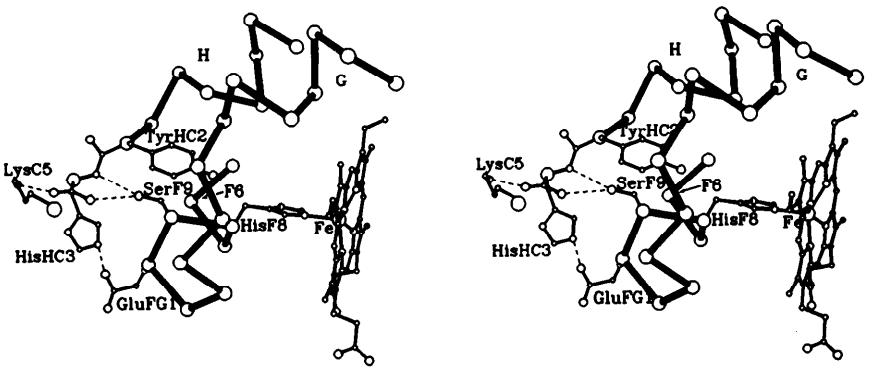

FIG. 5.—Stereo diagram showing the bonds at the C-terminus of the β-chain in the T structure of teleost fish hemoglobin. The amino group of Lys C5α donates a hydrogen bond to one of the carboxylate oxygens of His HC3β, and the OH of Ser F9β donates a hydrogen bond to the other oxygen. The imidazole donates a hydrogen bond to the carboxylate of Glu FG1. The main chain NH of His HC3 donates a hydrogen bond to the lone pair electrons of the OH of Ser F9. Note the direct link from the heme iron via His F8 and Ser F9 to the C-terminus, which would therefore feel the movement of the iron toward the porphyrin that occurs on oxygen binding (Perutz and Brunori 1982).

Table 1

Important Amino Acid Replacements Distinguishing Mammalian, Amphibian, and Fish Hemoglobins

Position of Amino Acid Residue[a]	Human	Carp	Trout IV	Trout I	Aquatic Frog *Xenopus*	Tadpole of *Xenopus*	Terrestrial Frogs: *Rana esculenta* (Europe)	Terrestrial Frogs: *R. catesbeiana* (America)	Tadpole of *R. catesbeiana*	Shark (*Heterodontus portusjacksoni*)	Lungfish (*Lepidosiren paradoxa*)
β-chain:											
NA2	His	Glu	Asp	Glu	Gly	His	–	–	His	His	His
EF6	Lys	Lys	Lys	Leu	Lys	Lys	Lys	Lys	Lys	Lys	Lys
F6	Glu	Val	Val	Glu	Lys	Thr	Glu	Glu	Glu	Lys	His
F9	Cys	Ser	Ser	Ala	Ser	Ala	Ser	Ser	Ala	Ala	Ser
FG1	Asp	Glu	Glu	Asn	Glu	Glu	Asn	Gly	Asn	Glu	Glu
H21	His	Arg	Arg	Ser	Lys	His	Lys	Lys	His	Lys	Arg
HC1	Lys	Gln	Gln	Arg	Gly	Gly	Ala	Gly	Ser	Glu	Glu
HC3	His	His	His	Phe	His	Phe	His	His	His	His	His
α-chain:											
NA1	Val	Ac-Ser	Ac-Ser	Ac-Ser	Leu	?	Gly	?	Ac-Ser	Ac-Ser	Met-Arg
C3	Thr	Gln	Gln	Gln	Lys	?	?	?	Gln	Ala	Gly
Root effect	–	+	+	–	+	?	–	–	–	–	–
Allosteric effector	DPG	ATP	ATP	–	DPG	?	?	?	?	?	ATP
Bohr effect	Normal	Strong	Strong	Absent	Normal	?	Weak	Weak-Normal	Reversed	Weak	Normal
Sources[b]	1	2	3	4, 15	5, 16	6	7, 8	9	10, 11	12, 13	14

[a] For meaning of the symbols, see figs. 1–6.

[b] Sources: 1 = Dayhoff 1972; 2 = Grujic-Injac et al. 1980; 3 = Bossa et al. 1976; 4 = Bossa et al. 1978; 5 = Williams et al. 1980; 6 = Banville et al. 1983; 7 = Chauvet and Acher 1972; 8 = Tentori et al. 1967; 9 = Baldwin and Riggs 1974; 10 = Maruyama et al. 1980; 11 = Watt et al. 1980; 12 = Fisher et al. 1977; 13 = Nash et al. 1976; 14 = Rodewald and Braunitzer 1983; 15 = Barra et al. 1983; 16 = Kay et al. 1983.

environment, so that less energy is needed to move His HC3β toward Asp FG1β on going from the R to the T structure (figs. 4, 5). The constellation of polar groups described here has been found in the hemoglobins of carp (Grujic-Injac et al. 1980), goldfish (Braunitzer and Rodewald 1980), trout IV (Bossa et al. 1976), and suckers (*Catostomus clarkii*) (Powers and Edmundson 1972), all of which exhibit strong Root effects.

Mammals and frogs use DPG as an allosteric effector, but teleost fish use ATP, GTP, or inositol pentaphosphate and probably also lactate (Gillen and Riggs 1977; Isaacks et al. 1977). While in mammals DPG lowers the oxygen affinity more than ATP, the opposite holds in teleost fish (Gillen and Riggs 1971). Mammalian hemoglobins whose oxygen affinity is regulated by DPG have a hydrogen donor side chain in position NA2β (His, Gln, or Asn) and they have His in position H21β (fig. 3). Teleost fish have either Glu or Asp in position NA2β and Arg at H21β. Substitution of those side chains in the atomic model of human deoxyhemoglobin produces a constellation of charged groups stereochemically complementary to strain-free ATP or GTP (fig. 6). The model suggests that, when ATP is bound, the carboxylate of either Glu or Asp NA2β_1 accepts a hydrogen bond from the N-6 amino group of adenine; the amino group of Val 1β_2 and the guanidinium group of Arg H21β_1 each donate a hydrogen bond to $(PO_4)_\gamma$, while Lys EF6β_1 and β_2 donate hydrogen bonds to either of the other two phosphates, thus neutralizing the four negative charges of ATP (Perutz and Brunori 1982).

Since this structure was first proposed, Braunitzer and his colleagues have determined the amino acid sequence of rhinoceros hemoglobin (Mazur et al. 1982). Its allosteric effector site shows only a single substitution compared with that of human hemoglobin: His Na2β ⟶ Glu, yet GTP lowers its oxygen affinity more than ATP, and ATP lowers it more than DPG, just as in teleost fish (R. Baumann, unpublished). This observation supports the hydrogen bonds between the purine and Glu NA2 proposed in figure 6; in fact, it can hardly be explained without these bonds. It also suggests that GTP is bound more tightly than ATP.

The stereochemistry of ATP and GTP in carp deoxyhemoglobin has recently been determined by nuclear magnetic resonance (Clore et al., unpublished) using

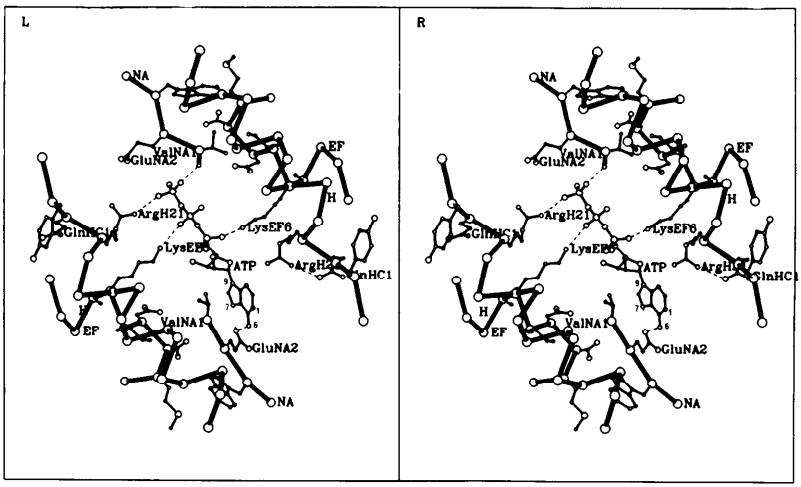

Fig. 6.—Suggested ATP binding site of fish hemoglobins

the time-dependent transferred nuclear Overhauser effect (Clore and Gronenborn 1983). This experiment showed the purine to be in the *anti* conformation with respect to the ribose in both trinucleotides; the ribose pucker is 3′ endo; the $O_{5'}$—$C_{5'}$—$C_{4'}$—$C_{3'}$ torsion angle is trans, P_α—$O_{5'}$—$C_{5'}$—$C_{4'}$ are coplanar (trans), P_1—O—P_β and P_β—O—P_γ are as in the free nucleotide, probably all trans, so that electrostatic repulsion between the phosphates is minimized. The results confirm the structure of ATP in carp deoxyhemoglobin proposed by Perutz and Brunori (1982), except for a small change in the angle of tilt of the purine (fig. 6). To convert the structure of ATP into that of GTP, the entire nucleotide has to be turned by roughly 180° about its long axis. This leaves the γ-phosphate bound to Val 1α of subunit β_1 and allows N_1 and N_2 of the guanine to donate hydrogen bonds to Glu NA2 of subunit β_2. The formation of two hydrogen bonds between the purine and Glu NA2 in GTP, compared with the single hydrogen bond in ATP, explains why GTP lowers the oxygen affinity of carp hemoglobin twice as much as ATP (Weber and Lykkeboe 1978).

It is thought that during fast movement the pH at the gills may drop too low for efficient oxygen uptake by those hemoglobins that exhibit the Root effect; to ensure a continued oxygen supply, trout and some other fast-swimming fish have two kinds of hemoglobin, those that respond to heterotropic ligands and those that do not. In trout, the hemoglobin that exhibits the Root effect has been assigned the number IV, while the two hemoglobins that fail to respond to heterotropic ligands are called I and II (Brunori 1975). Component III makes up only 3% of the total, and its ligand-binding properties are not known. In trout I all the polar residues involved in both the alkaline and acid Bohr effects and in phosphate binding are replaced by neutral ones: Lys EF6β is replaced by Leu, Ser F9β by Ala, Glu FG1β by Asn, Arg H21β by Ser, and His HC3β by Phe (table 1). The sequences of trout hemoglobins I and IV show many other replacements, but so far none of these is of a kind likely to have a significant effect on the affinity for heterotropic ligands.

The properties just described are not typical of all fish. Among elasmobranch, skate hemoglobin has no appreciable Bohr effect and chloride raises rather than lowers its oxygen affinity. Like trout I, torpedo hemoglobin fails to respond to organic phosphates (Riggs 1970, p. 209; Johansen and Weber 1976, p. 219). These properties cannot yet be interpreted for lack of amino acid sequences. Hemoglobin of the shark *Heterodontus portusjacksoni,* a cartilaginous fish without a swim bladder, exhibits a weak alkaline Bohr effect (Nash et al. 1976). Position F9β is occupied by Ala; residue FG1β is Glu, which could produce a normal alkaline Bohr effect by forming a strong salt bridge with His HC3β if it were not for a Lys in position F6β, one turn of π-helix away, competing with His HC3β for that salt bridge. The lungfish *Lepidosiren paradoxa* also lacks a swim bladder; its hemoglobin exhibits no Root effect and has an alkaline Bohr effect similar in magnitude to that of human hemoglobin (Phelps et al. 1979). It does have Ser at F9β and Glu at FG1β, but as in *Xenopus* and in the shark, its salt bridge with His HC3β is weakened by competition from residue F6β, in this instance a His.

The fish discussed so far are cold blooded, but fast-swimming sharks can maintain their bodies 7–10 C and tuna (*Thunnus thynnus*) up to 15 C above the water temperature (Carey and Teal 1969; Carey et al. 1971); cooling of the muscles is minimized by a countercurrent exchange system between the arterial and venous circulations that transfers metabolic heat from the veins to the cold blood arriving

in the arteries from the gills. In most species the oxygen affinity of hemoglobin drops with rising temperature because the reaction of heme with oxygen is exothermic; if this were true also in tuna, heating of the cold arterial blood would cause some of its oxygen to dissociate and be transferred to the nearby veins unused. To reduce this waste, tuna have evolved a hemoglobin in which the reaction with oxygen is endothermic (Carey et al. 1971; Gibson and Carey 1982). This reversal arises because the intrinsic heat of oxygenation of the hemes is exceeded by the heat absorbed in the T $\longrightarrow$ R transition. In human hemoglobin that transition absorbs about half of the -66 kcal released when four molecules of oxygen combine with one molecule of hemoglobin; in trout IV it absorbs about two-thirds. To produce a reversed temperature coefficient, the T $\longrightarrow$ R transition in tuna absorbs about 100 kcal (Gibson and Carey 1982). What is the source of the extra 30 kcal? The main source of heat in the allosteric transition comes from the formation of hydrogen bonds. Taking the enthalpy of hydrogen bond formation in the dimerization of N-methylacetamide in benzene of 3.6 kcal/mol as a measure (Davies and Thomas 1956), the T structure would have to contain four additional and the R structure four fewer hydrogen bonds to achieve the desired result. Taking the enthalpy of 1.5 kcal/mol for the disruption of a CO-HN bond in water as a measure (Kauzmann 1959), these numbers would rise to the improbable value of 10. Another enthalpy sink could be generated if the sum of the van der Waals interactions in the R structure was less than in the T structure, so that heat of fusion would be absorbed in the T $\longrightarrow$ R transition (Bello 1977).

Lest hemoglobin be regarded as indispensable to vertebrate life, let me draw attention to the existence of three species of large antarctic fish devoid of erythrocytes or blood pigment (Rund 1954). The amount of dissolved oxygen their very cold, colorless blood can carry is only one-eighth of that of two other antarctic fish with red blood, but that seems to be adequate for the sluggish existence of these large fish.

Experimental Tests of the Theory of the Root Effect

When X-ray analysis had indicated that a salt bridge formed by His HC3β in the T structure may be responsible for the major part of the alkaline Bohr effect in horse and human hemoglobins, Kilmartin set out to test that prediction by preparing a hemoglobin from which that C-terminal histidine had been enzymatically cleaved. X-ray analysis showed that this cleavage had no significant effect on the structure of the rest of the hemoglobin molecule. Nevertheless, it halved the alkaline Bohr effect (Kilmartin and Wootton 1970; Perutz and Ten Eyck 1971). This observation and many subsequent ones supported the dominant contribution of this one specific residue (Kilmartin et al. 1980). More recent is the discovery of an abnormal human hemoglobin with a drastically reduced alkaline Bohr effect, arising from the substitution Asp FG1β $\longrightarrow$ Asn which neutralizes the anionic arch of the salt bridge, as does the substitution Glu FG1β $\longrightarrow$ Asn that occurs between trout IV and I hemoglobins (Como et al. 1983).

If the Root effect in fish hemoglobins is due largely to the extra stabilization conferred by Ser F9β on the salt bridges of His HC3β, it should be inhibited by enzymatic cleavage of His HC3. Parkhurst, Goss, and Perutz tested that prediction by preparing des-His carp hemoglobin (Parkhurst et al. 1983). This halved the alkaline Bohr (Root) effect, lowered L, raised K_T, and also changed the kinetics of ligand binding. On approach to pH 6, native carp hemoglobin shows a sharp

rise in the rate of oxygen dissociation and fall in the rate of CO recombination characteristic of the Root effect. Both the rise and fall are much diminished in des-His carp hemoglobin. All these observations supported the theory.

A further test became possible when the hemoglobin of the South African frog *Xenopus* was found to have a serine in position F9β (Williams et al. 1980). According to the theory, this hemoglobin should exhibit a Root effect at low pH, and this was confirmed by Perutz and Brunori (1982). We found that it has a much lower oxygen affinity than human hemoglobin, consistent with a more stable T structure; its Hill's coefficient tends toward unity at pH 6, and its kinetics of CO recombination at low pH are similar to those of hemoglobins exhibiting a Root effect. However, its Root effect is not as strong as that of carp hemoglobin.

Amphibia

To produce a strong alkaline Bohr effect and a Root effect at acid pH, *Xenopus* hemoglobin has conserved the essential Ser F9β, Glu FG1β, and His HC3β, and it has Gly rather than Lys in position HC1β to prevent anchoring of the C-terminus in the R structure (table 1). However, it has a Lys at F6β, one turn of π-helix away from Glu FG1β and capable of competing with His HC3β for the essential salt bridge with that Glu (figs. 4, 5). This competition may weaken its alkaline Bohr effect compared with that of carp.

Xenopus hemoglobin is exceptional among amphibians for its strong alkaline Bohr effect; in 0.1 M Cl^- other amphibian hemoglobins show either weak alkaline or only weak acid Bohr effects. The major hemoglobin component of the large American bullfrog *Rana catesbeiana,* when stripped and in dilute solution, shows a Bohr effect of $\Delta \log p_{50}/\Delta pH = -0.16$, compared with -0.56 in *Xenopus* (Aggarwal and Riggs 1969). Position F9β is occupied by Ser, as in *Xenopus,* but its influence is counteracted by the substitution Gly for Glu in position FG1β. In consequence His HC3β cannot have its pK raised in the T structure by the usual strong salt bridge with Glu FG1β but can only interact more weakly with Glu F6β which is one turn of π-helix away from residue FG1β (table 1; figs. 4, 5). The two major fractions of the adult hemoglobin of the European frog *R. esculenta* also exhibit only weak alkaline Bohr effects (Brunori et al. 1968). The sequence of the major component of the β-chain is similar to that of *R. catesbeiana;* position FG1β is occupied by Asn rather than Gly, which again inhibits the strong salt bridge with His HC3β. His H21β, which contributes to the acid Bohr effect in human hemoglobin A, is replaced by Lys in all three adult frog hemoglobins (fig. 3).

Tadpole hemoglobin of *R. catesbeiana* in the absence of phosphates has only a reverse Bohr effect (Atha et al. 1979). Here Ser F9β is replaced by Ala, Glu FG1 by Asn, and Ser 1α, the other major source of the alkaline Bohr effect, is acetylated, so that the alkaline Bohr effect is completely inhibited. However, His H21β is present, consistent with its role in the acid Bohr effect of human hemoglobin. His H21β is also present in *Xenopus* tadpole hemoglobin; this has Phe in position HC3β, which would inhibit most of the alkaline Bohr effect contributed by the β-chains (D. Banville et al., unpublished). The amino acid sequence of the α-chain is not yet known. The β-chains of both tadpole species carry the same residues at the phosphate binding site as does adult human hemoglobin; organic phosphates, if present, would therefore be expected to overcome the acid Bohr effect and produce a weak alkaline one instead.

In another amphibian, *Amphiuma,* the adult hemoglobin has an acid Bohr effect at physiological pH, converted in vivo to a weak alkaline one by organic phosphate (Bonaventura et al. 1977). The adult hemoglobin of the crested newt, *Triturus cristatus,* also exhibits an acid Bohr effect at physiological pH, but in this amphibian it is maintained in the hemolysate; even so, the higher concentration of organic phosphates in the red cell may convert it to a weak alkaline Bohr effect in vivo (Condo et al. 1981). Blood of the frog *Telmatobius culeus* that lives in Lake Titicaca at an altitude of 3,800 m has a higher oxygen affinity than that of any other frog (Hutchinson et al. 1976), but the oxygen equilibrium curves of its purified hemoglobin are still unknown. It will be interesting to analyze the stereochemical bases of all these unusual properties once amino acid sequences become available.

Why is the alkaline Bohr effect of the bloods of the Port Jackson shark, of the newt, of *Amphiuma,* and of the anuran tadpoles so much weaker than that of mammals? In the blood of air breathers the partial pressure of CO_2 is about 40 mm Hg, and a substantial Bohr effect is needed for its transport from the lungs to the tissues, but in the blood of water breathers the partial pressure of CO_2 is only 2–4 mm Hg because it is 28 times more soluble in water than oxygen and diffuses out of the body through the gills and the skin. Having no swim bladder and no rete to pump oxygen into the eye, the shark and the tadpoles can therefore get along with only a weak alkaline Bohr effect. The same is true of the aquatic frog *Amphiuma,* which is regarded as a permanent larva, even though the adult has lost its external gills and shows other evidence of partial metamorphosis (Rahn 1966; Lenfant and Johansen 1967; Bonaventura et al. 1977; Shelton and Boutilier 1982). Judging by the strong acid Bohr effect, the switch from tadpole to adult hemoglobin may not have occurred in this amphibian. In contrast, the adult terrestrial bullfrog *R. catesbeiana* loses only a small part of its CO_2 through the skin even when submerged and relies on ventilation through its lungs. Its blood therefore needs a larger alkaline Bohr effect than that of *Amphiuma* (Lenfant and Johansen 1967). The same appears to be true of the South American lungfish *Lepidosiren paradoxa* which lives in hot, stagnant water and surfaces to breathe (Phelps et al. 1979; Rodewald and Braunitzer 1983). *Xenopus* is anomalous. It is an aquatic frog with a low blood CO_2, capable of respiration through the skin or even of switching to anaerobic metabolism (Shelton and Boutilier 1982). At this stage I have not found any satisfying explanation for why it needs a large alkaline Bohr effect at physiological pH, or a Root effect at acid pH.

Reptiles

Crocodilians are able to stay underwater for as long as an hour without coming up to breathe, but they lack the high concentrations of myoglobin that provide diving mammals and birds with large stores of oxygen. Instead, crocodilians reduce oxygen consumption by shutting off the circulation to their muscles so that oxygen supply is restricted to their brain and viscera. Even so, they need to use as much as possible of the oxygen stored in their lungs and their blood. This appears to be accomplished by the unusual allosteric properties of their hemoglobin, which hardly responds to the normal heterotropic ligands (protons, CO_2, chloride, or organic phosphates) but responds strongly only to bicarbonate ion (fig. 7). Crocodilian hemoglobins bind two equivalents of bicarbonate ion per tetramer which lower the oxygen affinity as much as P6-inositol (inositol-hexa-

phosphate) does in human hemoglobin (Bauer and Jelkmann 1977; Jelkmann and Bauer 1980*b*; Bauer et al. 1981).

Model building showed that the bicarbonate binding sites lie in the cavity between the two β-chains where organic phosphates or carbamino CO_2 are bound in other species; each HCO_3^- is bound by Lys EF6 and Glu HC1 of one β-chain together with the N-terminal residue of its partner chain (fig. 8). In caiman hemoglobin, the N-terminal sequence is Ser-Pro-Phe, compared with Val-His-Leu in human hemoglobin (table 2). The human tripeptide is straight (fig. 3), but the

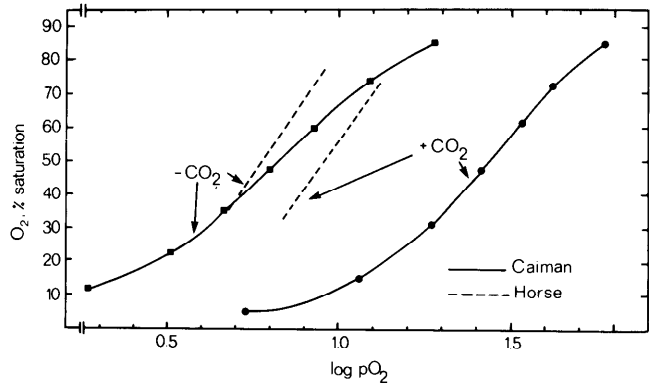

FIG. 7.—Oxygen equilibrium curves of caiman and horse hemoglobin with and without CO_2. P_{CO_2} = 40 torr, 25 C, I = 0.2 M, pH 7.2 for caiman (Bauer et al. 1981); pH 7.4 for horse (Kilmartin and Rossi-Bernardi 1969).

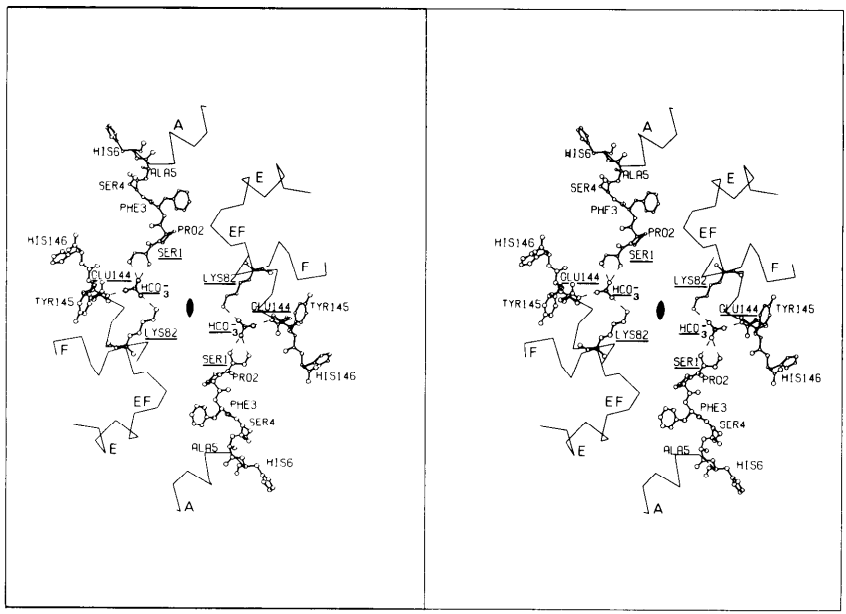

FIG. 8.—Stereo drawing of proposed bicarbonate binding site between the two β-chains of caiman deoxyhemoglobin. The central sign marks the dyad symmetry axis. Bicarbonates and their binding residues are underlined. Capital letters mark helical and interhelical segments (Perutz et al. 1981). Residues are marked in sequential, rather than structural notation. The following list gives the structural numbers with the sequential ones in parentheses: Ser NA1(1), Pro NA2(2), Phe NA3(3), Ser A1(4), Ala A2(5), His A3(6), Lys EF6(82), Glu HC1(144), Tyr HC2(145), His HC3(146), all β.

Table 2
Amino Acid Replacements for Allosteric Control in Crocodilian Hemoglobins

	Species				
Position	Human	Other Bony Vertebrates	Caiman	Nile Crocodile	Mississippi Crocodile
NA1β.....	Val	Val or Gly	Ser	Ac-Ala	Ac-Ala
NA2β.....	His	Gln, Asn, Glu, His, Asp, Met	Pro	Ser	Ser
EF6β	Lys	Lys	Lys	Lys	Lys
H21β	His	Lys, Arg, Ser, His	Ala	Ala	Ala
HC1β.....	Lys	Arg, Ala, Ser, Gln, Lys	Glu	Glu	Glu
H14α	Ser	Very variable	Ala	Ala	Ala

Note.—For full sequence, see Leclercq et al. (1981).

caiman tripeptide is forced to turn a corner at the proline. The turn brings the N-terminal serine within reach of the bicarbonate ion, so that one of the bicarbonate oxygens can form a salt bridge with the α-NH_3^+ and can also accept a hydrogen bond from the serine OH. The second bicarbonate oxygen can form a salt bridge with Lys EF6β, and the third oxygen can donate a hydrogen bond to one of the carboxylate oxygens of Glu HC1β. In the hemoglobins of the Nile crocodile and the Mississippi alligator, where the N-terminal sequence is acetyl-Ala-Ser-Phe, the α-NH of acetylalanine could donate a hydrogen bond to O_1 of the bicarbonate at the same distance as the α-NH_3^+ does in caiman, provided the chain bends at Ser 2 at the same angle as it does at Pro 2 in caiman.

Consider now the loss of allosteric inhibition by organic phosphates, carbamino CO_2, and chloride. The loss of affinity for organic phosphates is accounted for by the replacements His NA2β → Pro or Ser and His H21β → Ser (fig. 3). In human deoxyhemoglobin, carbamino CO_2 bound to Val 1α accepts a hydrogen bond from Ser H14α. In the three crocodilian hemoglobins this Ser is replaced by Ala. This replacement may also inactivate one of the strong chloride-binding sites which probably coincides with the CO_2 site. In human deoxyhemoglobin, another pair of CO_2 molecules competes with organic phosphates for binding to Val 1β; when bound, each carbamino CO_2 is stabilized by a salt bridge to Lys 82β. In caiman hemoglobin, the bend in the chain forced by Pro 2 inhibits that bridge; in the other two crocodilian hemoglobins, the blocking of Val 1β inhibits binding of CO_2 (Perutz et al. 1981).

The decrease in oxygen affinity brought about by the interaction of crocodilian hemoglobins with bicarbonate ensures that oxygen is released from the blood to the tissues at a relatively high partial pressure of oxygen. If the hemoglobin were insensitive to bicarbonate, the venous Po_2 would be only 7 torr; interaction with bicarbonate raises this to 27 torr, thus creating a large enough pressure head for the flow of oxygen from the blood to the tissues (Jelkmann and Bauer 1980*a*). In human hemoglobin, the same relative rise in Po_2 from 19 to 40 torr can be brought about only by the combined effects of CO_2 and DPG. It is surprising that the crocodilian hemoglobins' simple and direct reciprocating action between oxygen and one of the end products of oxidative metabolism has not been adopted by other vertebrates.

These results show that an entirely new function can evolve in a protein by no more than three amino acid substitutions (Val NA1β → Ser, His NA2β →

Pro, and Lys HC1β ⟶ Glu), requiring only four nucleotide base changes; just two more amino acid substitutions (His H21β ⟶ Ala and Ser H14α ⟶ Ala), or three base changes, are needed for inhibition of the old functions (oxygen-linked phosphate, carbamino CO_2, and Cl^- binding). Most of the other ~100 substitutions that distinguish crocodilian from human hemoglobins are conservative and would have little if any effect on the oxygen equilibrium. There are some significant substitutions in the heme pockets and at the subunit contacts, but none of these is unique or could play any part in the allosteric control by bicarbonate ions. All the residues which are essential for the formation of the characteristic T and R structures are either conserved or have been replaced by ones that can serve the same purpose equally well. A recent paper suggests that the hemoglobins of another class of diving reptiles, the sea turtles, have allosteric properties resembling those of the crocodilians (Isaacks et al. 1982).

Until recently investigators have searched in vain for an intermediate between the primitive dimeric hemoglobins of the lamprey and hagfish and the $\alpha_2\beta_2$ tetramers of the higher vertebrates. Such an intermediate has now been found in the reptile *Sphenodon punctatus* that inhabits islands off the shore of New Zealand and is a survivor from the Triassic period of the ancient order of "beakhead" reptiles. It closely resembles the rhynchocephalians living 200 million yr ago. The reptile's blood and its stripped hemolysate both exhibit hyperbolic oxygen equilibrium curves, the former with a p_{50} = 19 mm Hg and the latter with a p_{50} = 1.8 mm Hg. The alkaline Bohr effect was small: $\Delta p_{50}/\Delta pH = -0.16$. The erythrocytes contain 1.2 mol ATP per mole hemoglobin tetramer, and addition of ATP restored p_{50} of the hydrolysate to 14 mm Hg (Wells et al. 1983).

These properties pose a riddle. Organic phosphates have never been observed to influence the oxygen equilibrium curve of any hemoglobin other than $\alpha_2\beta_2$ tetramers that undergo an allosteric transition, yet *Sphenodon* hemoglobin looks as though it responded to ATP without such a transition, because its reaction with oxygen is devoid of cooperativity. The hemoglobin is tetrameric, but it is not clear yet whether the tetramer is made up of pairs of α- and β-like subunits. Conceivably it consists of two pairs of β-like chains, each pair capable of combining with ATP as in figure 6. The ATP would constrain the chains in their tertiary deoxy structure without being released by an allosteric transition on oxygenation. Further study of the hemoglobin may provide important clues to the evolution of cooperative oxygen binding.

Birds

Bird hemoglobins are functionally similar to mammalian ones but they use P_5-inositol (inositolpentaphosphate) in place of DPG as an allosteric effector. The residues at the phosphate-binding site are the same as in mammals except that position H21β is occupied by Arg rather than His, as in teleost fish. Two further replacements in the internal cavity are H13β Ala ⟶ Arg and H17β Asn ⟶ His (Takei et al. 1975; Oberthür et al. 1980; Oberthür et al. 1981; Braunitzer and Godovac 1982). These two basic residues may be too far removed from the P_5-inositol binding site to bond to its phosphates directly, but they would help to neutralize its negative charges and thus contribute indirectly to the binding energy.

One remarkable instance of adaptation has been reported in the hemoglobins of two related species of geese. The greylag goose (*Anser anser*) lives in the plains, and the oxygen affinity of its blood is normal; the bar-headed goose (*Anser indicus*)

migrates across the Himalayas at an altitude of 9,000 m, and the oxygen affinity of its blood is abnormally high (Petschow et al. 1977). In laboratory experiments the bar-headed goose proved more resistant to hypoxic stress than the Canada goose or the Pekin duck, neither of which flies at such high altitudes (Black and Tenney 1980). It was then found that the oxygen affinities of the purified hemoglobins of the two species differed only slightly: at pH 7.2 in 0.1 M NaCl at 37 C the hemoglobin of the greylag goose has a p_{50} of 5.8 mm Hg and that of the bar-headed goose one of 4.5 mm Hg. Their affinities for P_5-inositol were identical. How was this to be reconciled with the large differences between the oxygen affinities of their bloods? Rollema and Bauer (1979) demonstrated that by lowering the oxygen affinities of both hemoglobins 10-fold, P_5-inositol also amplified the differences between them 10-fold, raising p_{50} to 50 and 37 mm Hg, respectively: this decrease of 13 mm in p_{50} is sufficient to explain the high oxygen affinity of the bar-headed goose's blood.

The amino acid sequences of the two hemoglobins differ by only four substitutions, of which only one is unique among the bird sequences determined so far (Petschow et al. 1977; Oberthür et al. 1981). This is H2α Pro (greylag goose) ⟶ Ala (bar-headed goose). Pro H2α is invariant in nearly all other species, probably because its C_γ forms an important van der Waals contact at the $\alpha_1\beta_1$ boundary: in mammals with Met D6β and in birds with Leu D6β. Studies of abnormal human hemoglobins have shown that the loss of any interatomic contact at subunit boundaries is liable to loosen the constraints of the T structure, thereby lowering L and raising K_T (Fermi and Perutz 1981). The loss of this van der Waals contact in the bar-headed goose hemoglobin may therefore explain its high oxygen affinity.

Mammals

H. F. Bunn has shown that mammalian hemoglobins can be divided broadly into two groups: the great majority have an intrinsically high oxygen affinity, which is lowered in the red cell by DPG, while those of ruminants and cats (Cervidae, Bovidae, Felidae) and of one primate, the lemur, have an intrinsically low oxygen affinity that is little, or not at all, lowered by DPG (Bunn 1971; Bonaventura et al. 1974; Hamilton and Edelstein 1974; Taketa 1974; Scott et al. 1977). Typically, hemoglobins with high intrinsic oxygen affinity have p_{50} values of between 4 and 6 mm Hg, and those with low affinity have p_{50} values of between 10 and 20 mm Hg (measured in stripped hemoglobin solutions in 0.05 M bis-tris, 0.1 M NaCl, pH 6.5–7.5 at 20–25 C). The low affinity is due to lower values of K_T and higher values of L, while K_R remains the same in the two groups of species (Perutz and Imai 1980).

Nearly all the amino acid replacements that distinguish the high and low oxygen affinity hemoglobins are conservative and external, but consistent differences are found in position NA2β, which is occupied by hydrophilic residues, His, Gln, or Asn in hemoglobins with high affinity and by large hydrophobic residues, Leu, Met, or Phe in those with low affinity. What part could residue NA2β play in lowering the oxygen affinity? In the human T structure, DPG forms salt bridges with ValNA1 and HisNA2; these salt bridges pull the two helices A toward the center of the molecule so that they become locked more tightly to neighboring segments of the polypeptide chain (fig. 3). In bovine, sheep, and deer hemoglobins, residue NA1 is missing and residue NA2 is Met, which almost abolishes their affinity for organic phosphates. In these hemoglobins, the hydro-

phobic side chain of the N-terminal Met probably plays the same part in locking helix A tightly in place and thereby stabilizing the T structure as organic phosphates do in human and other hemoglobins with intrinsically high oxygen affinity. In cat hemoglobin this part would be played by Phe, and, in lemur Hb, by LeuNA2β (Perutz and Imai 1980).

Several mammals living at high altitude have bloods with high oxygen affinities. They include the llama, the golden-mantled ground squirrel (*Citellus lateralis*), and the yellow-bellied marmot (*Marmota flaviventris*). For example, at 35 mm Hg CO_2 and 37 C, p_{50} was about 20 mm Hg for the marmot, 30 mm Hg for the squirrel, and 50 mm Hg for a lowland rodent, the rat (Hall et al. 1936; Bartels et al. 1963; Bullard et al. 1966). Amino acid sequences have been reported only for llama hemoglobin (Braunitzer et al. 1977*a*, 1977*b*), which has a low oxygen affinity in the absence of phosphate, overcompensated by a low affinity for DPG, due to the replacement His NA2β ⟶ Asn (Bauer et al. 1980). The side chain of asparagine is 1.3 Å shorter than that of His or Gln. Figure 3 shows that this shortening places the amino groups of the asparagines further from the phospates of DPG than the NH of the histidine or the amino group of a glutamine. In consequence the affinity for DPG is weakened. Asn NA2β occurs also in the hemoglobin of the elephant (Braunitzer et al. 1982), whose blood shows a high oxygen affinity for an animal that lives at low and medium, rather than at high, altitudes (p_{50} = 22.4 mm Hg at 39 C) (Bartels et al. 1963); perhaps this helped the elephants who carried the supplies for Hannibal's army across the Alps in 218 B.C. In fact, there exists a somewhat weak correlation between body size and oxygen affinity of the blood, of which the elephant with p_{50} = 23 mm Hg and the mouse with p_{50} = 46 mm Hg are two extreme examples (Scott et al. 1977).

A similar adaptive mutation is found in human fetal hemoglobin. This has a lower oxygen affinity than the adult in the absence of phosphate, but it also has a lower affinity for DPG, mainly due to the replacement His H21(143)β ⟶ Ser. As a result, the oxygen affinity of fetal blood exceeds that of the adult, which helps the transfer of oxygen from the maternal to the fetal circulation across the placenta (Bunn et al. 1977).

On going to high altitudes, humans show an increased DPG concentration in their erythrocytes, which lowers the oxygen affinity. This used to be regarded as an adaptive response, lower oxygen affinity allowing a larger fraction of the oxygen carried to be discharged in the tissues. Theoretical and experimental studies have shown that this may be true at moderate altitudes (<3,000 m), but at higher altitudes a raised oxygen affinity is more advantageous, for the following reason. Suppose we mark the arterial and the venous oxygen pressure on the oxygen equilibrium curve and connect the two points by a line. Efficiency of oxygen transport will be greatest when the line lies on the steepest slope of the sigmoid curve. At moderate altitudes a shift to lower oxygen affinity was found to move the line to a steeper part of the curve, while at higher altitudes the reverse was true (Turek et al. 1973, 1978).

The mole (*Talpa europaea*) lives in its burrows under hypoxic conditions, to which it is adapted by having a blood with a high oxygen affinity, a high concentration of hemoglobin per unit volume, and a low body temperature (Quilliam et al. 1971). The high oxygen affinity of its blood is reported to be due to a low affinity of its hemoglobin for DPG, but how the amino acid sequence affects this remains unclear (Kleinschmidt et al. 1981).

Species Adaptation in Enzymes

I know of only two instances of adaptation of protein molecules other than hemoglobin that have been interpreted in atomic detail. They are the enzymes ferredoxin and glyceraldehyde phosphate dehydrogenase in thermophile bacteria. In these heat-resistant enzymes an entire range of adaptive mechanisms has been encountered, from 10 amino acid substitutions in the 55-residue chain of ferredoxin to create six more external salt bridges (Perutz and Raidt 1975), to a multitude of replacements in glyceraldehyde phosphate dehydrogenase of *Bacillus stearothermophilus* to produce extra salt bridges and van der Waals contacts (Walker et al. 1980). These enzymes have had many billions more generations to evolve than those of vertebrates, but even so their tertiary and quaternary structures have remained closely similar to those of mesophiles.

New enzymatic activities have evolved in the laboratory in bacteria subjected to selective pressures. For instance, growth of *Klebsiella aerogenes* on xylitol in place of its "natural" substrate ribitol led to a point mutation in the gene for ribitol dehydrogenase that increased that enzyme's affinity for xylitol (Hartley et al. 1976). Clarke has studied the evolution of an aliphatic amidase in *Pseudomonas aeruginosa* (Clarke 1980). The wild-type strain grows well on acetamide, but not on longer chain amides. Under selective pressure a single point mutation that substituted a serine for a phenylalanine in the enzyme's polypeptide chain produced a strain that grew on butyramide. A further single mutation produced a strain that grew on phenylacetamide and had lost the ability to grow on acetamide. Hall (1981) has studied the evolution of a minor β-galactosidase in a strain of *Escherichia coli* from which the gene for the major β-galactosidase had been deleted. He found spontaneous point mutations that altered the specificity of the enzyme, so that it hydrolyzed lactose and other sugars on which the original strain would not grow. The structures of the three enzymes used in these studies are unknown, so that we cannot unravel the stereochemical mechanisms underlying the evolution of new catalytic activities, but the fact that they did evolve by one or two point mutations is consistent with the conclusion from my hemoglobin studies that such evolution can be accomplished by very few amino acid substitutions.

There exists a large literature on enzyme polymorphism and species adaptation (Koehn et al. 1983), but none of it can as yet be interpreted in stereochemical terms. The best-studied species is *Drosophila melanogaster,* where the frequency of the two dominant alleles of alcohol dehydrogenase varies with latitude in several continents; one of these alleles has a consistently lower Michaelis constant for alcohols than the other (McDonald et al. 1980). The two enzymes have been found to differ by the single substitution of a lysine for a threonine at position 192 of the polypeptide chain (Thatcher 1981). Unfortunately the stereochemical meaning of the substitution remains unknown, because there is no sequence homology between the *Drosophila* enzyme and the horse heart enzyme whose structure has been determined (Bränden et al. 1973), but it is noteworthy that here again enzyme adaptation is the result of a single substitution. Little is known as yet about the chemistry of other polymorphic enzymes.

Discussion

We may now reconsider the questions raised in the introduction. Does adaptation involve changes in the tertiary and quaternary structure of hemoglobin? I have no firm answer, because the only structures that have been accurately determined are those of human and horse hemoglobin. Their tertiary and quaternary structures are the same within experimental error, despite 42 amino acid substitutions between them. The number of amino acid substitutions between human and fish hemoglobins is about 140 or half of the total number of amino acids; those between human and caiman number 110. Few of these lie at heme contacts or at contacts between the subunits. Comparison of the amino acid sequences of human and carp hemoglobins shows that of the residues in contact with the hemes in human hemoglobin listed by Fermi and Perutz (1981), 16 out of 20 are identical in the α-chains and 14 out of 20 in the β-chains. At the $\alpha_1\beta_2$ contact, which forms the switch between the deoxy and oxy structures, 27 out of 32 residues listed by Fermi and Perutz (1981) are identical in the two species. These homologous residues would be stereochemical misfits unless the tertiary and quaternary structures superposed with a standard deviation of less than 1 Å. I am confident that X-ray analysis will bear this out. Additions and deletions have occurred in nonhelical segments: for instance, in the Port Jackson shark, which is regarded as a living fossil, the α-chain contains 144 residues instead of the 142 in other fish, and the β-chain contains only 141 instead of the 147 in other fish (Nash et al. 1976; Fisher et al. 1977). The additional two residues in the α-chain are at the amino end and can be tucked away in the internal cavity without change of structure. The deletions in the β-chain occur in the CD segment, which is nonhelical and external; they require only small local adjustments in tertiary structure (fig. 1).

My proposal that the tertiary and quaternary structure of the hemoglobins has been conserved throughout evolution from fish to mammals has met with disbelief among biologists and others, but it comes as no surprise to protein crystallographers, who have found that homologous proteins in distant species have closely similar structures. A few examples are glyceraldehyde phosphate dehydrogenase from *Bacillus stearothermophilus* (Biesecker et al. 1977) and lobster (Buehner et al. 1974), phosphoglycerate kinase from yeast (Bryant et al. 1974) and horse (Banks et al. 1979), bovine and fungal catalase (Murphy et al. 1981; Vainshtein et al. 1981), human and hen lysozyme (Artymiuk and Blake 1981; Mioto et al. 1972), and cytochromes c (Dickerson 1972).

The tertiary structures of vertebrate myoglobins, of the monomeric lamprey hemoglobin, and of invertebrate and leguminous plant hemoglobins all resemble those of the α- and β-chains of vertebrate hemoglobin to the extent necessary to preserve the vitally important geometry of the heme pocket, but they vary in detail. The angles between helical segments differ by up to 30° and the points of contact between them by up to 7 Å. Many different combinations of side chains are found to produce helix interfaces that are comparably well packed, as if the tertiary structure had been conserved by a patchwork of improvisations (Lesk and Chothia 1980). In fact, the only invariant residues common to all globins are the proximal histidine F8 and phenylalanine CD1 which wedges the heme into its pocket. What maintains the tertiary structure is a set of sites that are invariably occupied by nonpolar residues (Perutz et al. 1965).

Has adaptation been brought about by the gradual accumulation of minor mutations, each producing a small shift in chemical affinity, or by a few amino acid substitutions in key positions? In the instances analyzed so far, new chemical functions appear to have evolved by only a few amino acid substitutions in key positions. Consider the evolution from cartilaginous fish to bony fish. The hemoglobin of the Port Jackson shark has only a weak alkaline Bohr effect and has Ala in position F9. It seems that the Root effect in teleost fish has evolved primarily by the substitution of this Ala by Ser. In the crocodilians, affinity for bicarbonate evolved by no more than three amino acid substitutions, requiring only four nucleotide base changes; just two more amino acid substitutions, or three base changes, were needed for inhibition of the affinity for the usual heterotropic ligands (carbamino CO_2, organic phosphate, and Cl^-). Five amino acid substitutions probably suffice to inhibit in trout I hemoglobin all the heterotropic interactions exhibited by trout IV hemoglobin. Conversion from an ATP to a DPG binding site needs only one amino acid substitution or one base change; modulation of the affinity for DPG in the human fetus, the llama, and the elephant also needs only a single base change.

In amphibia that do not need a Root effect, the alkaline Bohr effect is weakened by a variety of single substitutions: Ser F9 $\longrightarrow$ Ala in the tadpoles of *Xenopus* and *Rana catesbeiana*, Glu FG1β $\longrightarrow$ Asn or Gly in adult frogs and in one of the tadpoles, His HC3 $\longrightarrow$ Phe in another tadpole. It looks as though once a function is no longer needed or has become undesirable, evolution would permit any mutation that inhibits the function to become fixed (Kimura 1983).

What selective advantage does possession of multiple hemoglobin genes provide? In fish that possess two types of hemoglobin, one responsive and the other unresponsive to heterotropic ligands, such multiplicity offers a clear advantage. One of the hemoglobins assures the supply of oxygen to the swim bladder and the eye, while the other assures the fish of a continued supply of oxygen when low pH inhibits the uptake of oxygen by the first hemoglobin (Brunori 1975). The oxygen affinities of mammalian fetal blood are higher than those of the corresponding maternal blood, thus ensuring oxygen transfer across the placenta. In primates and ruminants this need is met by fetal hemoglobins that have higher oxygen affinities than the adult ones, but other species, among them the horse, manage with the same hemoglobin in both fetus and adult (Stockell et al. 1961). Studies of dogs, rats, and rabbits suggest that in species lacking fetal hemoglobins the oxygen affinity of the fetal blood is raised relative to that of the adult by a lower concentration of 2,3-DPG. This is achieved by an increased activity of pyruvate kinase, which helps to metabolize 1,3-DPG, the precursor of 2,3-DPG (Jelkmann and Bauer 1978; Jelkmann and Bauer 1980*b*; Mueggler and Black 1982).

Human adults possess two α- and two β-globin genes. The two α-chain genes code for identical amino acid sequences and are both efficiently expressed, so that humans have the advantage of being effectively tetraploid for α-globin. This benefits individuals born with a deletion or malfunction of one of the α-chain genes (e.g., α-thalassemia); like sickle cell heterozygotes such individuals are healthy and also have a better chance of surviving malaria than individuals with wild-type hemoglobin genes. The two polymorphic adult β-chains differ at 10 positions and are functionally indistinguishable, but one of them, known as the δ-chain, is made in only 1/40 of the quantity of the other, so that possession of the δ gene confers no significant advantage to either β-thalassemia or sickle cell

anemia patients. It may be a "fossil" of some past stage of evolution. Many vertebrates have multiple α and β genes, some coding for functionally equivalent and others for functionally different genes. It is not clear whether their possession offers any selective advantage or whether they have merely survived from selectively neutral gene duplications. H. F. Bunn has suggested to me that the presence of multiple hemoglobins might allow a higher total hemoglobin concentration to be maintained in the red cell than if there were only a single one. On the whole, the evidence supports Kimura's view that "in many cases polymorphism has no visible phenotypic effect and no obvious correlation with environmental conditions" (Kimura 1979).

How many of the amino acid substitutions between the hemoglobins of different species are functionally significant? The great majority of amino acid differences between hemoglobins of different species consist of conservative or semiconservative replacements of external polar and nonpolar residues and internal nonpolar ones. Comparison of normal and abnormal human hemoglobins, or of the hemoglobins of closely related species, shows that such replacements have little, if any, influence on the functional properties of hemoglobin. For example, human and horse hemoglobins differ in sequence at 42 out of 287 positions, yet their functional properties are the same. Only a minority of the 400 or so different amino acid replacements found in abnormal hemoglobins affect any of the hemoglobin functions. It could be argued that even an amino acid replacement that produces only a very small shift in the oxygen equilibrium curve may give an animal a selective advantage that would prove decisive over thousands of generations; against this it could be held that homeostatic mechanisms allow organisms to compensate efficiently for quite large shifts in the curve. Studies of abnormal hemoglobins have shown mutations that merely change surface charges to be without significant effect on the respiratory functions of hemoglobins, but recent work has revealed that such mutations may influence the rate of assembly of the α- and β-subunits into $\alpha_2\beta_2$ tetramers by electrostatic effects arising from their different isoelectric points ($\alpha^A = 8.0$; $\beta^A = 6.5$). At physiological pH free α-subunits exist as monomers and dimers carrying a net positive charge, while free β-chains exist as tetramers, dimers, and monomers carrying a net negative charge. The effect of these charges on the assembly is illustrated by the following example. The sickle cell mutation Glu A4(6)β $\longrightarrow$ Val reduces the negative charge on the β-subunits, thus reducing electrostatic repulsion in the β_4^S-tetramer and electrostatic attraction between α^A- and β^S-subunits. In consequence both the dissociation of β_4^S-tetramers and the association of β^S- and α^A-subunits is slower than the dissociation of β_4^A-tetramers and the association of β^A- and α^A-subunits. That difference in rates appears to account for the ratio of hemoglobin A to S in sickle cell heterozygotes being about 3:2 rather than 1:1. The higher ratio of hemoglobin A contributes to the fitness of the heterozygotes. It seems important, therefore, that the net charges of the subunits in an oligmeric protein be maintained (Bunn and McDonald 1984). With that proviso, the structural evidence suggests that most of the amino acid replacements between species are neutral or nearly so, caused by random drift of selectively equivalent mutant genes, and that adaptive mechanisms generally operate by a few replacements in key positions. Here again the evidence supports Kimura's view that "adaptive mutations are much

less frequent than selectively neutral or nearly neutral substitutions caused by random drift" (Kimura 1983).

R. Lewontin has pointed out to me that a mutation adapting a species to a new environment is likely to have preceded occupation of that environment. For example, a mutation that raised the oxygen affinity of the llama's blood would have occurred before llamas discovered that they were able to graze at altitudes barred to competing species. W. Bodmer suggested that once a large change in chemical affinities produced by one mutation had enabled a species to occupy a new environment, its effect might have been refined by later adaptive mutations, each contributing minor shifts, over a long period of time. The structure of glyceraldehyde phosphate dehydrogenase from *B. stearothermophilus* (Walker et al. 1980), referred to earlier, suggests that the heat stability of this enzyme may have evolved by such a process. This might be the molecular equivalent of punctuated equilibria in the evolution of species.

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