Crystal Structures of Deoxy- and Carbonmonoxyhemoglobin F1 from the Hagfish Eptatretus burgeri*

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Hagfish are extremely primitive jawless fish of disputed ancestry. Although generally classed with lampreys as cyclostomes (“round mouths”), it is clear that they diverged from them several hundred million years ago. The crystal structures of the deoxy and CO forms of hemoglobin from a hagfish (Eptatretus burgeri) have been solved at 1.6 and 2.1 Å, respectively. The deoxy crystal contains one dimer and two monomers in a unit cell, with the dimer being similar to that found in lamprey deoxy-Hb, but with a larger interface and different relative orientation of the partner chains. Ile(E11) and Gln(E7) obstruct ligand binding in the deoxy form and make room for ligands in the CO form, but no interaction path between the two hemoglobin subunits could be identified. The BGH core structure, which forms the αβ, interface of all vertebrate αβ, tetrameric Hbs, is conserved in hagfish and lamprey Hbs. It was shown previously that human and cartilaginous fish Hbs have independently evolved stereochemical mechanisms other than the movement of the proximal histidine to regulate ligand binding at the hemes. Our results therefore suggest that the formation of the αβ, tetramer using the BGH core and the mechanism of quaternary structure change evolved between the branching points of hagfish and lampreys from other vertebrates.

In human hemoglobin, the interactions between a ligand bound to a heme and the globin structure can be divided into proximal effects (interactions with the proximal histidine imidazole group and the heme upon ligand binding) and distal effects (interactions with the distal amino acids). The proximal effect moves the F helix and FG corner upon ligand binding and connects the heme to the structure change at the αβ, interface associated with the quaternary structure change. The distal effect is more important in the β subunit than in the α subunit (1). When the x-ray structures of two cartilaginous fish Hbs were solved (2, 3), the quaternary structure and its change upon ligand binding were preserved, as expected. The proximal effect linking the quaternary structure change to the hemes was also preserved, but the distal effect, especially the role of Val(E11), was altered. The Bohr proton sites and the 2,3-diphosphoglycerate-binding site were not conserved, indicating that stereochemical mechanisms other than the proximal effect have evolved independently in the different species.

Hbs from hagfish and lampreys are not of the αβ, tetramer type. The evolution of the tetramer, including the duplication and differentiation of the globin gene into the α and β genes, the formation of the subunit interfaces, and the mechanism of the quaternary structure change, took place within a comparatively short period between the branching points of hagfish and lampreys from cartilaginous fish. Some primordial globin structures may well have appeared before tetrameric hemoglobin that facilitated its development, and these might be found in modern hagfish and lamprey Hbs. In the case of lamprey Hbs, the dissociation of ligands promotes oligomerization of the protein. On the basis of a monomer structure of the cyanide-bound ferric form, Honzatko and Hendrickson (4) suggested that the subunit interfaces of the deoxy-Hb oligomer were the same as the α,β, interface of the αβ, tetramer. Recently, however, Heaslet and Royer (5) solved the x-ray structure of lamprey deoxy-Hb and found a dimer with an interface between the E helix and AB corner, totally different from any of the interfaces in the αβ, tetramer.

The evolutionary history of hagfish is a much disputed topic. Some sequence comparisons support the view that hagfish and lampreys belong in a single group, the cyclostomes, separate from jawed vertebrates (6, 7). Other morphological and sequence analyses consider hagfish and lampreys to be phylogenetically distant groups and do not class them together (8, 9). Hagfish are distinguished by their lack of bone or any trace of vertebrae, but other anatomical features support grouping them with lampreys. Whether or not the 45 living species of hagfish form a distinct monophyletic group (Myxiniformes) separate from all other craniates, it is clear that they diverged from lampreys (Petromyzontiformes) around 500 million years ago. Grouping hagfish and lampreys together has been supported by molecular studies of globin (10), but the similarities in these sequences may reflect a primitive form present before hagfish diverged (11).

Despite the apparent gulf of time separating them, Hbs from hagfish and lampreys show similar linkage relations between oligomerization and ligand binding (12, 13). According to Ban-nai et al. (14), Eptatretus burgeri has four Hb components designated F1, F2, F3, and F4. We have purified F1 and solved the x-ray structures of the CO and deoxy forms at 2.1 and 1.6 Å, respectively. In this study, we have analyzed the evolutionary changes that occurred in Hb around the branching point of...
The first eluted peak was F1, the second was F2 (with a slight contamination). There were no difficulties in modeling this region. Isolated electron density map of the main chain is maintained at 1.2σ. A 50% electron density map and a 1.3σ solvent flattening map were used to model the few remaining unassigned density regions. The electron density map was interpreted in the absence of the two residues, and some regions of the model were rebuilt at this stage. The electron density map was used to adjust the relative positions of the subunits. The final model contains a large number of conserved and unique residues. The electron density map was used to adjust the relative positions of the subunits. The electron density map was used to adjust the relative positions of the subunits.

### Table I

| Crystal data | Crystal data |
|--------------|--------------|
| Space group | C2 |
| Unit cell parameters | \(a = 104.02, b = 62.26, c = 66.53 \text{ Å}; \alpha = 90^\circ, \beta = 107.40^\circ, \gamma = 90^\circ\) |
| Observed reflections | 221,709 |
| Independent reflections | 52,908 |
| Completeness (%) | 99.2 |
| \(R_{merge} (%)\) | 5.0 |
| Refinement parameters | |
| Asymmetric unit | Two molecules |
| Resolution (Å) | 20 to 1.6 |
| \(R_{free} (%)\) | 23.6 |
| \(R_{merge} (%)\) | 20.6 |
| Deviation from ideality | 0.008 |
| Bonds (Å) | 1.1 |
| Angles | 93.3 |
| Most favored | 90.1 |
| Additional allowed | 6.3 |
| Generously allowed | 0 |
| Disallowed | 0.4 |
| Ramachandran plot (%) | 1.1 |

### Experimental Procedures

**Protein Purification—**Hagfish (*E. burgeri*) were captured in the sea near the Miura Peninsula in Japan. The hagfish studied by Bannai et al. (14) were captured in the same area. Blood was collected and lyophilized as described by Chong et al. (2). The hemolysate was equilibrated with 2 M Tris-Cl (pH 8.5) and then dialyzed against Amberlite MIF-3. After dialysis against 5 mM Tris-Cl (pH 8.5), the hemolysate was fractionated on a Whatman DE32-cellulose column equilibrated with the same buffer as described (14), there are four Hb components in this hagfish, designated F1, F2, F3, and F4. We have used the same nomenclature in other studies.

**Oxygen Equilibrium Measurement—**Oxygen equilibrium curves were measured as described by Imai et al. (15). Measurement conditions were 60 μM protein in 50 mM bis-Tris (pH 6.5) and 50 mM Tris buffer containing 100 mM chloride at 25 °C. The pH value was adjusted with concentrated NaOH. To minimize the autoxidation of hemoglobin during measurements, catalase and superoxide dismutase were added to each sample (16, 17). Deoxogenation curves were used to determine the \(p_{50}\) (partial pressure of oxygen at half-saturation) and Hill coefficient \(n_{H}\), the maximum slope of Hill plots of oxygen equilibrium curves.

**Analytical Gel Chromatography—**A 230 × 8-mm Superdex 75 column (Amersham Biosciences) was employed for analytical gel chromatography with 100 mM bis-Tris-Cl (pH 6.5) at 25 °C, the same pH as that used for crystallization. In the case of deoxy-Hb, the same column was used with 1 mM Na, 0.3 M NaCl, 21% polyethylene glycol 4000, 10% glycerol, 50 mM bis-Tris-Cl (pH 6.5), 20 μM 2-mercaptoethanol, 100 mM NaCl, and 1.5% protein. Data were collected from a cryo-cooled crystal at RIKEN beam line-2 (BL44B2) of the SPring8 Synchrotron in Harima, Japan (18), using a MARCCD detector. The data processing was performed with MOSFLM (19) and scaled with SCALA (20). The CO crystals were obtained with 21% polyethylene glycol 4000, 10% glycerol, 50 mM bis-Tris-Cl (pH 6.5), 20 μM 2-mercaptoethanol, 10 mM NaCl, and 1.5% protein. Data were collected at 0.3 M NaCl, 21% polyethylene glycol 4000, 10% glycerol, 50 mM bis-Tris-Cl (pH 6.5), 20 μM 2-mercaptoethanol, 100 mM NaCl, and 1.5% protein. Data were collected at 1.5 M NaCl, 21% polyethylene glycol 4000, 10% glycerol, 50 mM bis-Tris-Cl (pH 6.5), 20 μM 2-mercaptoethanol, 100 mM NaCl, and 1.5% protein.

**Structure Determination and Refinement—**Both deoxy and CO crystals were grown at 27 °C by batch crystallization under an atmosphere of nitrogen or carbon monoxide, respectively, using screw-top vials. Deoxy-Hb crystals were obtained with 19% polyethylene glycol 4000, 10% glycerol, 50 mM bis-Tris-Cl (pH 6.5), 20 μM 2-mercaptoethanol, 100 mM NaCl, and 1.5% protein. Data were collected from a cryo-cooled crystal at RIKEN beam line-2 (BL44B2) of the SPring8 Synchrotron in Harima, Japan (18), using a MARCCD detector. The data processing was performed with MOSFLM (19) and scaled with SCALA (20). The CO crystals were obtained with 21% polyethylene glycol 4000, 10% glycerol, 50 mM bis-Tris-Cl (pH 6.5), 20 μM 2-mercaptoethanol, 100 mM NaCl, and 1.5% protein. Data were collected at 1.5 M NaCl, 21% polyethylene glycol 4000, 10% glycerol, 50 mM bis-Tris-Cl (pH 6.5), 20 μM 2-mercaptoethanol, 100 mM NaCl, and 1.5% protein.

**Data collection and structure refinement statistics**

| Deoxy-Hb | HbCO |
|----------|-------|
| \(R_{merge} = \frac{\sum_h(i(h)) - (i(h))}{\sum_h(i(h))}\), where \(i(h)\) is the intensity value of the \(i\)th measurement of \(h\) and \(\langle i(h) \rangle\) is the corresponding mean value of \(i(h)\) for all \(i\) measurements; the summation is over the reflections with \(I(h)>1.0\). \(R_{merge}\) factor calculated for 5% of reflections that were randomly selected and were excluded from the refinement. \(R_{factor} = \frac{\sum_h(F_o - |F_c|)^2}{\sum_h(F_o)^2}\), where \(F_o\) is the observed structure factor and \(F_c\) is that calculated from the model. | |
 modeled as water molecules if the locations were sterically reasonable. Crystallographic and refinement data are shown in Table I.

Comparison of Hb Structures—The coordinates of different Hbs were obtained from the Protein Data Bank: human deoxy-Hb A, code 1bz0; human deoxy-Hb β, code 1bch; human HbCO β, code 1bch; shark deoxy-Hb, code 1a1y; shark HbCO, code 1agc; sperm whale (Physeter catodon) deoxymyoglobin, code 1bqx; sperm whale MbCO, code 1bzx; sea lamprey (Petromyzon marinus) deoxy-Hb, code 3hbi; sea lamprey ferricyan-Hb, code 2hbg; and marine blood worm (Glycera dibranchiata) deoxy-Hb, code 2hbg; and marine blood worm HbCO, code 2hbg. The high-resolution model of human HbCO A has been refined by Park et al.3

The buried surface area between intermolecular interfaces (half the difference in solvent accessibility between the independent and assembled partner molecules) was calculated using AREAIMOL, part of the CCP4 suite (20). To compare Hbs, the coordinates were superimposed either on the heme or on appropriate parts of helices. The heme frame contains 25 atoms: 4 nitrogen and 20 carbon atoms of the porphyrin ring and 4 methyl carbons. To compare human and hagfish Hbs, we defined a helix frame consisting of the central regions of the helices, A5–A15, B8–B16, C1–C7, D1, E7–E18, F1–F9, G4–G14, and H8–H17 in human α- and β-globins. F1–F9 and H8–H17 in human Hb correspond to F7–F15 and H1–H10 in hagfish and lamprey Hbs (see Table II), respectively. The BGH frame was originally defined by Baldwin and Chothia (25) as the conserved structural core of human Hb at the αβ interface. For Glycera Hb and sperm whale Hb, the sequences were aligned according to Lesk and Chothia (26), and for blood clam, according to Royer et al. (27).

RESULTS

Overall Structure—Although according to Bannai et al. (14) E. burgeri Hb has four components, we were able to identify only three cDNAs, including F1. We have determined the sequence of F1, which is consistent with the crystal structure. It has 146 amino acid residues, 2 less than Hb III from another hagfish, Myxine glutinosa (Table II). The sequence identity to M. glutinosa Hb III is 61%; that to the α and β chains of human Hb is ~20%; and that to lamprey (P. marinus) Hb V is ~40%. The EF corner and G and H helices are shorter than their counterparts in human Hb by 2, 3, and 7 residues, respectively; the F helix is longer by 6 residues, starting earlier in the sequence. In common with lamprey Hb V (28), hagfish Hb has a long N-terminal tail (NA region) with 11 residues attached to the A helix and a 10-residue deletion from the tail of the G helix to the start of the H helix. The NA region is only 2 or 3 residues long in all other vertebrate Hbs. Sea cucumber (Caudina arenicola) and blood clam (S. inaequivalvis) Hbs also have long N-terminal tails, but they are dissimilar to hagfish and lamprey Hbs in both primary and tertiary structure (29, 30). F1 has only a short D helix (residues 61–63 inclusive), whereas lamprey Hbs have a D helix with 7 residues (28).

The notable mutations among F1 heme contact residues are Gln71(E7), Ile75(E11), and Phe107(FG3), which replace well conserved His, Val, and Leu residues, respectively, among vertebrate αβ-type Hbs. In M. glutinosa Hb III, E7 is also Gln; but in lampreys, it is His. Ile15(E11) and Phe205(FG3) are common to all known sequences of lamprey and hagfish Hbs (13).

Properties in Solution—The average molecular mass of F1 was found by analytical gel filtration to be 19 kDa in the CO form and 23 kDa in the deoxy form (data not shown). The oxygen equilibrium characteristics are presented in Table III. The hagfish hemolysate showed lower oxygen affinity than the human Hb hemolysate, and purified F1 showed lower oxygen affinity still. Although for the hemolysate the apparent Hill coefficient (nH) is 1.0, for F1, the maximum nH is ~1.3 at pH 7.4, which shows that a small but significant cooperativity is present in this Hb. The Bohr effect is weak. Bannai et al. (14)

| Table II: Sequence alignment of F1 with hemoglobins from the hagfish M. glutinosa (Hbs I, II, and III) and lamprey P. marinus (Hb V) and human α- and β-globins |
|---|
| F1 | II | III | V | H | α | β |
| M. glutinosa | 92.0 | 92.0 | 92.0 | 92.0 | 92.0 | 92.0 |
| P. marinus | 92.0 | 92.0 | 92.0 | 92.0 | 92.0 | 92.0 |
| Human | 92.0 | 92.0 | 92.0 | 92.0 | 92.0 | 92.0 |

| Table III: Oxygen equilibrium characteristics of E. burgeri Hb | pH | 50 | H (max) |
|---|---|---|---|
| F1 | 7.4 | 28.96 | 1.3 |
| 8.5 | 23.23 | 1.1 |
| 8.5 | 8.85 | 1.0 |
| Hemolysate | 6.5 | 11.43 | 1.2 |
| 7.4 | 8.93 | 1.0 |
| 8.5 | 8.85 | 1.0 |

reported oxygen equilibrium properties of the same Hb under different conditions (neutral pH in 0.1 m phosphate buffer at 22 °C); and therefore, it is not possible to compare their results with ours directly, but they also showed the Bohr effect to be comparatively small and that F1 had lower oxygen affinity than the hemolysate. The oxygen affinity of M. glutinosa Hb seems to be a little higher (12).

Crystal Packing.—The overall structure of hagfish hemoglobin F1 is very similar to that of lamprey Hb V. In the deoxy crystal, the asymmetric unit consists of two F1 monomers. One of them, designated d1, forms a large contact (855 Å2) with a symmetry-related partner in the unit cell (d1’); and the other one, designated d2, has a smaller contact (330 Å2) with d1 using a different patch of the surface. d2 has only minor contacts with its partner in the same unit cell (Fig. 1). No compact tetramers are formed; instead, the unit cell apparently consists of a dimer and two monomers of F1. F1 does not strongly

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self-associate in solution (14), in common with Hbs from the _M. glutinosa_ hagfish (12).

In the CO form, the asymmetric unit of the crystal consists of four F1 monomers, designated c1–c4 (Fig. 2). In this crystal form, there are no pairs of closely fitting monomers related to each other by 2-fold symmetry, but the contact surface areas between monomer molecules are comparatively large (31). The largest is that between c3 of one asymmetric unit and c2 of another (c2–c3) with a contact area of 494 Å². Hb forms a variety of crystal types containing 6–12 monomers in an asymmetric unit. In the case of lamprey deoxy-Hb V, the asymmetric unit contains six dimers arranged around two approximate 3-fold screw axes into hexamers (5), so the crystal packing of both lamprey and hagfish Hbs is rather unusual.

There are relatively large differences between d1 and d2 in the deoxy form and between c1, c2, c3, and c4 in the CO form compared with the differences between crystallographically nonequivalent α or β subunits in human Hb A (32). Comparing monomers in the CO form, the r.m.s. deviation of all the main chain atoms varies from 0.25 Å (c1–c4) to 0.42 Å (c1–c3). The main chain r.m.s. deviation between d1 and d2 in the deoxy form is 0.54 Å. Most of these differences probably arise from the large number of non-crystallographic contacts with neighboring molecules. In this study, c1 and d1 have been used as canonical structures to draw figures and to make tables.

Comparison between the Deoxy-Hb Dimers of Lampreys and Hagfish—Hagfish deoxy-Hb F1 and lamprey deoxy-Hb V form dimers using a similar interface, which includes the AB corner and the N terminus of the E helix. In the hagfish dimer, the distance between the two F helices is shorter than found in lamprey Hb (Fig. 3). The distance relates the partner chains of the hagfish dimer. From the structure, the hagfish dimer seems rather more stable than the lamprey dimer. However, cooperativity of oxygen binding will obviously depend on the change in oxygen affinity between the monomer and dimer forms as well as the stability of the dimer in the absence of heme ligands.

The self-association of different Hb components from _M. glutinosa_ has been studied using analytical ultracentrifugation by Fago et al. (33). These experiments showed that Hbs I and III do not self-associate, but that Hb IIA forms dimers with itself, with Hb I, and with Hb III. Both Hbs I and IIA from _M. glutinosa_ have Trp at E6, and both also lack Lys(F5) and Asp(F9). Hb III has leucine at E6 and alanine at E3. Because Hb IIA has histidine (instead of tyrosine) at A16, Asp (instead of Glu) at E5, and His (instead of Asn) at E13 and has Trp at the key E6 position, it seems unlikely that any of the dimers formed among the _M. glutinosa_ Hbs will resemble the F1 dimer.

Both sea cucumber and blood clam Hbs also have a dimer interface using the E and F helices (29, 30). Although the contact region overlaps that found in hagfish Hb, the direction of the 2-fold symmetry axis is very different. The evolutionary advantage of a dimer interface that permits ready communication between hemes has resulted in more than one dimer form associated through the E and F helices that can sense the presence of ligand at the heme (34).

Tertiary Structure Comparison with Vertebrate Globins—Comparison of vertebrate deoxyglobins including F1 shows that the similarity (in terms of the r.m.s. deviation by residue)
between the hagfish and lamprey Hbs is clearly greater than that between human $\alpha$- and $\beta$-globins, although the level of amino acid sequence identity is about the same for both pairs ($\sim 40\%$). The $\alpha_1\beta_1$ subunit interface of vertebrate tetrameric Hb is formed by the B, G, and H helices, which are conserved among vertebrate Hbs (2, 3, 35, 36) and which form a relatively
rigid core that moves little upon ligand binding (25). Table IV
presents the r.m.s. deviations of all the helix core residues that
indicate that the BGH core structure is relatively fixed among
different deoxyglobins, including hagfish Hb. Deoxy-Hbs were
used in Table IV because better structures are available for
them, but similar results were obtained when CO forms were
compared. It can be concluded that the BGH core structure
evolved before the branching point of the hagfishes, before it
began to serve as a subunit interface of the
\[ \text{H9251} \]
\[ \text{H9252} \]
\[ \text{H2} \]
tetramer.

Table IV

| Protein       | Hb Aα | Hb Aβ | Shark Hb a | Shark Hb b | Hagfish Hb | Mb | Clam Hb | Glycera Hb |
|---------------|-------|-------|------------|------------|------------|----|---------|------------|
| Hb Aα         | 0.5   | 0.4   | 0.6        | 0.7        | 0.8        | 1.4| 1.4     | 0.7        |
| Hb Aβ         | 1.1   | 0.5   | 0.6        | 0.6        | 0.9        | 1.5| 1.5     | 1.0        |
| Shark Hb a    | 1.0   | 1.1   | 0.5        | 0.6        | 0.6        | 1.3| 1.3     | 0.8        |
| Shark Hb b    | 1.1   | 1.1   | 1.1        | 0.6        | 0.6        | 1.4| 1.1     | 0.9        |
| Hagfish Hb    | 1.4   | 0.9   | 1.4        | 1.4        | 1.7        | 1.5| 1.5     | 1.0        |
| Mb            | 1.0   | 0.9   | 1.0        | 1.4        | 1.9        | 1.5| 1.2     | 1.0        |
| Clam Hb       | 1.4   | 1.5   | 2.0        | 1.6        | 1.1        | 1.5| 1.3     | 1.6        |
| Glycera Hb    | 1.2   | 1.3   | 1.2        | 1.3        | 1.3        | 1.6| 1.6     | 1.6        |

Four key parameters describing the link between ligand
binding and tertiary structure change are given in Table V.
Myoglobin is included because its branching point is near that
of hagfish and lamprey Hbs (10). On the distal side, the
positions and movements of His(E7) and Val(E11) (Gln and Ile,
respectively, in hagfish) are rather variable among different
animals, more so for E11, but with no apparent pattern. This

Structure Changes at the Heme upon Ligand Binding—Fig. 5
shows a stereo view of the heme region of the deoxy and CO
forms of hagfish Hb superposed by least-squares fitting the
helical core residues. E11 is Ile rather than Val in all known
sequences of hagfish and lamprey Hbs (13) and may be one of
the reasons for their low oxygen affinity (Table III). E7 is His in
most vertebrate Hbs, including lamprey Hb. Both Ile(E11) and
His(E7) occupy positions obstructing CO binding in the deoxy
form and move away in the CO form by a concerted tilting of
the heme and displacement of the residues, just as for the β
subunit of human Hb (1). The movements of residues on the
proximal side are relatively small.

Four key parameters describing the link between ligand
binding and tertiary structure change are given in Table V.
Fe–Pn, the distance between iron and the center of the four porphyrin nitrogen atoms. This parameter represents the heme doming upon ligand binding. F8(C–δ-N3)–[C– N-1], the difference of two distances representing a tilting of the imidazole of the proximal His. One is between C–δ of the imidazole of the proximal His (F8 in human Hb and shark Hb and Mb and F14 in hagfish Hb) and nitrogen of pyrole 3 of porphyrin, and the other is between C–δ of the imidazole of the proximal His and nitrogen of pyrole 1. This tilting links the shift of the F helix toward the P corner upon ligand binding. Val(E11) C–H9253 O(CO), the distance between Val(E11) C–H9253 O(CO), and the oxygen atom of the ligand CO. For the deoxy form, the ligand has been inserted into the model by overlapping the CO form of the same protein on the heme atoms. His(E7) N–H9255 O(CO), the distance between His(E7) N–H9255 O(CO), and the oxygen atom of the ligand CO, treating the deoxy form as for Val(E11) C–H9253 O(CO).

The electron density maps show that the distal histidine appears to be highly flexible and cannot be modeled in a single conformation, unlike the clear density seen for this residue in the deoxy and ligated monomer structures. The changes seen by Heaslet and Royer (5, 39) show that ligand affinity is controlled by distal effects, principally movements of the E helix and distal histidine, just as in hagfish Hb F1.

**Cooperative Oxygen Binding in Cyclostome Hbs**—F1 shows small but significant cooperativity in oxygen binding. Like human hemoglobin, deoxy-Hb F1 crystals crack when exposed to the air, indicating that some intermolecular interactions (not necessarily the interaction between subunits of the dimer) are oxygen-linked. Because the distal Ile(E11) and Gln(E7) obstruct oxygen binding in the deoxy form, then if the dimer is more stable in the deoxy form than in the oxy form, as suggested by the crystal packing described in this study, then oxygen binding must be cooperative.

In lamprey Hb V, dimer formation displaces the E helix, pushing the distal His closer to the ligand-binding site and reducing the oxygen affinity of the deoxy conformation (5). The most marked conformational change takes place at Trp72(E6). As discussed earlier, this residue is mutated to His in hagfish Hb, but similar distal residue movements take place upon ligand binding in both proteins (Fig. 5). Overall, the deviations of the proximal side chains are smaller than those of distal residues, and there is no apparent connection between the movement of the proximal His and the hydrogen bonds between Lys(F5) and Asp(F9) at the deoxy dimer interface. The only apparent link between heme ligation and dimerization involves a heme propionate. Ligation flattens the heme, pressing against Phe107 on the proximal side and moving it -0.5 Å across the face of the proximal histidine side chain. In turn, the benzene ring pushes against Lys102, breaking the salt bridge that this residue forms with the heme propionate in the deoxy form.
form. This group lies within 4 Å of Glu^81 on the partner chain of the dimer, so breaking the bond with Lys^102 destabilizes the dimer in the liganded form. The 2F_o - F_r electron density map of the heme region of the molecule in the deoxy form is shown in Fig. 6.

The lamprey deoxy-Hb dimer has a comparatively small subunit contact area of 478 Å^2 (5), which may not completely explain the linkage properties of subunit assembly and ligand binding (40) because oligomers larger than dimers have been observed (41, 42). In the case of hagfish deoxy hemolysate, Bannai et al. (14) found Hb with a sedimentation coefficient corresponding to tetramers. Moreover, they showed by ultracentrifugation that a 1:1 mixture of F3 and F4 was tetrameric. More studies of cyclostome Hbs under conditions that stabilize higher molecular mass species are necessary to characterize cooperative oxygen binding in these Hbs.

**Heterotropic Effects**—Lamprey and hagfish Hbs show markedly different heterotropic effects, with the former lacking regulation by organic phosphates and the latter showing a very weak Bohr effect. *M. glutinosa* hemolysate shows a slightly greater Bohr effect in the absence of organic phosphates and chloride, both of which favor dissociation (12, 13). In contrast, lamprey Hb has a Bohr effect as strong as that found in Root effect Hbs of teleost fish (43); mutating Glu^75 to Gln cuts this Bohr effect in half (44). Glu^75 lies very close to its symmetry mate and Glu^45 in the lamprey deoxy-Hb dimer. Because hagfish Hb F1 has a valine residue at the equivalent position, it is expected that its Bohr effect will be small.

The oxygen affinity of hagfish (*M. glutinosa*) hemolysate is strongly lowered by bicarbonate ions (45), an unusual property that is shared with crocodile Hbs (46). The structure of F1 does not suggest an obvious binding site, although this may be because other hagfish Hb components are responsible for the effect.

**DISCUSSION**

As noted by Fago and Weber (13), the two main differences between the red cell of hagfish and higher vertebrates are (i) the inability of the Hbs to form stable tetramers and (ii) the lack of an active anion exchanger in the red cell membrane. This leads to high concentrations of bicarbonate inside the red cell, which may be transported to the gills with the help of Hbs, whose oxygen affinity is linked to bicarbonate binding. The low metabolic rate of hagfish does not seem to require a highly cooperative oxygen carrier, and our structures confirm that F1 is unable to form monomer-monomer interactions of the kind found in higher vertebrate Hbs. The long N-terminal extension and the deletion between the G and H helices make both αβαβ- and αββα-type interactions impossible (4). It has been suggested that hagfish Hbs represent an intermediate between invertebrate and vertebrate Hbs (47), but we see no evidence of direct heme-heme interaction as found in the Hb of the clam *S. inequivalvis* (27). From the structures described in this study, it appears that the BHG core structure of the globin fold evolved before the branching point on the evolutionary tree where hagfish diverged from vertebrates. The αββα tetramer, with its two quaternary structures, arose between the branching points of hagfish and lampreys from cartilaginous fish. The movement of the F helix commonly found upon ligand binding to vertebrate αββα-type Hb is not seen in F1 and appears to be linked more firmly to quaternary structure change than to the ligation state of the heme. The stereochemical mechanisms regulating heme and non-heme ligand binding have clearly evolved independently among hagfish and lampreys and other vertebrates since their divergence. The BHG core structure was apparently present in ancestral globins before this occurred, but the switching function of the proximal histidine appears to have arisen later. The structural basis of the heterotropic effects of protons, chloride, and bicarbonate ions in hagfish Hb remains unclear, which is perhaps not surprising given the number of mutant forms that have been studied to achieve our present understanding of these effects in human Hb.

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Crystal Structures of Deoxy- and Carbonmonoxyhemoglobin F1 from the Hagfish *Eptatretus burgeri*

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