Mutual Effects of Protons, NaCl, and Oxygen on the Dimer-Tetramer Assembly of Human Hemoglobin

THE DIMER BOHR EFFECT*

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The dimer-tetramer equilibrium constants of human oxyhemoglobin ($K_o$) and deoxyhemoglobin ($K_d$) have been determined at 21.5°C as a function of pH and chloride concentration. In buffers containing 0.1 M NaCl, 1 mM EDTA, the apparent numbers of protons released upon assembly of dimers into tetramers were determined from the pH dependencies of $K_o$ and $K_d$. At pH 7.4, the assembly of unliganded tetramers is accompanied by the absorption of 0.9 ± 0.1 mol of $H^+$. For assembly of oxyhemoglobin, there are 0.8 ± 0.1 mol of $H^+$ released per mol of tetramer formed. From these results and the value of 2.1 mol of $H^+/4O_2$ for the tetramer Bohr effect, a Bohr effect for dimers is determined as 0.2 ± 0.8 mol of $H^+$ released upon binding 2 mol of $O_2$. Thus, the dimer Bohr effect is approximately 20% as large as the tetramer Bohr effect.

At pH 7.4, the value of $K_d$ is insensitive to [Cl$^-$], whereas $K_o$ varies inversely with [Cl$^-$]. At pH 8.95, both $K_o$ and $K_d$ decrease with increasing [Cl$^-$]. These and previous results indicate that salt bridges are not the dominant energetic factor in stabilizing the deoxy quaternary structure of hemoglobin.

In buffer conditions of 0.1 M Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4, we estimate 1.8 mol of Cl$^-$ bound upon dissociation of 1 mol of oxy tetramers into oxy dimers, whereas the deoxy molecules dissociate without any change in bound chloride. From the [Cl$^-$] dependence of oxygenation curves, we estimate 1.8 mol of [Cl$^-$] released upon binding 4 mol of $O_2$ to tetramers. Thus, oxygenation of dimers at pH 7.4 apparently involves no change in bound chloride.

The functional properties of human hemoglobin include the cooperative binding of oxygen and protons, carbon dioxide, organic phosphates, and other anionic species, particularly chloride. Understanding the self-regulatory mechanism of this allosteric system requires a resolution of the mutual influence of these small molecules upon their interactions with the hemoglobin tetramer and also of their effect on the subunit-subunit interactions within the quaternary structures of the functional molecule. In a series of thermodynamic studies, of which this work forms a part, we have been pursuing a comprehensive approach to the energetic aspects of this problem. Experimental studies on normal human hemoglobin have been reported, describing (a) the linkage between dimer-tetramer assembly and oxygen binding (1-10), (b) the self-association and oxygenation properties of isolated $\alpha$ and $\beta$ chains (11-14), and (c) the assembly of hemoglobin tetramers from isolated chains (15). The resulting set of energetic properties, obtained under comparable conditions for these processes, has permitted an evaluation of the possible sources of dominant thermodynamic effects (16-18), and provides a basis for rigorously testing mechanistic theories of hemoglobin (10, 20). Corresponding studies of mutant and chemically modified hemoglobins (21-23) provide a basis for considering the possible roles of specific amino acid residues in the cooperative mechanism, and also for testing concepts of the roles of local effects versus general system properties in protein chemistry (23).

In this paper, we report experimental studies on the effects of pH and chloride concentration on the dimer-tetramer assembly of human hemoglobin in both the unliganded and fully oxygenated states. The results, in combination with those of previous studies, provide new insight into the mutual influence of the three ligands upon subunit interactions in the hemoglobin system. A recent study by Atha and Riggs (21) on the pH dependence of the dimer-tetramer equilibria led these authors to conclude that the dimers had essentially no Bohr effect. However, Rollema et al. (24) reached the opposite conclusion from their measurements of assembly-linked proton absorption combined with the Atha-Riggs results. Their analysis leads to a negative Bohr effect for the dimers, i.e., an absorption of protons upon oxygenation. By contrast, Gray (25) interpreted the kinetic behavior in flash photolysis experiments with CO-hemoglobin to indicate a positive Bohr effect for the $\alpha\beta\delta$ dimers. Results of the present study clearly establish that oxygenation of the dimers is accompanied by the release of protons at pH 7.4.

MATERIALS AND METHODS

Hemoglobin Preparation—Fresh blood was obtained from normal adult nonsmoking donors. The major component of hemoglobin, Hb $\alpha_2\beta_2$, was isolated as oxyHb using the method of Williams and Tsay (26). This method also removes organic phosphates.

Haptoglobin Preparation—Human haptoglobin types 1-1, 2-1, and 2-2 were prepared according to the method of Connell and Shaw (27) with minor modifications as described in an earlier paper (3).

Buffers—Buffers used in the pH dependence studies consisted of 0.1 M bis-Tris (pH 6.5), 0.1 M Tris (pH 7.2 to 8.5), or 0.1 M glycine (pH 9.85 to 9.5) with 10 mM Na$_2$EDTA and 0.1 M NaCl, titrated to the desired pH with concentrated HCl. The total Cl$^-$ concentration of these buffers was 0.18 M. For the chloride dependence studies, buffers were prepared by titrating the free base form of Tris or glycine with concentrated HCl and adding enough NaCl to bring the buffer to the desired total anion concentration. Titration and pH measurements on

The abbreviation used is: bis-Tris, [bis-(2-hydroxyethyl)amino]tris(hydroxymethyl)methane.

1980)
each buffer were carried out at 21.5°C. Tris, glycine, bis-Tris, and EDTA were purchased from Sigma; NaCl and HCl were obtained from Fisher.

**RESULTS**

**Dimer Bohr Effect**

**pH Dependence of the Dimer-Tetramer Association Constants**

**Oxyhemoglobin**—The weight average partition coefficients (G_\text{av}) measured as a function of oxyhemoglobin concentration and pH are shown in Fig. 1. From the numerical analysis of these data, the association reaction was found to be in close conformity with a dimer-tetramer stoichiometry under all conditions. The equilibrium constants (K_2) at the various pH values, calculated from the respective data sets of Fig. 1, are shown as the lower data points in Fig. 2. It can be seen that the value of K_2 increases with decreasing proton concentration (increasing pH). The slope at each pH can be interpreted in terms of the linked function relationship:

\[
\frac{dn}{d\text{pH}} = \frac{2}{K_2} \Delta n_{H^+}.
\]

where \(\Delta n_{H^+}\) is the change in the number of moles of protons bound by 1 mol of tetramers compared with the 2 mol of dimers from which it is formed. For oxyhemoglobin (i = 4) at pH 7.4 in 0.1 M Tris, 0.1 M NaCl, and 1 mM EDTA, the data of Fig. 2 yield 0.8 ± 0.1 mol of protons released upon formation of 1 mol of oxy tetramers from 2 mol of oxy dimers. These data for oxyhemoglobin are in good agreement with those of Atha and Riggs (21) obtained under comparable conditions. Their data yielded a value of 0.6 ± 0.2 mol of H\(^+\)/mol of tetramer formed in the region of pH 6.5 to 8.5. The function \(K_2\) exhibits a biphasic character with a maximum near pH 8.5. Our results and those of Atha and Riggs for liganded hemoglobin (CO or oxygen) do not agree with those reported by Barksdale and Rosenberg (29), who reported a trisphic pH dependence of \(K_2\) with a maximum near pH 7.0.

**Deoxyhemoglobin**—Table I lists the pH dependence of the deoxyhemoglobin dimer-tetramer association rate constants (k_1) and the dissociation rate constant (k_2). The association rate constant does not vary much with pH, whereas the reverse rate constant (k_2) is seen to increase substantially with increasing pH. The three morphologically different types of haptoglobin (types 1-1, 2-1, and 2-2) yield very similar kinetic results as shown in Table II. The deoxy dimer-tetramer association equilibrium constants (K_2) (calculated from those rate constants) are found to decrease from 2.31 \times 10^{-11} \text{ M}^{-1} at pH 6.5 to 4.69 \times 10^{-10} \text{ M}^{-1} at pH 9.5 (see Table I and Fig. 2). The slope at pH 7.4 indicates (by Equation 5) that the association of deoxy dimers to tetramers is accompanied by the absorption of 0.9 ± 0.1 mol of protons/mol of tetramer formed. From combination of these results with the corresponding ones for oxyhemoglobin, we have calculated, at each pH, the net free energy of stabilization for deoxy tetramers as compared with oxy tetramers: \(\Delta G_{\text{SO}} = \Delta G_2 - \Delta G_{\text{O}}\). It can be seen (Table III) that the total linkage energy, \(\Delta G_{\text{SO}}\), decreases markedly with increasing pH.

Atha and Riggs (21) analyzed a combination of data from various laboratories according to Equation 5 and obtained a value of \(\Delta n_{H^+} \approx -1.8\), i.e. 1.6 mol of protons apparently were absorbed per mol of tetramer formed. The high pH data used in their analysis, taken from a variety of literature sources, represent experiments performed mostly at considerably lower chloride concentrations than those of the present study, and in the absence of EDTA. In order to investigate the chloride concentrations and EDTA effects as possible sources of the discrepancy between our results and theirs, we carried out a series of experiments in buffers containing 0.1 M Tris or glycine, titrated to each pH but with no additional anions added. The results are shown in Fig. 2 as the dashed line. By comparison of these results with the solid line of Fig. 2 for
deoxyhemoglobin, it can be seen that the altered anion composition has little effect on \(K_2\) in the pH region near neutrality. However, at high pH, the lower anion composition results in a significant decrease in \(\log K_2\). The slope of the dashed line corresponds to 1.4 mol of protons absorbed per mol of deoxyhemoglobin tetramer formed. These results, obtained at low anion concentration, are similar to those analyzed by Atha and Riggs (21) for unliganded hemoglobin. It thus appears that the apparent discrepancy in results obtained in this study and those of Atha and Riggs (21) can be attributed to the previously unknown sensitivity of \(K_2\) to anion concentration at high pH.

Further experiments were conducted to investigate the effect of EDTA on \(K_2\) in glycine buffer in the absence of added chloride. At pH 8.95 and with no EDTA, a value of \((3.99 \pm 0.71) \times 10^{-9} \text{ M}^{-1}\) was obtained for \(K_2\). In the same buffer to which 1 mM EDTA was added, \(K_2\) increased to \((2.01 \pm 0.43) \times 10^{-9} \text{ M}^{-1}\). Thus, the addition of EDTA was found to be capable of producing essentially all of the difference between the high pH values for \(K_2\) of Fig. 2. By contrast, at pH 7.4 in Tris buffer, the corresponding values of \(K_2\) were: \((4.77 \pm 0.22) \times 10^{-9} \text{ M}^{-1}\) with no EDTA, and \((3.32 \pm 0.68) \times 10^{-9} \text{ M}^{-1}\) in the presence of 1 mM EDTA. The greater effect of EDTA in stabilizing the deoxy tetramer at high pH may be associated with increased ionization of the EDTA anion. A systematic investigation of chloride effects on \(K_2\) at pH 7.4 and 8.95 at constant EDTA concentration is presented in a later section of this paper.

The Dimer Bohr Effect

The experimental results described in the previous section provide values of \(\Delta_{\text{dimer}}\) and \(\Delta_{\text{tetramer}}\), derived from pH dependence measurements obtained under constant concentration of total chloride. In combination with the value of 2.1 \(\pm 0.1\) mol/4O_2 obtained for the tetramer Bohr effect (30, 31), these results permit an estimate to be made for the oxygenation-linked change in protonation of dimers at pH 7.4. The linkage relationships for subunit assembly and ligation are depicted in the scheme:

\[
\begin{align*}
    \frac{3}{2} \beta_1 & + \alpha_2 \xrightarrow{\text{IV}} 2(a\beta) \xrightarrow{\text{VI}} (a\beta_2) \\
    1_x & \xrightarrow{L} 2X \xrightarrow{\text{II}} 4X \xrightarrow{\text{III}} 4X \\
    \frac{3}{2} (\beta_4 X_4) + \alpha_2 X_2 & \xrightarrow{\text{V}} 2(a\beta) X_1 \xrightarrow{\text{VII}} (a\beta_2) X_1
\end{align*}
\]

where \(X\) represents a heme-site ligand, either O_2 or CO. The change in proton ionization accompanying Reaction II is calculated from the experimentally determined values for Reactions III, VI, and VII:

\[
    n_{\text{dimer}}^{\text{oxygenated}} = n_{\text{dimer}}^{\text{unliganded}} + \Delta_{\text{dimer}} = \Delta_{\text{tetramer}}.
\]

where \(n_{\text{dimer}}^{\text{oxygenated}}\) and \(n_{\text{dimer}}^{\text{unliganded}}\) are the number of oxygenation-linked protons for the dimers and tetramers, respectively. Using values of the terms on the right side of Equation 6 pertaining to pH 7.4, we obtain a value of 0.4 \(\pm 0.2\) for \(n_{\text{dimer}}^{\text{oxygenated}}\). Thus, the dimer Bohr effect at pH 7.4 is approximately 20% as great as the tetramer Bohr effect under the same conditions, and operates in the same direction, i.e. 0.2 mol of protons are released upon fully oxygenating 1 mol of dimers.

From these results and those of previous studies on the Bohr effect of isolated \(\alpha\) and \(\beta\) chains, carried out under buffer conditions comparable to those of this study but at 25°C (32), we have listed (Table IV) the known changes in proton binding
it is possible to interpret the slope of the data in Fig. 3 in terms of the difference \( \Delta n_{\text{Cl}} \) in the number of moles of Cl\(^-\) bound upon formation of 1 mol of tetramer from its constituent dimers. At pH 7.4, the region where [Cl\(^-\)] = 0.07 to 0.2 M, the data for oxyhemoglobin yield slopes corresponding to the release of 1.8 mol of chloride/mol of oxyhemoglobin tetramer formed.

\[ \Delta n_{\text{Cl}} = \Delta n_{\text{Cl}}^{\text{ag}} + \Delta n_{\text{Cl}}^{\text{ag}} + \Delta n_{\text{Cl}}^{\text{ag}} = \Delta n_{\text{Cl}}^{\text{ag}} + \Delta n_{\text{Cl}}^{\text{ag}} \]

yields \( \Delta n_{\text{Cl}}^{\text{ag}} = 0 \). Thus, the oxygenation of dimers (Reaction II) is apparently accompanied by no appreciable change in bound chloride.

**TABLE II**

<table>
<thead>
<tr>
<th>pH of buffer</th>
<th>Haptoglobin type</th>
<th>( k )</th>
<th>( k_1 )</th>
<th>( k_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>1-1</td>
<td>( (2.2 \pm 0.1) \times 10^{-5} )</td>
<td>( (2.2 \pm 0.2) \times 10^{-5} )</td>
<td>( (2.1 \pm 0.3) \times 10^{-5} )</td>
</tr>
<tr>
<td>8.0</td>
<td>1-1</td>
<td>( (4.5 \pm 0.1) \times 10^{-5} )</td>
<td>( (3.0 \pm 0.1) \times 10^{-5} )</td>
<td>( (1.2 \pm 0.1) \times 10^{-5} )</td>
</tr>
<tr>
<td>8.5</td>
<td>1-1</td>
<td>( (1.2 \pm 0.1) \times 10^{-5} )</td>
<td>( (1.3 \pm 0.1) \times 10^{-5} )</td>
<td>( (5.3 \pm 0.1) \times 10^{-5} )</td>
</tr>
<tr>
<td>8.95</td>
<td>1-1</td>
<td>( (4.6 \pm 0.1) \times 10^{-5} )</td>
<td>( (5.0 \pm 0.3) \times 10^{-5} )</td>
<td>( (9.5 \pm 0.1) \times 10^{-5} )</td>
</tr>
<tr>
<td>9.5</td>
<td>1-1</td>
<td>( (9.5 \pm 0.1) \times 10^{-5} )</td>
<td>( (9.5 \pm 0.1) \times 10^{-5} )</td>
<td>( (9.5 \pm 0.1) \times 10^{-5} )</td>
</tr>
</tbody>
</table>

**TABLE III**

<table>
<thead>
<tr>
<th>pH</th>
<th>( \Delta G_1 )</th>
<th>( \Delta G_2 )</th>
<th>( \Delta G_{\text{OxyHb}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>-15.3 \pm 1.0</td>
<td>-7.0 \pm 1.0</td>
<td>8.3 \pm 0.2</td>
</tr>
<tr>
<td>7.2</td>
<td>-14.3 \pm 1.0</td>
<td>-7.7 \pm 1.0</td>
<td>6.6 \pm 0.2</td>
</tr>
<tr>
<td>7.4</td>
<td>-14.4 \pm 1.0</td>
<td>-8.1 \pm 1.0</td>
<td>6.3 \pm 0.2</td>
</tr>
<tr>
<td>8.0</td>
<td>-13.9 \pm 1.0</td>
<td>-8.9 \pm 1.0</td>
<td>5.1 \pm 0.1</td>
</tr>
<tr>
<td>8.5</td>
<td>-13.2 \pm 1.0</td>
<td>-9.2 \pm 1.0</td>
<td>4.0 \pm 0.2</td>
</tr>
<tr>
<td>8.95</td>
<td>-12.3 \pm 0.2</td>
<td>-9.0 \pm 0.1</td>
<td>3.3 \pm 0.3</td>
</tr>
<tr>
<td>9.5</td>
<td>-11.6 \pm 0.1</td>
<td>-8.5 \pm 0.1</td>
<td>3.1 \pm 0.2</td>
</tr>
</tbody>
</table>

**Effect of Chloride Concentration on the Dimer-Tetramer Association Constants**

Oxyhemoglobin—In order to clarify the relation between anion binding and the pH-dependent dimer-tetramer association, the equilibrium constants \( K_2 \) and \( K_3 \) were measured at different chloride concentrations but constant EDTA concentration (1 mM) at two pH values (7.4 and 8.95). The results for oxyhemoglobin are shown in Fig. 3B. At both pH values, \( K_2 \) is found to decrease with increasing chloride concentration. Based on the linkage relationship

\[
\frac{d \ln K_2}{d \ln [\text{Cl}^-]} = \Delta n_{\text{Cl}}
\]

which accompany the various reactions of Scheme 1. The values listed as \( \Delta n_{\text{Cl}} \) refer to the number of moles of protons released.
which exists in the mutual influence of the various interacting
Bohr effect, leads to the requirement that protons are released
and Edelstein
we have presented here clearly demonstrates that the proton-
reactions are dependent on chloride concentration. The work
upon oxygenation of dimers. Previously, Atha and Riggs
assembly reactions to be proton-linked as well. Thomas and
resolution of the various effects.
NaCl, along with the well established value for the tetramer
components of the hemoglobin system. The work of Thomas
and points to the need for much more extensive experimental

TABLE IV

<table>
<thead>
<tr>
<th>Reaction</th>
<th>( \Delta n'_{1+} )</th>
<th>( \Delta n'_{2+} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ia</td>
<td>0.1*</td>
<td>ND*</td>
</tr>
<tr>
<td>Ib</td>
<td>0.0*</td>
<td>ND</td>
</tr>
<tr>
<td>II</td>
<td>0.4*</td>
<td>0.9*</td>
</tr>
<tr>
<td>III</td>
<td>2.1*</td>
<td>1.8*</td>
</tr>
<tr>
<td>IV</td>
<td>0.0*</td>
<td>ND</td>
</tr>
<tr>
<td>V</td>
<td>0.0*</td>
<td>ND</td>
</tr>
<tr>
<td>VI</td>
<td>-0.9*</td>
<td>0.0*</td>
</tr>
<tr>
<td>VII</td>
<td>0.8*</td>
<td>-1.8*</td>
</tr>
</tbody>
</table>

* From Ref. 31. Values pertain to 25°C.
* ND, not determined to date.
+ From results of this study, in combination with the value of \( n_{1+} \), from Refs. 30 and 31.
* Estimated from values from Reactions III, VI, and VII.
* From Refs. 30 and 31.
+ From data of Refs. 33 and 34.
* Values derived from results of this study and that of Ref. 32.
* From the variation of \( K_c \) (\( i = 0.4 \)) with concentration of ligand (H⁺ or Cl⁻).

**DISCUSSION**

The results of this study illustrate the delicate balance which exists in the mutual influence of the various interacting components of the hemoglobin system. The work of Thomas and Edelstein (35), Atha and Riggs (21), and Rollema et al. (24) have previously demonstrated the oxygenation-linked assembly reactions to be proton-linked as well. Thomas and Edelstein (35) also found that the dimer-tetramer assembly reactions are dependent on chloride concentration. The work we have presented here clearly demonstrates that the proton-oxygen assembly linkages are in turn dependent upon chloride concentration. This study provides an initial delineation of some of the interrelationships between these linked functions, and points to the need for much more extensive experimental resolution of the various effects.

**The Dimer Bohr Effect**—The results in this study provide strong evidence for a Bohr effect in dimers of human hemoglobin. The finding of 0.9 mol of H⁺ absorbed in Reaction VI and 0.8 mol of H⁺ released in Reaction VII at pH 7.4, 0.1 M NaCl, along with the well established value for the tetramer Bohr effect, leads to the requirement that protons are released upon oxygenation of dimers. Previously, Atha and Riggs (21) concluded that the dimers have no appreciable Bohr effect, based on their estimates of \( \Delta n'_{1+} = -1.6 \), and \( \Delta n'_{2+} = 0.6 \).

These values, however, were derived from data representing different concentrations of anion. As shown in this study, the effect of anions on the pH dependence of \( K_c \) is sufficient to account for the difference between \(-1.6\) and \(-0.9\) in values of \( \Delta n'_{1+} \).

Recently, Rollema et al. (24) have measured directly the total changes in proton binding which accompany assembly of oxy and deoxy tetramers from \( \alpha \) and \( \beta \) chains. Their measurements were made at 25°C under buffer conditions otherwise comparable to those of the present study. They found 0.9 mol of H⁺ absorbed upon total assembly of deoxy tetramers. For total assembly of liganded hemoglobin, they measured a release of 0.7 mol of H⁺/mol of tetramer formed. These experimental values for total assembly are identical with the values estimated in the present study for \( \Delta n'_{1+} \) and \( \Delta n'_{2+} \), respectively. Thus, to within experimental errors (which may arise in part from the 3.5°C temperature difference), it appears that the assembly-linked change in protonation arises entirely from the dimer-tetramer stage of assembly in both the oxy and deoxy states. This conclusion is in agreement with that of Rollema et al. (24) as regards the assembly of oxyhemoglobin, but is in disagreement with their conclusions regarding assembly of deoxy hemoglobin. They interpreted their data, in combination with the result of Atha and Riggs (21) \( \Delta n'_{1+} = -1.6 \) to imply a release of 0.7 mol of H⁺ upon formation of two deoxy dimers from isolated chains, i.e. Scheme 1, Reaction IV.

Using the values of \( n_{1+} = 0.7 \) and 0.0 for Reactions IV and V, respectively, and the previously determined values for the Bohr effect of isolated chains (32) (see Table IV), Rollema et al. (24) concluded that the dimers have a Bohr effect. Use of the proton balance around the left side of Scheme 1 (Reactions I, II, IV, and V) to calculate the protonation change for Reaction II would lead to a negative Bohr effect, i.e. an absorption of 0.5 mol of protons upon oxygenation of dimers.

When we consider the positive dimer Bohr effect established by the present studies in relation to the other reactions of Scheme 1, we find consistency with all known values. Estimating the uncertainties in values of \( n_{1+} \) for Reactions IV and V conservatively to be ±0.1 mol of H⁺, and taking the value of 0.4 ± 0.2/40°C mol of H⁺ for the dimer Bohr effect, we find considerable uncertainty in the calculated value for the average Bohr effect in isolated chains: \( n_{1+} = 0.4 ± 0.1 \) mol of H⁺/40°C. This result is consistent with the experimentally determined Bohr effects for the chains (Table IV). It should be noted that the apparent Bohr effects of isolated chains determined by Rollema et al. (32) do not take into account
the possibility of protonation-linked changes in subunit association, concerning which almost no information presently exists. The same factor may contribute to the directly measured values of protonation change upon mixing solutions of the separate chains (22). The conclusion that neither Reactions IV nor V are proton-linked is consistent with the finding of essentially identical enthalpies of assembly for the dimers in their unliganded and fully oxygenated states (14, 15). Protonation-linked reactions may be accompanied by appreciable heat effects arising from the protonation reactions themselves (14).

Studies by Chiancone et al. (36) using $^{35}$Cl NMR indicate that there are at least two classes of chloride-binding sites, one of high affinity, the other of low affinity. The high affinity sites are oxygen-sensitive. Since the numbers of bound chloride do not change with oxygenation of dimers at pH 7.4, the observed dimer Bohr effect cannot be explained simply by a difference in interaction of chloride ions with the two forms of the protein. These considerations suggest that the dimer Bohr effect results from changes in tertiary structure upon dimer ligation.

Effects of Chloride on Stability of the Deoxy Quaternary Structure—In human hemoglobin, the intersubunit contact region between $\alpha^{\beta} \beta$ and $\alpha^{\alpha} \beta$ dimers contains specific chemical linkages (salt bridges, hydrogen bonds) and other interactions believed responsible for stabilizing the constrained quaternary structure of the molecule in its unliganded state (37). Crystallographic analyses indicate a total of six interchain bridges involving protonation of the amino acid side chains within the dimer-dimer contact region of the deoxy molecule. Perutz (37) has proposed that these salt bridges are the dominant factor in stabilizing the deoxy quaternary structure in relation to the oxy structure. If salt bridges were the dominant factor in accounting for the increased stability of deoxy tetratomers, the value of $K_2$ would be expected to decrease with increasing salt concentration, and the decrease in $K_2$ should be more pronounced than that of $K_1$. However, our chloride dependence studies (Fig. 3) show that at pH 7.4, the deoxyhemoglobin subunit association constant is not sensitive to chloride concentration, whereas in the oxy state, where the intersubunit salt bridges are presumably broken, $K_2$ varies inversely with anion concentration. At pH 8.95, where some of the salt bridges are broken, both $K_1$ and $K_2$ decrease significantly with increasing anion concentration. The results demonstrate that the quaternary interactions in deoxyhemoglobin other than salt bridges are less sensitive to NaCl than their counterparts in liganded Hb. These findings are consistent with the results of previous investigations (35, 38, 39) obtained at higher concentrations of NaCl. The enthalpic and entropic results determined in this laboratory (7, 10) have also suggested strongly that salt bridges are not the prime contributor to stabilization of the deoxy quaternary structure. Rollema and co-workers (31) have recently shown that the suppression of the alkaline Bohr effect by higher concentrations of neutral univalent salt is not caused by a weakening of the salt bridges in deoxyhemoglobin, but is due to an interaction of chloride ions with oxyhemoglobin. Our results, like those of Rollema et al. (31) show that at pH 7.4, deoxyhemoglobin has a higher affinity for chloride than does oxyhemoglobin.

Recent calculations of Gurd et al. (40) provide an estimate of the electrostatic contribution to tetramer formation from monomers. These and more recent calculations indicate that the total contribution of electrostatic free energy to the dimer-tetramer assembly process for deoxyhemoglobin is approximately $-4.2$ kcal/mol of tetramer at pH 7.4, and that this value is essentially independent of chloride concentration. Thus, electrostatic free energy may account for approximately one-third of the $-14.3$ kcal found for the total free energy of stabilization of the $a^{\beta}\beta$ contact in human hemoglobin. For oxyhemoglobin, the electrostatic contribution to $\Delta G$ is estimated to be $-6.7$ kcal, as compared with a total of $-8.0$ kcal (pH 7.4).

The effects of chloride and EDTA found in this study may, for the most part, represent general behavior of anionic species in their interactions with hemoglobin. In general, the effects of anions on the hemoglobin system may be classified in two categories: (a) specific binding at particular sites of the hemoglobin molecule, and (b) nonspecific binding and ionic strength effects. An ion which exhibits both types of effects might stabilize the tetramer at low concentrations but destabilize it at higher concentrations.

Molecular Interactions and the Bohr Effects—A number of recent studies suggest that the molecular mechanisms of the Bohr effects in human hemoglobin depend on the experimental conditions. Experimental results of the present study and that of Rollema et al. (31) on the anion dependence of the tetramer Bohr effect, as well as the elegant NMR studies of Russu et al. (41) provide experimental evidence for this concept. The electrostatic calculations of Matthew et al. (42) indicate that as many as 11 ionizable groups may contribute to the tetramer Bohr effect and that their relative contributions are strongly modulated by chloride anions.

There are now six Bohr effects identifiable in the hemoglobin system, i.e. those associated with Reactions I, I, II, III, VI, and VII. It is not clear from results presently available to what extent these separate Bohr effects may involve the same ionizable groups. However, it is worth noting some interesting observations related to this issue. Recent x-ray diffraction and solution studies by O’Donnell et al. (34) indicate that two inorganic anion-binding sites are associated with each a chain NH$_2$ terminus and are strongly linked to oxygen and proton affinity of hemoglobin. These intrasubunit salt bridges are formed by the binding of anions with the $\alpha$-amino group of Val 140 and the $\beta$-hydroxyl group of Ser 131a. It is possible that these groups may be responsible for the dimer Bohr effect. The possibility of His 122 of the $\alpha^{\beta}$ interface being a Bohr group has been suggested by Perutz (43), and the interaction of bound chloride with certain residues in deoxyhemoglobin, leading to a change in pK values upon oxygenation, is another possibility. A conspicuous possibility for contribution to the assembly-linked Bohr effects (Reactions VI and VII) is the His 97$\beta$ group, since this residue lies at the $\alpha^{\beta}\beta$ interface. One possibility is that assembly of tetratomers leads to masking of partially ionized groups. It is clear, in any case, that a number of further studies on the mutual influence of the various interacting components as well as additional structural information will be required in order to make definite assignments of the roles of specific groups under any given set of conditions.

REFERENCES

Dimer Bohr Effect