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.

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 (Pe-truzzelli 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 protein and after selective acylation of 1 or 2 reactive lysine residues (Boffi et al., 1987). More recently, the specific role of anions was confirmed in a study of the ion binding properties of the two S. inaequivalvis hemoglobins by \(^{35}\)Cl and \(^{23}\)Na NMR. The HbII molecule possesses high affinity chloride-binding sites, and binding of the anion to these sites gives rise to a chloride-linked polymerization process that manifests itself in an unusual dependence of the \(^{35}\)Cl excess line width on the concentration of the anion. In fact, the residence time of the transiently bound chloride ions on the hemoglobin tetramer is such that the \(^{35}\)Cl excess line width is proportional to the correlation time of the molecule; hence, the \(^{35}\)Cl excess line width provides a very sensitive probe of polymerization but cannot be used for the determination of the chloride ion-binding constant. Polymer formation is more marked in the deoxygenated form of the protein but can be observed readily also in the oxygenated derivative where it had never been detected by means of 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 \textit{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 \textit{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 oxygenated form.

Sedimentation velocity experiments were carried out on a Spinco model E ultracentrifuge equipped with an RTIC unit at 44,000 or 59,000 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 ensures complete deoxygenation; the dithionite concentration in such buffers was <2 \(\times 10^{-7}\) 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 pattern, at protein concentrations around 6 mg/ml; usually the same deoxy-derivative was used for all experiments. 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 (1961). 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 equilibria 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 \(K_0\) and \(L_0\) were taken from Ikeda-Saito et al. (1983) and were held constant in all the experiments; in particular, the value of \(L_0\) has been calculated by fitting the data measured at 0.16 mg/ml where polymerization is negligible. The values of \(L_0\), relative to the n-mers, were calculated according to the relationship \(L_n = L_0^n (A_n/B_n)\) where \(A_n\) and \(B_n\) are the association constants of the T and R states, respectively. The fitting procedure was carried out with the Newton-Raphson method on a Hewlett-Packard 87.

**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 \(^{35}\text{Cl}\) 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 \(s_{20,w}\) 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 dimercianion 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 shown as a function of the NaCl concentration (in mM) at pH 6.3 and 20 ± 1 °C.

**Table I**

<table>
<thead>
<tr>
<th>(C_{NaCl}) (mM)</th>
<th>(T^\circ) (°C)</th>
<th>(s_{20,w} (X 10^9))</th>
<th>(d_{20} (X 10^7))</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>8–12</td>
<td>4.15 ± 0.04 (8)</td>
<td>6.74 ± 0.16 (8)</td>
</tr>
<tr>
<td>20</td>
<td>8–12</td>
<td>4.25 ± 0.04 (8)</td>
<td>7.14 ± 0.21 (8)</td>
</tr>
<tr>
<td>5</td>
<td>20–25</td>
<td>4.24 ± 0.01 (8)</td>
<td>7.10 ± 0.27 (7)</td>
</tr>
<tr>
<td>20</td>
<td>20–25</td>
<td>4.32 ± 0.06 (7)</td>
<td>7.70 ± 0.23 (6)</td>
</tr>
</tbody>
</table>

**Table II**

<table>
<thead>
<tr>
<th>(C_{NaCl}) (mM)</th>
<th>(K_{1,5}) (mM/mg)</th>
<th>(K_{1,5} (mL/mg^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>3 \times 10^{-2}</td>
<td>6.5 \times 10^{-4}</td>
</tr>
<tr>
<td>10</td>
<td>4 \times 10^{-2}</td>
<td>1.1 \times 10^{-4}</td>
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<tr>
<td>20</td>
<td>5 \times 10^{-2}</td>
<td>1.0 \times 10^{-4}</td>
</tr>
<tr>
<td>100</td>
<td>1 \times 10^{-2}</td>
<td>1.2 \times 10^{-4}</td>
</tr>
</tbody>
</table>

In the simulations, \(s^\circ\) was taken as 4.435 S, and the sedimentation constants of the n-mers were calculated using the relationship \(s_n^\circ = s^\circ / \sqrt{n}\); \(D_n\) values were calculated using the equation \(M_n = s_n^\circ RT / [D_n (1 - GP)]\) using \(M_n = 64,800\) and \(V = 0.745\).
FIG. 1. Sedimentation velocity of the faster component of deoxygenated S. inaequivalvis HbII as a function of chloride ion concentration (C) and temperature at pH 6.2–6.5. The sedimentation velocity of the slower component corresponds to 4.3 ± 0.2 S and is indicated by the arrow. Type of cation: ○, Na⁺; □, BisTris. Different symbols refer to different preparations. Temperature, 20–24 °C (open symbols); 10–13 °C (filled symbols). Protein concentration (in mg/ml): ○, 6.2–7; □, 4.6–5.3; ○, 4.8; □, 6.4. The insets show relevant Schlieren patterns taken 52 min after reaching full speed of 52,000 rpm at 23 °C.

Moreover, the system responds to changes in temperature as expected on the basis of the data obtained in phosphate buffer (Chiancone et al., 1981); namely, both the extent of polymerization and the sedimentation velocity of the polymer decrease at the lower temperature. Fig. 1 also shows that the same extent of polymerization is obtained when BisTris¹ rather than sodium is used as cation. Again, representative Schlieren patterns obtained at varying chloride concentrations were compared with simulated ones. The formation of tetramers as the highest polymers of the native 4.3 S structure, which 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 representation of the observed sedimentation behavior of deoxy-HbII is obtained. The dependence of the equilibrium constants on chloride concentration given in Table II shows that polymerization follows the same trend as the maximum ordinate of the fast peak in Fig. 1; the latter value therefore provides a good estimate of the extent of polymerization in particular for comparative purposes.

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 Fig. 7, the observed peak has $S_{20,w} = 4.4$ S and $D_{20} = 9-10 \times 10^{-7}$ cm²/s; on the basis of the equilibrium constants given in Table II, at 5 mM chloride only 5% of the protein (by weight) is associated into tetramers, and no octamers are formed, whereas at 20 mM chloride, 9% tetramers and 1% octamers are formed.

In a further series of experiments performed only on 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

1 The abbreviation used is: BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.

FIG. 2. Sedimentation velocity patterns of deoxygenated S. 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): ○, 2.2; □, 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.

FIG. 3. Sedimentation velocity of the faster component of deoxygenated S. inaequivalvis HbII as a function of the concentration of phosphate buffer at pH 7.0 (A) or NaClO₄, at pH 6.6–6.9 (B). The sedimentation velocity of the slower component corresponds to 4.3 ± 0.2 S and is indicated by the arrow. The concentration (C) of phosphate is expressed also in terms of the concentration of negative charges (charge equivalents/liter) calculated from the dissociation of phosphate anions at pH 7.0. Different symbols refer to different preparations. Temperature, 20–23 °C (open symbols); 6–11 °C (filled symbols). Protein concentration (in mg/ml) in A: ○, 4.8; □, 6.2–6.6; △, 4.2–5.3; in B: ○, 6.2–6.4. The insets show relevant Schlieren patterns taken 67 min after reaching full speed of 52,000 rpm at 20 °C.

phosphate and perchlorate concentration are presented in Fig. 3, where the sedimentation coefficient of the polymer again has been taken as a measure of the extent of polymerization. In the presence of phosphate, polymerization goes through a maximum, as already observed in the case of chloride. In contrast, in the presence of perchlorate, only a monotonic decrease in polymerization is observed over the range covered, namely at concentrations above 5 mM. It should be mentioned that in perchlorate, oxy-HbII sediments as a tetrameric protein.

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
brought out by the sedimentation velocity study regarding the values, namely 6.3 and 5.5, both at high (5-7 mg/ml) and low 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 \(p_{1/2}\) 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

\[\log p_{1/2}\] 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 erythrocrurin (Frigon and
that oxy-HbII sediments as a single peak whose sedimentation velocity experiments reconcile the 35C1 polymerization pattern is observed in the presence of chloride, presence of chloride ions irrespective of whether sodium or phosphate, or perchlorate when Na⁺ is the common cation. Since ionic interactions, like hydrophobic interactions, are predominantly entropic (Kauzmann, 1959), there is no justification for 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 35C1 NMR experiments showed that the HbII molecule binds sodium ions in quadrupole relaxation NMR experiments. These measurements 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 anion was shown to bring about an oxygen-linked polymerization process in both the liganded and unliganded forms of the protein (Chiancone et al., 1988). In line with the different affinity of HbII for 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 ions irrespective of whether sodium or phosphate, or perchlorate when Na⁺ is the common cation (Figs. 1 and 3). Also, the effect of temperature can be rationalized in the framework of an anion-dependent polymerization. Since hydrophobic interactions, like hydrophobic interactions, are predominantly entropic (Kauzmann, 1959), there is no need to assume that the cold lability of HbII polymers reflects hydrophobic 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 35C1 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 35C1 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 35C1 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 derivative 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 formation 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 polymerization 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 cooperativity than the tetrameric structure as anticipated 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 obtained by maintaining the allosteric parameters Kₚ and L₀ constant and by varying K₇ as a function of chloride concentration. This choice entails that K₇ 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 K₇ 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 pHi exerts on the chloride concentration dependence of polymer formation. Thus, at pH 6.3, polymerization 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 reciprocal way, a decrease in pH from 6.3 to 5.5 at a constant concentration of 50 mM chloride results in a significant decrease in 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 tetrameric molecules. Binding of a single chloride ion to this site at low enough chloride concentrations could bridge the positively charged residues and thus favor polymer formation, whereas binding of two anions at higher chloride concentra-
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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, 0.025 (50 mM NaCl, ■—■); c, 0.050 (100 mM NaCl, ◊—◊).

The so-called second type of high affinity chloride-binding site may be involved in the acid Bohr effect. This possibility is suggested by the oxygen-binding data of Fig. 7 which were obtained at a low protein concentration, where the extent of polymerization of deoxy-HbII is negligible. Under these conditions, the size of the acid Bohr effect decreases by a very 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., 1985) appears as an appealing candidate for the first type of binding site also 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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