Anion-linked Polymerization of the Tetrameric Hemoglobin from *Scapharca inaequivalvis*

**CHARACTERIZATION AND FUNCTIONAL RELEVANCE**

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The anion-linked polymerization of the tetrameric hemoglobin from *Scapharca inaequivalvis* has been characterized by means of sedimentation velocity experiments in terms of its dependence on the binding of other protein ligands, oxygen, and protons. The linkage with oxygen manifests itself at any given anion concentration in the markedly different sedimentation behavior of the oxygenated and deoxygenated protein; whereas the former sediments always as a single peak ($\approx 4.3$ S), the latter exhibits bimodal Schlieren patterns with a fast peak that attains $\approx 10$ S. A comparison of experimental and computer-simulated (Cox, D. J. (1971) *Arch. Biochem. Biophys.* 146, 181–195) boundaries shows that the behavior of oxyhemoglobin can be represented by a rapidly reversible dimerization of the native $4.3$ S molecule, whereas the behavior of the deoxygenated protein can be described adequately in terms of the polymerization of the native structure into tetramers and octamers.

The interplay between the binding of protons and anions is brought out by the different dependence of polymer formation on anion concentration at different pH values. In the case of chloride, polymerization goes through a maximum at around 20–50 mM Cl$^-$ at pH 6.3 but decreases monotonically above 5 mM at pH 5.5. On the basis of these data and of the effect of other anions such as phosphate and perchlorate, a tentative picture of the high affinity anion-binding sites has been proposed.

From a functional point of view, the hemoglobin polymers are characterized by a lower oxygen affinity and a higher cooperativity than the tetrameric structure; hence, polymerization results in a shift of the lower asymptote of the Hill plots while the upper asymptote is unaltered. The effect of polymerization on oxygen binding has been analyzed in terms of the polysteric linkage scheme (Colosimo, A., Brunori, M., and Wyman, J. (1974) *Biophys. Chem.* 2, 338–343). The data obtained at pH 6.3 as a function of chloride concentration could be fitted satisfactorily by taking the self-association behavior of the protein into account.

The dimeric and tetrameric hemoglobins extracted from Arcid mollusks like *Scapharca inaequivalvis* represent an interesting model system in the study of hemoglobins since cooperativity in oxygen binding is achieved in the Arcid proteins by means of a unique mode of assembly of the globin chains. Thus, the heme-linked E and F helices are not exposed to solvent as in vertebrate hemoglobins but form the inter-subunit contact region in the homodimeric protein HbI; in the tetrameric one, HbII, two heterodimers, assembled in the same "inside-out" fashion relative to vertebrate hemoglobins, give rise to the tetramer by contact between the A helices and the nonhelical segments preAA and GH (Royer et al., 1985). A further difference between the molluskan and vertebrate hemoglobins lies in the nature of the forces that stabilize the native quaternary structure. In vertebrate hemoglobins, as exemplified by human hemoglobin, salt bridges and hydrogen bonds play a predominant role. In contrast, in the Arcid proteins, hydrophobic interactions are of major importance, as indicated by the presence of highly conserved residues in the hydrophobic patch formed by the E and F helices (Petruzelli et al., 1985; Royer et al., 1989) and by the stability of both HbI and HbII toward dissociation into subunits at high salt concentrations (Gattoni et al., 1983).

However, a most interesting property of the tetrameric HbII revealed in its initial characterization by sedimentation velocity, namely the strong tendency to polymerize upon removal of oxygen (Chiancone et al., 1981; Furuta et al., 1981), appears to be determined by ionic interactions and in particular anion binding. The first indication in this respect was provided by the observation that polymer formation in S. *inaequivalvis* HbII is drastically decreased in the deionized form of the protein but can be observed readily in its initial characterization by sedimentation velocity experiments (Chiancone et al., 1988).

The polymerization of HbII from a functional viewpoint is reflected in a slight decrease of the oxygen affinity and a slight increase in cooperativity (Chiancone et al., 1981).

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In the present work, the effect of chloride on the state of association and on the oxygen-binding properties of HbII from Scapharca S. inaequivalvis has been studied in a systematic way. In addition, the effect of chloride has been compared with that of other anions, namely phosphate and perchlorate. The body of the results obtained provides a fairly complete picture of the linkage between the anion-dependent polymerization of HbII and the binding of oxygen and protons and allows one to arrive at a tentative description of the relevant anion-binding sites.

**MATERIALS AND METHODS**

HbII was purified from the red cells of the mollusk S. inaequivalvis as reported previously (Chiancone et al., 1981). Hemoglobin concentrations (in heme) were determined at 578 nm using \( E = 14,200 \text{ M}^{-1} \text{cm}^{-1} \) for the oxyhemoglobin form.

Sedimentation velocity experiments were carried out on a Spinco model E ultracentrifuge equipped with an RTIC unit at 44,000 or 50,000 rpm at low (6-12 °C) and high (20-25 °C) temperature and at protein concentrations between 1 and 7 mg/ml. In the experiments involving the deoxy-derivative, the hemoglobin solutions were degassed in a tonometer and were transferred into the ultracentrifuge cells, flushed extensively with nitrogen by means of a syringe washed with deoxygenated buffer containing dithionite. The small volume of dithionite-containing buffer that remains in the needle ensured complete deoxygenation; the dithionite concentration in such buffers was <2 × 10⁻³ M as determined by means of titrations with a known ferricyanide solution at pH 7.0 (Dixon, 1971). The sedimentation coefficients were evaluated from the maximum ordinate of the Schlieren peak and were reduced to \( s_{20w} \) by standard procedures. The diffusion coefficients were calculated from the areas of the Schlieren diagrams in the sedimentation velocity experiments and corrected for radial dilution and for the movement of the boundary in the centrifugal field according to Elias (1981). The boundary shape during sedimentation has been simulated using the computational model developed by Cox (1971) which treats a rapidly equilibrating self-associating solute as a single component with concentration-dependent sedimentation and diffusion coefficients.

Oxygen equilibria were measured on a Cary 219 spectrophotometer at 20 ± 1 °C using a tonometer (Rossi Fanelli and Antonini, 1958). The oxygen-binding curves were fitted to a simple two-state model. Polymerization was taken into account following the basic principles of the polymeric linkage scheme (Colosimo et al., 1974). The values of the polymerization constants for the monomer-tetramer-octamer equilibrium of deoxy-HbII obtained from the sedimentation velocity experiments were approximated to the association constant of the protein in the T state. The values of the allosteric parameters \( K_R \) and \( L_0 \) were taken from Ikeda-Saito et al. (1983) and were held constant over the range covered. The diffusion coefficients likewise increase by a very significant amount and hence point to increasing polydispersity. The latter finding in turn indicates that the Schlieren patterns are sensitive to polymer formation and in principle can be used to extract quantitative information regarding the stoichiometry of association and the interaction constants of the oxygenated protein. To this end, representative experimental boundaries measured at various chloride concentrations have been compared with simulated ones calculated by means of the computer model developed by Cox (1971) for systems in rapid self-association equilibrium which take both sedimentation and diffusion into account. The experimental boundary shapes of oxy-HbII correspond to those simulated for a simple dimerization equilibrium of the native 4.3 S molecule. The equilibrium constants depend on chloride concentration, as indicated in Table II.

The behavior of the deoxygenated derivative as a function of NaCl concentration is illustrated in Fig. 1, where the sedimentation velocity of the faster component in the Schlieren diagrams is plotted as a function of the NaCl concentration.

**RESULTS**

**Sedimentation Velocity**—As mentioned above, previous sedimentation velocity studies performed in phosphate buffer have given clear evidence of polymerization only for the deoxygenated derivative whose Schlieren patterns, at protein concentrations above 3 mg/ml, are characterized by the presence of a polymer peak of 8-10 S in addition to the tetramer one. These studies also showed that the extent of polymerization and the sedimentation velocity of the polymer decrease with decreasing temperature in the range 20-8 °C, are hardly affected by a change in ionic strength between 0.02 and 0.1 M, but diminish markedly at ionic strengths above 0.25 M (Chiancone et al., 1981).

In the light of the recent ³¹Cl NMR data that point to a significant polymerization of both oxy- and deoxy-HbII when the chloride concentration is around 20 mM at pH 6.5 (Chiancone et al., 1988), the majority of experiments were carried out in NaCl at this pH value. A first series was performed at protein concentrations around 6 mg/ml; usually the same solution was analyzed at low (8-12 °C) and high (20-25 °C) temperatures.

The behavior of the oxygenated derivative is given in Table I. Under all the conditions studied, oxy-HbII sediments as a single peak whose velocity closely corresponds to that of tetrameric hemoglobins, in agreement with published data (Chiancone et al., 1981; Furuta et al., 1981). As a matter of fact, the \( s_{20w} \) values do display a small but significant increase with an increase in chloride concentration and temperature over the range covered. The diffusion coefficients likewise increase by a very significant amount and hence point to increasing polydispersity. The latter finding in turn indicates that the Schlieren patterns are sensitive to polymer formation and in principle can be used to extract quantitative information regarding the stoichiometry of association and the interaction constants of the oxygenated protein. To this end, representative experimental boundaries measured at various chloride concentrations have been compared with simulated ones calculated by means of the computer model developed by Cox (1971) for systems in rapid self-association equilibrium which take both sedimentation and diffusion into account. The experimental boundary shapes of oxy-HbII correspond to those simulated for a simple dimerization equilibrium of the native 4.3 S molecule. The equilibrium constants depend on chloride concentration, as indicated in Table II.

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had been suggested by Chiancone et al., (1981), does not suffice to achieve the observed sedimentation velocity of the fast peak. Upon addition of a further association step, namely the formation of octamers of the native molecule, an adequate polymer formation goes through a maximum, as already observed in the case of the deoxygenated derivative, the dependence of polymer formation on the type and concentration of anion was studied while keeping Na⁺ as cation. The results obtained as a function of chloride only 5% of the protein (by weight) is associated into tetramers, and no octamers are formed. In Table II, at 5 mM chloride, 9% tetramers and 1% octamers are formed.

Polymerization of Scapharca Hemoglobin

It is worth pointing out that due to the interplay of temperature, protein, and chloride concentration, situations are encountered in which the deoxygenated derivative sediments as a single peak since the amount of polymers formed is very low (Fig. 2). For example, at 1 mg/ml and 20 °C, namely under the conditions used in the oxygen equilibrium experiments of Scapharca inaequivalvis HbII at low protein concentration in 5 and 20 mM NaCl. Left Schlieren patterns, 20 mM NaCl; right Schlieren patterns, 5 mM NaCl. Protein concentration (in mg/ml): a, 2.2; b, 1.0. Temperature: a, 12 °C; b, 20 °C. Photographs were taken a, 51 min after reaching full speed of 52,000 rpm; b, 100 min after reaching full speed of 44,000 rpm.

Lastly, a few experiments were carried out to study the combined effect of proton and chloride concentration. The experiments were carried out in parallel at pH 5.5 and 6.3...
since previous studies in 0.1 M acetate buffers indicated that at the lower pH polymerization is drastically decreased (Chiancone et al., 1981). Fig. 4 shows that the extent of polymerization depends on chloride concentration in a markedly different way at the two pH values. Thus, at pH 5.5, the degree of polymerization is highest at 5 mM Cl\(^-\) and decreases with an increase in anion concentration, whereas at pH 6.3, polymerization is greater at 50 than at 5 mM Cl\(^-\) in agreement with the data of Fig. 1. A few experiments were carried out also with the oxygenated derivative; the sedimentation and diffusion coefficients measured at pH 5.5 do not appear to differ significantly from those at pH 6.3 reported in Table I.

Oxygen Equilibria—In the light of the complex pattern brought out by the sedimentation velocity study regarding the linkage between the polymerization of HbII and the binding of oxygen, chloride ions, and protons, oxygen equilibria were measured as a function of chloride concentration at two pH values, namely 6.3 and 5.5, both at high (5–7 mg/ml) and low (1 mg/ml) protein concentration. All the experiments were carried out at 20 ± 1 °C. Fig. 5 depicts the changes in oxygen affinity and cooperativity (as measured by \(n_{1/2}\)) in the experiments carried out at high protein concentration between 5 and 100 mM NaCl. The values of log \(p_{1/2}\) and \(n_{1/2}\) vary in a different way depending on pH: they go through a maximum around 20–50 mM Cl\(^-\) at pH 6.3 but decrease monotonically at pH 5.5 and hence follow the same pattern as the polymerization of the deoxy derivative (Figs. 1 and 4).

The results of a similar set of oxygen equilibria measured at 1 mg/ml, in which the contribution of polymer formation is less marked, are given in Fig. 6. Again, the dependence of the oxygen affinity and cooperativity on chloride concentration differs at pH 6.3 and 5.5. At the higher pH value, the two parameters characterizing the oxygen equilibrium are hardly affected by changes in the concentration of chloride over the range studied, whereas at the more acid pH, both log \(p_{1/2}\) and \(n_{1/2}\) decrease sharply with an increase in chloride concentration above 5 mM.

In the light of these results, a further set of experiments was performed as a function of pH. Two chloride concentrations were used, i.e. 5 and 20 mM; protein concentration was around 1 mg/ml in order to minimize the influence of polymerization. Fig. 7 shows that the acid Bohr effect is more marked at the higher chloride concentration, where its magnitude is comparable to that measured in \(I = 0.1\) M ionic strength phosphate buffers (Chiancone et al., 1981).

Lastly, the effect of perchlorate was studied under experimental conditions similar to those used in the sedimentation velocity runs, namely slightly acid pH values and protein concentrations around 6 mg/ml. The measured values of log

**Fig. 4** Sedimentation velocity patterns of *S. inaequivalvis* deoxygmetated HbII at different pH values in 5 mM (a) and 50 mM (b) BisTris HCl buffer, pH values: 6.3 in left patterns; 5.5 in right patterns. Protein concentration, 6.2 mg/ml. Temperature, 22 °C. Photographs were taken 40 min after reaching full speed of 52,000 rpm.

**Fig. 5** Influence of chloride and perchlorate ion concentrations on the oxygen affinity and degree of cooperativity of *S. inaequivalvis* HbII. Temperature, 20 °C. Different symbols refer to different preparations. Protein concentration (in mg/ml): ●, 0; ▲, 1.2; ◊, 0.9–1.3. The continuous line at pH 6.3 has been traced using the log \(p_{1/2}\) values obtained from the fitted curves of Fig. 8.

**Fig. 6** Influence of chloride ion concentration on the oxygen affinity and degree of cooperativity of *S. inaequivalvis* HbII at different pH values and low protein concentration. Temperature, 20 °C. Different symbols refer to different preparations. Protein concentration (in mg/ml): ●, 1.2; ○, 0.9–1.3. The continuous line at pH 6.3 has been traced using the log \(p_{1/2}\) values obtained from the fitted curves of Fig. 8.

\(p_{1/2}\) and \(n_{1/2}\) are included in Fig. 5; they decrease monotonically with an increase in perchlorate concentration above 5–10 mM.

**DISCUSSION**

One of the most intriguing characteristics of the tetrameric hemoglobin from Arcid mollusks is represented by its marked tendency to polymerize upon removal of oxygen (Chiancone et al., 1981; Furuta et al., 1981). The present paper sheds further light on this linkage and in particular brings out a most unusual feature of the polymerization process, namely its dependence on the binding of anions. To our knowledge, *S. inaequivalvis* HbII is the only anion-linked associating system described so far, whereas specific cation-linked associations have been observed in a number of macromolecules, e.g. tubulin, hemocyanins, and erythrocyropeptins (Frigon and
that oxy-HbII sediments as a single peak whose sedimentation polymerization pattern is observed in the presence of chloride, specific since their effect on the state of association and on the oxygen-binding properties of HbII manifests itself at fairly polymerization process that is both oxygen and proton linked complete picture was obtained with chloride; this anion was formation also in the oxygenated protein. In this respect, the study just mentioned which allowed the detection of polymer NMR data with the old ultracentrifuge ones. They confirm al., function of oxygenation. The polymers formed by the deoxy- and electrostatic interactions, given the effect of temperature, role of anions was then demonstrated by measuring in a direct way the interaction among HbII, sodium ions, and chloride ions in quadrupole relaxation NMR experiments. These meas- both the liganded and unliganded forms of the protein (Chian- to bring about an oxygen-linked polymerization process in chloride ions. In addition, binding of the anions was shown anion binding was proposed (Boffi et al., 1987) based on the behavior of the deionized or selectively acetylated protein. The role of anion was then demonstrated by measuring in a direct way the interaction among HbII, sodium ions, and chloride ions in quadrupole relaxation NMR experiments. These measures- showed that the HbII molecule binds sodium ions with low affinity (K < 10 M⁻¹) but interacts strongly with chloride ions. In addition, binding of the anions was shown to bring about an oxygen-linked polymerization process in both the liganded and unliganded forms of the protein (Chian- cations and anions, in the present sedimentation velocity study the same degree of polymerization is attained in the presence of chloride ions irrespective of whether sodium or BisTris is used as counterion and, conversely, a different polymerization pattern is observed in the presence of chloride, phosphate, or perchlorate when Na⁺ is the common cation (Figs. 1 and 3). Also, the effect of temperature can be ration- alized in the framework of an anion-dependent polymeriza- tion. Since ionic interactions, like hydrophilic interactions, are predominantly entropic (Kauzmann, 1959), there is no need to assume that the cold lability of HbII polymers reflects hydrophilic bonding, as proposed previously (Chiancone et al., 1981).

The binding of all the anions studied appears to be rather specific since their effect on the state of association and on the oxygen-binding properties of HbII manifests itself at fairly low concentrations, namely in the millimolar range at the pH values tested. The high affinity binding of anions promotes a polymerization process that is both oxygen and proton linked and differs in detail depending on the anion used. The most complete picture was obtained with chloride; this anion was used in the majority of experiments in view of the ³⁵Cl NMR study just mentioned which allowed the detection of polymer formation also in the oxygenated protein. In this respect, the present sedimentation velocity experiments reconcile the ³⁵Cl NMR data with the old ultracentrifuge one. They confirm that oxy-HbII sediments as a single peak whose sedimentation velocity (≈4.3 S) is close to that of tetrameric hemoglobins. However, under experimental conditions similar to those used for the ³⁵Cl NMR experiments, namely 5–20 mM NaCl at pH 6.3, a qualitative indication of polymer formation is provided by the finding that the boundary broadens more than it would by diffusion alone (Table I). A quantitative description of the relevant association-dissociation equilibria has been obtained by comparing the experimental Schlieren boundaries with simulated ones calculated with the realistic computer model of Cox (1971). The experimental sedimentation patterns of oxy-HbII conform to a simple dimerization equilibrium in line with theoretical predictions (Gilbert, 1969; Gilbert and Gilbert, 1973). A similar analysis carried out for deoxy-HbII under the same conditions of pH and chloride concentration showed that the experimental Schlieren patterns of this deriva- tive can be represented adequately by a polymerization of the native 4.3 S molecule into tetramers and octamers. The concentration of chloride ions affects polymerization of both oxy- and deoxy-HbII in a similar way in that polymer for- mation is at a maximum around 20 mM chloride; in the case of deoxy-HbII, polymerization into octamers appears to be influenced by changes in chloride concentration more than the tetramerization step (Table II).

From a functional viewpoint, the consequence of polymer- ization is to change both the oxygen affinity and cooperativity. Polymer formation is paralleled by a shift to the right of the lower asymptote of the Hill plots while the upper asymptote remains constant (Fig. 8). This finding requires that the HbII polymers be characterized by a lower oxygen affinity and a higher cooperativity than the tetrameric structure as antici- pated in previous studies (Chiancone et al., 1981; Furuta et al., 1981). The linkage between oxygen and chloride binding and polymerization at constant pH has been analyzed quantitatively in terms of the polysteric linkage scheme (Colosimo et al., 1974), making use of the polymerization constants determined at pH 6.3 (Table II). The fit to the experimental data shown in Fig. 8 was obtained by keeping the allosteric parameters Kₗ and L constant and by varying K₉ as a function of chloride concentration. This choice entails that K₉, at each chloride concentration, represents a weighted average among a set of values characteristic of the various n-mers. It should be stressed that a change in K₉ is necessary in order to mimic the observed shift in the lower asymptote of the Hill plots.

The linkage between the binding of chloride and protons is brought out clearly in the sedimentation velocity experiments carried out on the deoxy-derivative where it manifests itself in the effect that pH exerts on the chloride concentration dependence of polymer formation. Thus, at pH 6.3, polymer- ization goes through a maximum at about 20–50 mM chloride, whereas at pH 5.5, polymer formation decreases above 5 mM chloride, the lowest anion concentration analyzed. In a recip- rocal way, a decrease in pH from 6.3 to 5.5 at a constant concentration of 50 mM chloride results in a significant de-polymerization, a finding that is consistent also with previous observations made in sodium acetate buffers (Chiancone et al., 1981). These observations are of interest as they provide new insight into the possible nature and number of the high affinity chloride-binding sites. One may envisage that the HbII molecule is endowed with two such sites. One type may result from the juxtaposition of two positively charged amino acid residues, most likely lysines, belonging to different tetra- meric molecules. Binding of a single chloride ion to this site at low enough chloride concentrations could bridge the posi- tively charged residues and thus favor polymer formation, whereas binding of two anions at higher chloride concentra-

FIG. 7. Influence of pH on the oxygen affinity and cooper- ativ. Temperature, 20 °C. Different symbols refer to different prepara- tions. Temperature, 20 °C. Different symbols refer to different prepara- tions. Temperature, 20 °C. Different symbols refer to different prepara- tions. Temperature, 20 °C. Different symbols refer to different prepara- tions. Temperature, 20 °C. Different symbols refer to different prepara- tions.
Polymerization of Scapharca Hemoglobin

**FIG. 8. Hill plots of S. inaequivalvis HbII as a function of chloride ion concentration at pH 6.3–6.5.**

Temperature, 20 °C. Protein concentration (in mg/ml): left three panels, 6.4; right panel, 1. NaCl concentration (in mM, from left to right): first panel, 5; second panel, 50; third panel, 100; fourth panel, 5, 50, 100. The value of $K_n$ was taken from Ikeda-Saito et al. (1983). The parameters used were: $K_n$, 0.055 torr$^{-1}$; $L_o$, 800; c, 0.053 (5 mM NaCl), □□□□; c, 0.020 (50 mM NaCl, ■■■■); c, 0.008 (100 mM NaCl, ·····).

Significant amount upon lowering the chloride concentration from 20 to 5 mM. The observed effect appears to be too large to be accounted for by differences in the extent of polymer formation. A more likely explanation is that the acid Bohr effect depends on the oxygen-linked binding of chloride, and one may speculate that both phenomena result at least in part from the involvement of the same sites. The modulation of the Bohr effect by an allosteric effector is not a novel finding; in some respiratory pigments, like mammalian hemoglobins, the oxygen-linked binding of anions, in particular organic phosphates, simply enhances the intrinsic Bohr effect of the deionized protein (Riggs, 1971; De Bruin et al., 1973; Antonini et al., 1982), whereas in others, like the giant erythrocruorin from Octolasium complanatum, the Bohr effect is due totally to the oxygen-linked binding of cations (Santucci et al., 1984). How much of the acid Bohr effect is due to the allosteric binding of anions in S. inaequivalvis HbII one cannot establish due to the difficulties encountered in performing detailed oxygen equilibrium experiments with the deionized protein.

At this point it becomes tempting to speculate on the possible location of the high affinity anion-binding sites. The requirement these sites have to meet is that they be located on the surface of the molecule where they can feel conformational differences between the oxy- and deoxy-derivatives. The cluster of positively charged residues at the end of the pre-A helix in the contact region between two dimers in the tetramer formed by Lys 15 and Arg 19,20 in the IIA chain and by Lys 17 and Arg 21 in the IIB chain (Petruzzelli et al., 1989) appears as an appealing candidate for the first type of binding site because the pre-A and H helices form an extensive contact in the HbII crystals (Royer et al., 1985). No suggestions as to the location of the second type of binding site can be put forward in the absence of a high resolution structure.

In conclusion, the characterization of the unusual anion-linked polymerization of S. inaequivalvis HbII has brought new insight into its linkage with the binding of other ligands, namely oxygen and protons. The data obtained allow a detailed description of the complex linkage pattern that can be accounted for by the presence of two different types of high affinity anion-binding sites, one resulting from the juxtaposition of lysine residues belonging to different tetramers, and the other consisting of a salt bridge between a carboxylate and a lysyl residue. The latter site may be involved also in the modulation of the acid Bohr effect and hence may be of special functional relevance.

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Polymerization of Scapharca Hemoglobin

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