Studies on Hemoglobin from the Hagfish Eptatretus burgeri

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SUMMARY

Ultracentrifugation studies on hemolysates from the erythrocytes of the hagfish, Eptatretus burgeri, showed that in dilute solution oxygenated hemoglobins were in a monomeric form with a molecular weight of approximately 18,000. In deoxygenated hemolysates or in concentrated solutions of oxygenated hemoglobins, an aggregate product appeared which was considered to be a tetramer, as judged from its sedimentation coefficient. Contrary to a previous report, the hemolysate of the hagfish showed weak but significant heme-heme interaction (n = 1.2) depending on its hemoglobin concentration and pH.

In the hemolysate, four hemoglobin components were found by electrophoresis, and other hemoglobins, if any, were very small in amount. The four components were preparatively separated by chromatography on DEAE-cellulose and hydroxyapatite. Among them only one component, F3, aggregated slightly by itself, whereas the other three hemoglobins did not. No components in their isolated state showed significant heme-heme interaction. The hemoglobins of E. burgeri could be divided into two classes; one (F1 and probably F2) hardly aggregated with any other components, and the components of the other (F3 and F4) aggregated with each other, especially in the deoxygenated state, and formed a hybrid tetramer which showed heme-heme interaction.

Hagfish are undoubtedly a very archaic form of life. They belong to the class Cyclostomata, together with lampreys. Whereas the molecular weight of vascular hemoglobins of all known nongyrocystome vertebrates corresponds to four hemes per molecule, the hemoglobins of both hagfish and lampreys consist of but a single heme per molecule (1, 2). Yet lamprey hemoglobin shows small heme-heme interaction with an interaction of but a single heme per molecule (1, 2). Yet lamprey hemoglobin shows small heme-heme interaction with an interaction of but a single heme per molecule (1, 2). Yet lamprey hemoglobin shows small heme-heme interaction with an interaction of but a single heme per molecule (1, 2).

However, little is known about the hemoglobin of the hagfish, the other group of cyclostomes. The oxygen equilibria of the hemolysate of hagfish Eptatretus stoutii and Mixine glutinosa have been studied previously, and an absence of heme-heme interaction in Eptatretus hemoglobin and an extremely slight interaction (n < 1.1) in Mixine hemoglobin have been reported (5, 6). These results did not necessarily mean that no hemoglobin molecule showed molecular interaction, because the hemolysate consisted of heterogeneous hemoglobin molecules (6, 7). In this paper we describe this heterogeneity, a method of separating each hemoglobin component, as well as the aggregation behavior and functional aspects of the hemoglobin of the hagfish, Eptatretus burgeri.

MATERIALS AND METHODS

Preparation of Hagfish Hemoglobin—Hagfish (E. burgeri) were captured in the sea near Miura Peninsula in Japan. Blood was drawn through a needle inserted in a large vessel near the heart. From thrice-washed erythrocytes, a freeze-thaw hemolysate was prepared in distilled water. Nuclei and stroma were removed by centrifugation at 10,000 x g for 15 min. The supernatant was dialyzed against 0.005 N Tris-HCl, pH 8.0, and stored at -15°.

Determination of Hemoglobin Concentration—The concentration of hemoglobin was estimated after conversion to pyridine hemochrome. Reduced pyridine hemochrome was prepared by adding 0.8 ml of pyridine and 1.2 ml of 0.1 N sodium hydroxide solution to 2 ml of the hemoglobin solution. Then a small amount of solid sodium dithionite was added and the absorbance at 557 nm was measured. The concentration of heme was calculated by means of a millimolar extinction coefficient of 32.2 nm⁻¹ cm⁻¹.

Electrophoresis—Electrophoresis was carried out on 4% acrylamide gel in the discontinuous system of Poulak (8). Acrylamide gel was made by mixing 20 ml of a 10% aqueous acrylamide solution (acrylamide-ethylene bisacrylamide, 19:1) with 12.5 ml of Tris-citrate buffer (pH 8.9), 0.04 ml of N,N,N',N'-tetramethylethylenediamine, 40 mg of ammonium persulfate, and 17.5 ml of water. After electrophoresis, gels were stained by Amido black 10B.

Amino Acid Analysis—Prior to amino acid analysis, heme was removed from hemoglobin by cold acid acetone (acetone-2 N HCl, 1000:2). Globin was dissolved in constant boiling HCl and hydrolyzed in sealed, evacuated tubes for 24 and 48 hours at 105°. Hydrolysate was analyzed on a Hitachi KLA-3B automatic amino acid analyzer, essentially according to the method of Spackman, Stein and Moore (9). Values for serine and threonine were extrapolated to zero time hydrolysis. Tryptophan was determined spectrophotometrically (10). Half-cystine was determined as cysteic acid after oxidation by performic acid (11).

Sedimentation Experiments—Sedimentation velocity and equi-
librium sedimentation experiments were performed with a Spinco model E analytical ultracentrifuge. Sedimentation velocity measurements were carried out at 59,780 rpm at 20°. Deoxyhagfish hemoglobin was prepared by the addition of a few grains of solid sodium dithionite to the hemoglobin solution just before injection into the cell. The cell was filled almost to the top to minimize an amount of air left in it. Before and after centrifugation, the cell was placed in a Hitachi EPR-2 recording spectrophotometer and absorption spectra between 500 and 650 nm were measured. It was confirmed that the hagfish hemoglobin was completely deoxygenated and that no significant change of deoxymoglobin to oxyhemoglobin or methemoglobin occurred during the course of the sedimentation experiments. Sedimentation constants were calculated from the movement of the median of the boundary. Equilibrium measurements were made with a 12-mm double sector synthetic boundary cell on 0.1 mM hemoglobin at 15,220 rpm. The rotor was stopped after the final equilibrium picture was taken. The cell was removed from the rotor and shaken gently. Solvent was then added to the reference side, and a synthetic boundary experiment was carried out. The apparent molecular weight was calculated from the equation

$$M_{app} = 2.303 \frac{d \log f}{d(r)^2} \frac{2RT}{(1 - \bar{v})w^2}$$

where \(\bar{v}\) is the partial specific volume (0.743 was used), \(\rho\) the solution density, and \(\omega\) the angular speed of rotation.

Oxygen Equilibrium—The method of Askam et al. (12) was used with minor modifications. Spectra between 500 and 700 nm were obtained at 22° with a Hitachi EPR-2 recording spectrophotometer. Fractional saturation was calculated from extinction at two wavelengths, the \(\beta\)-band and the minimum between the \(\alpha\)- and \(\beta\)-bands, to cancel small baseline shifts. When concentrations of hemoglobin were used, spectra between 740 and 810 nm were obtained with a Cary 14 recording spectrophotometer, and fractional saturation was calculated from extinctions at 705 nm. The value of \(n\), the slope of the line relating \(\log y/(1 - y)\) to \(\log p\), where \(y\) is the fractional saturation and \(p\) is the oxygen pressure, was estimated at 50% oxygenation.

RESULTS

Molecular Weight and Heterogeneity of E. burgeri Hemoglobin—The molecular weight of \(E.\ burgeri\) hemoglobin was estimated by sedimentation equilibrium. Plots of \(\log f\) versus \(r^2\) for the oxygenated hemolysate are shown in Fig. 1. The molecular weight of oxygenated hemoglobin was calculated by means of the slope of the straight line of Fig. 1, and the value of 18,000 was obtained. Where \(\log f\) is larger than 0.9, meaning that the protein concentration was higher than 0.18 g per dl, the slope became steeper, probably because of aggregation of the protein. The ratio of heme to protein was determined. The concentration of heme was measured as described under “Materials and Methods,” and protein was estimated by the method of Lowry (13), with human hemoglobin as a standard. A ratio of 19 mg of protein per micromole of heme was found, indicating that a single heme prosthetic group was present on each molecule.

In the hemolysate which was obtained from more than 30 hagfish, at least four types of hemoglobin were found by electrophoresis (Fig. 2a). We designated these four hemoglobins as F1, F2, F3, and F4, according to the order of electrophoretic mobility from the origin. A very faint band was also found between F1 and F2. Hemolysates of four hagfish which were studied individually showed almost the same electrophoretic pattern as seen in Fig. 2a, but among them, two hemolysates were completely lacking in the band between F1 and F2. Thus, it seems reasonable to state that \(E.\ burgeri\) commonly possesses at least four different hemoglobin molecules and some individuals have another hemoglobin, which is present in very small amounts. The relative amounts of these four hemoglobins were estimated by densitometric measurements, and the ratio of 17:13:35:35 for F1:F2:F3:F4 was obtained.

Fractionation of E. burgeri Hemoglobins—Hemoglobin solution of about 1 mM heme in 5 mM Tris-HCl, pH 8.0, was applied on a DEAE-cellulose column (2 × 30 cm) previously equilibrated with the same buffer and eluted with a linear gradient of KCl from 0 to 0.1 M in 5 mM Tris-HCl, pH 8.0. As shown in Fig. 3, \(E.\ burgeri\) hemoglobin was separated into four peaks. Electrophoresis showed that the first and the second peaks contained only F1 and F2, respectively. The third peak, however, was a mixture of F2 and F3 and the fourth was a mixture of F3 and F4. Components other than these four were not found in these four peak fractions. To separate F3 from F2, and F4 from F3, the third and the fourth peak fractions were collected and concentrated. They were dialyzed against 0.005 M phosphate buffer, pH 7.0, and applied to hydroxyapatite columns (1.2 × 5 cm) equilibrated with 0.005 M phosphate buffer, pH 7.0. Hemoglobins were separated by elution with a linear gradient from 0.005 to 0.1 M phosphate buffer, pH 7.0. As shown in Fig. 4, F3 was successfully separated from F2. The separation of F4 from F3 was not complete, but the fractions eluted later than No. 31 in Fig. 4b were essentially free from F3, and pure F4 was obtained by collecting these fractions. Electrophoretic patterns of separated components are shown in Fig. 2b, 5 to e.

Amino acid analyses (Table I) were performed on all four
hemoglobins, showing some differences in the composition of Fl, F3, and F4, and almost identical results for hemoglobins Fl and F2. Preliminary experiments indicated that the NH₂-terminal amino acid residue of Fl and F2 was proline and the COOH-terminal was tyrosine. These results suggest that Fl and F2 are similar to each other in primary structures. Although the possibility remains that one is the denatured product of the other, this seems not to be the case, because both purified Fl and F2 show a single band with the same electrophoretic mobility as that before purification.

Aggregation of E. burgeri Hemoglobins—Results of sedimentation velocity experiments on the hagfish hemolysate are given in Table II. The schlieren pattern of the oxygenated hemolysate at the heme concentration of 0.11 mM showed a single peak, the sedimentation coefficient of which was about 2.0 S. All oxygenated hemoglobins were considered to be in a monomeric form.
at that concentration. However, when the concentration was increased, the S value was increased and the peak became broad, and finally two boundaries appeared. The slowly moving boundary represented a monomer and the fast moving one seemed to be an aggregated form. This phenomenon was more remarkable when the hemoglobins were deoxygenated. In the deoxygenated state, the hemoglobins aggregated at lower concentrations than in the oxygenated state. Comparison of the sedimentation coefficients of the fast moving boundary with those of human hemolysate indicated that the aggregate product was a tetramer. At the concentration of 2.54 mM in heme, both oxy- and deoxygenated hemolysates showed the schlieren pattern with a fast moving peak and a slowly moving shoulder. A large part of the hemoglobin was considered to be in aggregated form.

When E. burgeri hemolysate was applied to a short column of DEAE-cellulose equilibrated with 0.01 M Tris-HCl, pH 8.0, and washed with the same buffer, only F1 was eluted from the column unadsorbed. In this way, it was easily removed from the hemolysate. The remainder, consisting of F2, F3, and F4, was analyzed in both the oxy- and deoxygenated form by ultracentrifugation. The amounts of F3 and F4 in this preparation were nearly equal, as can be seen in Fig. 2d, and their sum accounted for 85% of all hemoglobins, whereas F2 was estimated to be only 15%. Although the schlieren pattern of the oxyhemoglobin in concentrated solution showed two peaks corresponding to that of monomer and tetramer, the deoxyhemoglobin gave only one asymmetric peak, whose S value was higher than 4.0 and seemed to be the same as that of a tetramer. It was seen that the slowly moving peak of the deoxyhemoglobin disappeared when F1 was removed. These results, therefore, suggested that F1 was hard to aggregate with any other components, whereas F2, F3, and F4 aggregated each other to form a tetramer in the deoxygenated state. To elucidate what combination of these three components was essential to form a tetramer, mixtures of the two components from F2, F3, and F4 in equal amounts were investigated for their sedimentation profile in the deoxygenated state.

The sedimentation coefficients of isolated F1, F2, F3, and F4 hemoglobin were also measured, and the results are summarized in Table III. The values for F1, F2, and F4 were all approximately 2.0 S in both the oxy- and deoxygenated states, whereas that of F3 was significantly higher than 2.0 S, especially in the deoxygenated state. These results indicate that F1, F2, and F4 did not aggregate by themselves but existed in a monomeric form both in the oxy- and deoxygenated states, whereas F3 was in an

### Table I

Results of amino acid analysis of E. burgeri hemoglobins

| Amino acid | F1 | F2 | F3 | F4 |
|------------|----|----|----|----|
| Aspartic acid | 10.27 | 10.70 | 10.55 | 8.29 |
| Threonine | 3.54 | 3.48 | 2.12 | 4.47 |
| Serine | 9.08 | 8.82 | 8.47 | 6.36 |
| Glutamic acid | 12.48 | 13.09 | 11.75 | 10.00 |
| Proline | 4.95 | 4.38 | 5.35 | 4.04 |
| Glycine | 3.02 | 3.32 | 3.44 | 5.20 |
| Alanine | 4.19 | 4.05 | 5.47 | 9.06 |
| Leucine | 4.70 | 4.96 | 6.59 | 8.16 |
| Methionine | 0.78 | 0.70 | 1.98 | 2.07 |
| Isoleucine | 9.91 | 10.35 | 0.19 | 5.17 |
| Valine | 9.32 | 9.14 | 11.38 | 8.39 |
| Tyrosine | 2.74 | 2.63 | 2.01 | 3.39 |
| Phenylalanine | 6.74 | 6.83 | 8.40 | 5.02 |
| Lysine | 12.31 | 12.43 | 9.00 | 8.46 |
| Histidine | 1.52 | 1.39 | 1.18 | 2.43 |
| Arginine | 1.56 | 1.54 | 4.31 | 5.51 |
| Half-cystine | 1.25 | 1.17 | 1.39 | 0.82 |
| Tryptophan | 1.27 | 1.31 | 1.42 | 1.77 |

- *Values were obtained by extrapolation to zero hour hydrolysis.
- *Value for 48-hour hydrolysis only.
- Determined as cysteic acid after oxidation by performic acid.
- Determined spectrophotometrically.

### Table II

Sedimentation coefficients of E. burgeri hemolysate and human hemolysate

| Sample     | Concentration of hemoglobin | pH  | Form     | Slowly moving peak | Intermediate peak | Fast moving peak |
|------------|-----------------------------|-----|----------|-------------------|------------------|-----------------|
| E. burgeri hemolysate | 0.110 | 7.0 | Oxygenated | 2.01 | 2.29 | Shoulder* |
|             | 0.365 | 7.0 | Oxygenated | 2.03 |     | 3.25 |
|             | 0.696 | 7.0 | Oxygenated | 1.66 | 4.12 |     |
|             | 2.54 | 6.5 | Oxygenated | 2.01 | 4.30 |     |
| Human hemolysate   | 0.28  | 6.0 | Deoxygenated | 4.20 |     |     |
|             | 1.20  | 6.0 | Deoxygenated | 3.79 |     |     |
|             | 2.30  | 6.0 | Deoxygenated | 3.13 |     |     |

- *All sedimentation coefficients were arranged conveniently in three classes, those corresponding to monomer, tetramer, and their intermediate values.
- *When two boundaries were observed and one did not separate completely from the other, a shoulder appeared in schlieren optics.
alone. The mixture of F2 and F3 and that of F2 and F4 did not as the hemoglobin concentration was increased. Therefore, the action. The p:\ of that mixture was higher than that of F3 or F4 efficient of approximately 2.0 S, the aggregate product appeared

...cause of the experimental difficulties involved.

F3, and F4 were investigated. As shown in Table IV, only the more complicated. Although oxygenated hemolysates, n-hendications of...hemoglobins. These results strongly suggest molecular interaction between them. It is possible that F2 takes part in the equilibrium state of association-dissociation, probably monomer-

eq.00-0.051 7.8 6.0 0.97

-0.051 7.0 6.6 0.08

-0.051 5.8 11.2 1.01

-0.22 7.8 8.1 1.09

-0.22 7.0 10.2 1.03

-0.61 6.0 13.0 1.19

-0.61 7.8 8.6 1.02

-0.61 7.1 11.4 1.20

-0.61 6.0 19.0 1.19

-0.043-0.051 7.0 5.1 1.08

-1.04 4.3 0.10

-0.02-0.16 7.0 10.2 1.28

-0.12-0.16 5.8 18.5 1.29

-0.12-0.16 7.0 11.0 1.36

-0.039 7.0 5.1 1.08

F2 + F3 0.248 7.0 22.0 1.01

F3 0.227 7.0 6.7 1.09

F4 0.217 7.0 4.8 1.05

F2 + F3 0.394 7.0 11.8 1.09

F2 + F4 0.200 7.0 6.0 1.00

F3 + F4 0.216 7.0 8.0 1.31

E. burgeri hemolysate was passed through a DEAE-cellulose column equilibrated with 0.01 M Tris-HCl, pH 8.0 and the adsorbed fraction was eluted with 0.1 M KCl-0.01 M Tris-HCl, pH 8.0. The eluted fraction, which was lacking in F1, was dialyzed against 0.1 M phosphate buffer, pH 7.0.

Each fraction was mixed in equal amount.

DISCUSSION

Hagfish, E. burgeri, were found to have at least four different hemoglobin molecules. All four individual hagfish tested showed similar electrophoretic patterns. Although another hemoglobin was found in two individuals, its amount was very small. Ohno and Morrison (7) found that the hagfish, E. stoutii, captured at the east coast of the United States had four to six hemoglobin phenotypes, and they postulated that these hemoglobins were controlled by genes at four loci. The relationship between the hemoglobins of these two species of hagfish is uncertain, but E. burgeri, captured at the Pacific coast of Japan, seems to be genetically more homogeneous.

In previous reports, the heme-heme interaction and Bohr effect of hagfish hemoglobin were described as extremely slight or undetectable (5, 6) and their sedimentation coefficient was approximately 2.3 S (1, 2). However, these must be revised because of the fact that hagfish hemoglobins are heterogeneous and their components are different from each other both in structure and function.

Sedimentation behavior of the hemolysate from E. burgeri was more complicated. Although oxygenated hemolysates, when diluted to less than 0.1% in heme, showed a sedimentation coefficient of approximately 2.0 S, the aggregate product appeared as the hemoglobin concentration was increased. Therefore, the sedimentation coefficient of 2.3 S is interpreted to be that of the mixture of the monomer and the aggregate product. The he-

| TABLE III | Sedimentation coefficients of E. burgeri hemoglobin components |
|-----------|---------------------------------------------------------------|
| Component | Concentration of hemoglobin | Form       | S   |
|           | mS in heme            |            |     |
| F1        | 0.220                 | Oxygenated | 2.05|
| F2        | 0.230                 | Deoxygenated| 2.05|
| F3        | 0.180                 | Oxygenated | 1.96|
| F4        | 0.182                 | Deoxygenated| 1.88|
| F3        | 0.105                 | Oxygenated | 1.80|
| F4        | 0.248                 | Oxygenated | 2.18|
| F3        | 0.500                 | Oxygenated | 2.00|
| F3        | 0.105                 | Deoxygenated| 2.22|
| F3        | 0.373                 | Deoxygenated| 2.44|
| F3        | 0.690                 | Deoxygenated| 2.44|
| F4        | 0.404                 | Oxygenated | 1.90|
| F4        | 0.310                 | Deoxygenated| 1.91|
| F2 + F3  | 0.386                 | Deoxygenated| 2.71|
| F2 + F4  | 0.328                 | Deoxygenated| 2.05|
| F3 + F4  | 0.400                 | Deoxygenated| 4.03|

a All components were dissolved in 0.1 M phosphate buffer, pH 7.0.

b Two components were mixed in equal amount.

equilibrium state of association-dissociation, probably monomer-dimer, and this equilibrium was changed by the binding of oxygen. As shown in Table III, it is clear that the mixture of F3 and F4 formed a tetrameric molecule, but there seems to be no interaction between F2 and F4, and between F2 and F3. Because F3 aggregated by itself, the relatively high S value of 2.7 S for the mixture of F2 and F3 did not necessarily imply an interaction between them. It is possible that F2 takes part in the formation of the tetramer in such a form as (F2F3F4F4) or (F2F3F4F4). However, we did not study this possibility because of the experimental difficulties involved.

Functional Aspects of E. burgeri Hemoglobin...brium of E. burgeri hemoglobin were measured. The oxygen pressure at 50% oxygenation, p\textsubscript{50}, and n, the slope of Hill’s plot, were obtained and are summarized in Table IV. It was found that E. burgeri hemolysate showed weak but significant hemeheme interaction at high hemoglobin concentrations and at pH values below 7.0. A slight Bohr effect was observed. Oxygen affinity also changed, depending upon the concentration of hemoglobins. These results strongly suggest molecular interactions of E. burgeri hemoglobins. When we used the hemoglobin preparation lacking F1, whose properties were described in the preceding section, the heme-heme interaction and Bohr effect became more remarkable (Table IV).

In Table IV, the functional properties of isolated F1, F2, F3, and F4 are also shown. All of them showed no significant heme-heme interaction. Oxygen affinity of F1 and F2 were nearly equal and very low as judged from p\textsubscript{50}, whereas F3 and F4 exhibited relatively high affinity. It is interesting that the monomeric hemoglobins, F1 and F2, showed such a low affinity.

Oxygen equilibria of the combination of two fractions from F2, F3, and F4 were investigated. As shown in Table IV, only the mixture of F3 and F4 showed a significant heme-heme interaction. The p\textsubscript{50} of that mixture was higher than that of F3 or F4 alone. The mixture of F2 and F3 and that of F2 and F4 did not show heme-heme interactions, and p\textsubscript{50} values were approximately the same as the average of those of the components.

| TABLE IV | Oxygen equilibria of E. burgeri hemoglobins |
|-----------|-------------------------------------------|
| Sample    | Concentration of hemoglobin | pH | p50 | n  |
|           | mS in heme            |    |     |    |
| F1        | 0.137                 | 7.0| 20.0| 1.00|
| F2        | 0.248                 | 7.0| 22.0| 1.01|
| F3        | 0.277                 | 7.0| 6.7 | 1.09|
| F4        | 0.217                 | 7.0| 4.8 | 1.05|
| F2 + F3  | 0.394                 | 7.0| 11.8| 1.09|
| F2 + F4  | 0.200                 | 7.0| 6.0 | 1.00|
| F3 + F4  | 0.216                 | 7.0| 8.0 | 1.31|

* E. burgeri hemolysate was passed through a DEAE-cellulose column equilibrated with 0.01 M Tris-HCl, pH 8.0 and the adsorbed fraction was eluted with 0.1 M KCl-0.01 M Tris-HCl, pH 8.0. The eluted fraction, which was lacking in F1, was dialyzed against 0.1 M phosphate buffer, pH 7.0.

Each fraction was mixed in equal amount.
Hemoglobin of Eptatretus burgeri showed heme-heme interaction depending upon the concentration and pH. It should be emphasized that significant heme-heme interaction is observed only when hemoglobin molecules aggregate sufficiently. Heme-heme interaction was evident much more when F1 was removed from the hemolysate. F1 did not aggregate with any other hemoglobins and remained in a monomeric state under the experimental conditions.

Hemoglobins of E. burgeri studied here can be divided into two classes; those of the first exist always as monomer, and those of the second aggregate with one another to form tetrameric molecules. F1 belongs to the first class, and although the precise aggregation properties of F2 are still obscure, F2 probably belongs to this class also. There is close resemblance between F1 and F2 in their amino acid compositions, sedimentation behaviors, and functional properties. Chromatographic and electrophoretic properties distinguished them, probably because of their different surface charges due to a few amino acid replacements. Their low oxygen affinity is interesting, for the oxygen affinity of all other vertebrate hemoglobins, including lampreys, is known to be relatively high when they are in a monomeric state. Physiological meaning of these hemoglobins, however, is obscure at present.

The second class of E. burgeri hemoglobins consists of F3 and F4. The amounts of F3 and F4 in the hemolysate were nearly equal and the mixture of F3 and F4 formed tetrameric molecules and showed heme-heme interaction. The tetramer of E. burgeri hemoglobin was a hybrid molecule, and all hemoglobin fractions in their isolated state neither formed tetramers nor had any significant heme-heme interaction. Sea lamprey, Petromyzon marinus, was shown to have six species of hemoglobin (14). Among them, Fractions III, IV, V, and VI aggregated in the deoxygenated state to form a self-tetramer or a hybrid tetramer (15). The self-tetramer showed heme-heme interaction (16). Another lamprey, Entosphenus japonicus, has essentially one species of hemoglobin which is self-associated to form a tetramer.1 Although in the deoxygenated form both E. burgeri and lamprey hemoglobin showed a marked tendency toward aggregation, in the oxygenated form lamprey hemoglobin hardly aggregated (4), whereas in E. burgeri the aggregate product of oxygenated hemoglobin is easily observed at high hemoglobin concentrations. This suggests that E. burgeri hemoglobin tends to aggregate more easily than lamprey hemoglobin. In this respect E. burgeri hemoglobin, F3 and F4, are more closely related to higher vertebrate hemoglobin than the lamprey’s. It may be postulated that F3, which aggregated slightly by itself, corresponds to the β chain of human hemoglobin and F4 to the α chain. Of course, there are differences between F3-F4 and higher vertebrate hemoglobins; heme-heme interaction of the latter are more extensive and their association-dissociation equilibria are more favorable to association in comparison with E. burgeri hemoglobins. These differences, however, may be in degree rather than in quality. Although our experimental conditions were not physiological, it may be said that tetrameric hemoglobins both in oxygenated and deoxygenated form exist in E. burgeri erythrocytes where the hemoglobin concentration is more than 10 mm in heme.

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