Characterization of the Ionizable Groups Interacting with Anionic Allosteric Effectors of Human Hemoglobin*

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Benzenehexacarboxylate binds preferentially to the deoxy form of human hemoglobin and lowers its oxygen affinity. Saturating amounts of this anionic cofactor produce changes in the oxygen affinity of hemoglobin A which are intermediate between those produced by 2,3-diphosphoglycerate and inositol hexaphosphate. Between pH 6 and 9 the interaction of deoxyhemoglobin A with benzenehexacarboxylate results in the absorption of protons. The proton absorption may be ascribed to three pairs of groups having pK values near 5.59, 6.78, and 7.66 where the pK values are shifted by 1.26 pH units upon interaction with benzenehexacarboxylate. In deoxyhemoglobin Deer Lodge (β2His → Arg) only two pairs of groups appear to be responsible for the proton absorption. Their apparent pK values are 5.55 and 7.18, respectively, with a ΔpK = 1.19 upon the interaction with benzenehexacarboxylate. These results are compared with the crystallographic models of Arnone (Arnone, A. (1972) Nature 237, 146-149) and Arnone and Perutz (Arnone, A., and Perutz, M. F. (1972) Nature 249, 54-56). It is proposed that in hemoglobin A the groups with pK = 5.59 are the β143 histidines; those with pK = 6.78 are the β1 valines and those with pK = 7.66 are the β2 histidines. In hemoglobin Deer Lodge, the groups with pK = 7.18 are inferred to be the β1 valines. Substitution of an aspartyl residue for a lysine in deoxyhemoglobin Providence-Asp(β82Lys → Asp) greatly reduces the interaction with the effector above pH 7.5 as anticipated by the mentioned models of Arnone and Arnone and Perutz.

The liganded form of hemoglobin A differs from its deoxy derivative in the interaction with benzenehexacarboxylate. In the liganded derivative the NH2 terminus of the β chain and the β2 histidine do not appear to experience pK changes upon addition of benzenehexacarboxylate. Thus the liganded derivatives of hemoglobins A and Deer Lodge (β2His → Arg) show very similar proton absorption. These findings suggest that the site at which anionic effectors are bound differs in liganded and unliganded hemoglobin. The difference could arise from conformational changes which make different groups available for interaction with the effector.

The CD spectrum in the Soret region of the carbon monoxide derivative of hemoglobin A is sensitive to benzenehexacarboxylate and inositol hexaphosphate while the same derivatives of hemoglobins Deer Lodge and Providence-Asp are not. In contrast, comparable CD spectra of the deoxy derivatives of the hemoglobins are all similar and do not show sensitivity to either benzenehexacarboxylate or inositol hexaphosphate.

Crystallographic models of Arnone (1) and Arnone and Perutz (2) indicate that the interaction of human deoxyhemoglobin with polyanion effectors is regulated by the electrostatic interaction of the negative charges of the effector with a cluster of 8 positive groups in the αβ2α tetrameric molecule. Some of these groups lose their positive charge when the pH is increased from pH 7 to 9. Studies on the interaction of human hemoglobin with BPC1 (3) showed that the carboxyl groups of the effector do not undergo changes in ionization state in this pH range. Consequently, measurement of the protons absorbed upon the interaction of deoxyhemoglobin and polycarboxylates was seen as a possible tool for studying the characteristics of the ionizable groups of hemoglobin which interact with anionic effectors. It has previously been shown that polycarboxylate effectors modulate hemoglobin oxygen affinity in a manner analogous to 2,3-diphosphoglycerate and other polyphosphate effectors (4). In this study we used BHC rather than BPC because it has a higher affinity for hemoglobin, making it possible for liganded hemoglobin to be approached (3, 4).

This paper reports a study of the ionization characteristics of side chains which in liganded and unliganded hemoglobin interact with polyanions. We used BHC as the effector, and Hb A plus two mutant hemoglobins as model proteins. The...
two mutant forms were hemoglobin Deer Lodge (αL-β2Liss-A166) and hemoglobin Providence-Asp (αL-β2Llls-Asp199). These two hemoglobins were chosen because their mutations involve the polyphosphate binding sites. Both of these hemoglobins show altered interaction with anionic effectors (5–7).

**EXPERIMENTAL PROCEDURES**

**Materials and Methods**

The various hemoglobins were prepared from washed red cells of freshly drawn blood. The hemolysates were treated with chloroform (5% v/v) or with ammonium sulfate (up to 20% saturation) (8), in order to remove the lipids and the stroma. Hemoglobin Deer Lodge and Providence-Asp were purified from the respective hemolysates by column chromatography as described. The final hemoglobin solutions were denatured, “stripped,” by recycling for 30–40 min through a mixed bed ion exchange column in the cold. Comparison with past records and a few ad hoc experiments indicated that the functional and optical properties of at least hemoglobins A and Deer Lodge were not affected by the various procedures used for their preparation.

The amount of ferric form in the solutions varied. The presence of residual ferric form was practically absent in the solutions of hemoglobin Deer Lodge and was approximately 40% in the solutions of hemoglobin Providence-Asp. In this case the ferric form was removed by filtration through Sephadex G-25 after reduction with sodium dithionite in 0.1 M phosphate buffer at pH 7.0. The amount of ferric form in solutions of hemoglobin Deer Lodge was disregarded when potential dynamic measurements were performed. The presence of residual amounts of ferric form in the mutant hemoglobins was the reason for performing spectropolarimetric measurements of their carbonmonoxy derivatives in the presence of 0.1 mg/ml of sodium dithionite.

Protein concentrations were measured spectrophotometrically using \( \epsilon = 14,000 \text{ cm}^{-1} \text{ M}^{-1} \text{heme} \) for the carbonyl derivatives at 540 nm, \( \epsilon = 13,000 \text{ cm}^{-1} \text{ M}^{-1} \text{heme} \) for the deoxy derivatives at 430 nm. These solutions were kept under nitrogen at all times.

Spectra of the carbonmonoxy derivatives were recorded with samples of normal hemoglobin. In such cases the same amount of sodium dithionite was added to the samples of normal hemoglobin.

Protein concentrations were measured spectrophotometrically assuming a stoichiometric relationship between hemoglobin and BHC. The BHC solutions were kept under carbon monoxide, in the presence of 0.1 mg/ml of sodium dithionite.

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Stoichiometry of Interaction—The stoichiometry of the reaction of deoxyhemoglobin with BHC was measured as described for the interaction of deoxyhemoglobin with BPC (5), i.e., monitoring the pH changes that accompany a stoichiometric addition of effector to hemoglobin solutions. Alternatively, the amount of HCl necessary to restore the original pH after each addition of effector was recorded. In the pH range where the high affinity of hemoglobin for the effector produced a stoichiometric reaction, a sharp break in the titration curve indicated the end point.

Analysis of Interaction—The difference in the number of protons bound by hemoglobin in the presence and absence of effector is given by Equation 1 (3):$
\ln \frac{[E]}{[C]} = \frac{\Delta H}{R} \frac{1}{T} - \frac{\Delta S}{R}
$
in which \( \Delta H \) is the number of protons absorbed upon interaction; \( Y \) is the fractional saturation of hemoglobin with effector; \( [E] \) and \( [C] \) are the concentrations of the free protein and of the complex, respectively. Thus at each intermediate step of the titration of hemoglobin with the effector.

Measurement of \( H_{max} \)—The proton binding behavior of BHC indicates that between \( \phi = 6 \) and \( 7 \) a small number of protons are bound by the reagent. The interaction of BHC with deoxyhemoglobin produces a complex that is produced upon formation of the hemoglobin BHC complex. This assumption was supported by the evidence that the interaction between deoxyhemoglobin and BHC produces a large \( H_{max} \) (higher than \( 1 \)) in the groups involved in the formation of salt bridges. Consequently the measured \( H_{max} \) values were corrected for the amount of protons liberated by BHC. In order to keep this correction within tolerable limits titrations were not performed below pH \( \phi = 6.0 \). Similar evidence could not be obtained for the interaction of BHC with oxyhemoglobin. In the case of liganded hemoglobin only raw data are reported, uncorrected for the number of protons liberated by BHC in the system below pH \( \phi = 7.0 \).

\( \phi \) Dependence of \( H_{max} \)—The \( \phi \) dependence of \( H_{max} \) is a function of the ionization of the positive groups in hemoglobin that interact with the effector. Because hemoglobin is a polyampholyte, we should take into consideration the electrostatic interactions produced by all of the other charges present in the protein, when studying the ionization characteristics of specific groups. Several thermodynamic treatments have been proposed for simulating these reciprocal interactions (13–15). It is questionable whether any of these apply to the case in point.

For example the treatment proposed by Lindström-Lang (13) is based on the assumption that the net charge of the protein is evenly distributed on the surface of the molecule. The process we are studying is \( \text{O}_{2} \)-binding on a small portion of the molecular surface, which is characterized by an excess of positive charges. Other treatments take into consideration the actual reciprocal distance between the charges (14, 15). In these treatments the
dielectric constant of the environment and the ionic strength of the solution play a major role. These two parameters are not available to us, because the cluster of positive groups will produce a redistribution of the anions in the surrounding solution, and possibly a restructuring of solvent molecules. Additionally, the models referred to assume the protein to be an impenetrable sphere. We are dealing with a cavity very well permeated by the solvent.

In the absence of an appropriate model, in the treatment that follows we took into consideration only the electrostatic interactions which produced salt bridges between the effector and the protein. As shown below, this over-simplification results in a notable internal consistency of the data.

Simulations of the pH dependence of $H_{\text{max}}$ were performed using the equation

$$\Delta H^\circ = \sum \left( \frac{K_i'}{(H^+ + K_i')^{-1} + (H^+ + K_i')^{-1}} \right)$$

where $K_i$ and $K_i'$ refer to the ionization constants of the various ionizable groups in the hemoglobin molecule in the presence and absence of BHC respectively; and $(H^+)$ is the proton activity. The data were processed with the help of the Marquardt algorithm (16); the Linear-Taylor-Differential-Correction procedure developed by McCulla (17) gave very similar results. Both these procedures require the input of initial estimates for the unknown parameters. These are then refined by the algorithms so as to minimize the sum of square residuals. In all cases the results obtained were independent from the initial estimates.

Fittings between simulated curves and experimental determinations were subjected to the following considerations. The number of groups involved in the interaction cannot be less than the maximum value of $H_{\text{max}}$, experimentally obtained. In addition, the models of Arnoff (1) and Arnoff and Perutz (2) indicate that eight positively charged groups in hemoglobin interact with the polyanions. These are the four pairs represented by the $\beta$1 valines, $\beta$2 histidines, $\beta$82 lysines, and $\beta$143 histidines. The absorption of protons produced by the $\beta$82 lysines is not detectable in the pH range investigated because of the high pK (near 10.2) of lysines in hemoglobin (18). Therefore the maximum number of groups detectable in our experiments could not be more than 6. Moreover the symmetrical structure of hemoglobin implies very similar or identical pK values for the groups involved in each pair. Consequently simulations were produced considering three pairs of interacting groups. In the case of hemoglobin Deer Lodge, where the $\beta$2 histidines are substituted by arginines, the high pK value of arginine decreases the maximum number of groups detectable in our experiments from the initial estimates.

This is the equation used for simulating the pH dependence of the affinity constant of hemoglobin for the effector.

### RESULTS

**Interaction of Deoxyhemoglobin A with BHC**—The pH dependence of oxygen affinity of stripped hemoglobin A in the presence and absence of DPG, BHC, or IHP is shown in Fig. 1. The effect of BHC is intermediate to that of DPG and IHP. The value of $n_{1/2}$, in Hill plots of the oxygen binding, remained between 2.5 and 2.8 in all cases. Representative Hill plots at neutral pH are shown in Fig. 2.

In Fig. 3 the titration of deoxyhemoglobin A with BHC was followed potentiometrically, monitoring the amount of HCl necessary to restore the original pH of the solution after each addition of BHC. The titration was performed near pH 7.0 where the reaction was essentially stoichiometric. The sharp break of the titration curve indicates a stoichiometric ratio of 1 mol of effector/mol of tetrameric hemoglobin.

The interaction of deoxyhemoglobin with BHC above pH 7.4 is nonstoichiometric. This makes it possible to produce a plot of the log $Y/(1 - Y)$ against the logarithm of the free...
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effector concentration. Fig. 4 shows such a plot for deoxyhemoglobin at pH 8.5. The slope of the curve is close to 1.0 (0.99) as expected for a simple noncooperative binding. The graphical interpretation of this plot gives a value of \( \log P = 4.62 \).

In Fig. 5 the open circles indicate the pH dependence of \( H_{max} \) for the interaction of deoxyhemoglobin with BHC. The interpolating line represents the best fit obtained with Equation 1, assuming that three pairs of identical groups were interacting with the effector with a common pK shift. The pK values assigned to the groups before the interaction with BHC were 5.59, 6.78, and 7.66, with a \( \Delta P_k \) of 1.26. Table I lists these parameters and the 95% confidence limits obtained for them. We will call the groups with pK = 5.59 the acid groups, and those with pK = 6.78 and pK = 7.66 the neutral groups.

In Fig. 6 the circles correspond to the association constants measured for the formation of the complex between deoxyhemoglobin A and BHC. Below pH 7.4 the reaction was essentially stoichiometric and no free effector was detectable in solution. At pH 9.5 the value for the association equilibrium constant was near 3000 M\(^{-1}\). At this pH only 1% of the neutral groups are protonated. Therefore this value was assigned to \( P_0 \) in Equation 7. The line fit to the data points in Fig. 5 was computed using this equation and the parameters listed in Table I.

![Fig. 3. Stoichiometric titration of deoxy- (O) and oxyhemoglobin (□) with BHC. Deoxyhemoglobin was titrated at pH 7.0 and oxyhemoglobin at pH 6.5, in 0.05 M NaCl at 20º. Heme concentration was 3.1 \( \times \) 10\(^{-4}\) M for deoxyhemoglobin and 2.5 \( \times \) 10\(^{-4}\) M for oxyhemoglobin.](image)

![Fig. 4. Interaction of deoxyhemoglobin with DHC at pH 8.5, in 0.05 M NaCl at 20º. Heme concentration 2.1 \( \times \) 10\(^{-4}\) M.](image)

![Fig. 5. Dependence on pH of \( H_{max} \) for deoxyhemoglobin A (O) and deoxyhemoglobin Deer Lodge (□).](image)

![Fig. 6. Dependence on pH of the overall affinity \( P \) of deoxyhemoglobin A (O) for BHC.](image)

| Protein                  | Parameters | 95% confidence limits |
|--------------------------|------------|-----------------------|
|                          |            | Lower | Upper |
| Deoxyhemoglobin A        | \( P_{K_1} \) | 5.59  | 5.46  | 5.73  |
|                          | \( P_{K_2} \) | 6.78  | 6.71  | 6.86  |
|                          | \( P_{K_3} \) | 7.66  | 7.57  | 7.75  |
|                          | \( \Delta P_k \) | 1.26  | 1.22  | 1.31  |
| Deoxyhemoglobin           | \( P_{K_1} \) | 5.55  | 5.41  | 5.71  |
| Deer Lodge               | \( P_{K_2} \) | 7.18  | 7.11  | 7.25  |
|                          | \( \Delta P_k \) | 1.49  | 1.45  | 1.54  |
Interaction of Oxyhemoglobin A with BHC—In Fig. 3 a stoichiometric titration of oxyhemoglobin with BHC is shown. Also in this case, as in deoxyhemoglobin A, the stoichiometry of the reaction proved to be 1:1. The pH dependence of $H_{\text{max}}$ for the interaction of oxyhemoglobin with BHC is shown by the open circles in Fig. 7. It is substantially different from that of deoxyhemoglobin A. Above pH 7, the low affinity of liganded hemoglobin for BHC made it impossible to objectively determine the end point of the titration. Above pH 7.5 practically no pH change was observed when BHC was added to the protein solutions. The data presented in Fig. 6 are raw values, therefore not corrected for the number of protons liberated by BHC. Since the data obtained covered only a limited pH range, no numerical simulation was conducted.

Interaction of BHC with Deoxyhemoglobin Deer Lodge—The reaction of deoxyhemoglobin Deer Lodge with BHC was studied in the same way as for hemoglobin A. In this case the stoichiometry was also 1:1. The pH dependence of $H_{\text{max}}$ is represented in Fig. 5. It appears that in the entire pH range investigated the absorption of protons was less than in the case of deoxyhemoglobin A. The interpolating line is the best fit obtained with Equation 1, assuming that two pairs of identical groups interact with the effector with a common pK shift. The assigned pK values were 5.55 and 7.18, with a ΔpK = 1.49. Table I lists those parameters and the 95% confidence limits obtained for them.

The affinity of the effector for the protein appeared to be higher than in the case of deoxyhemoglobin A. The reaction was essentially stoichiometric at all pH values investigated.

Interaction of Oxyhemoglobin Deer Lodge with BHC—The stoichiometry of the reaction was measured at pH 6.5 as previously described. Also in this case the titration showed a stoichiometry of 1:1. The pH dependence of $H_{\text{max}}$ for the reaction is presented in Fig. 7 together with the data obtained for normal hemoglobin. This parameter appeared to be very similar to that obtained for oxyhemoglobin A. Also in this case the interaction with BHC at pH near and above 7.5 produced a negligible or totally absent absorption of protons.

Interaction of Deoxyhemoglobin Providence-Asp with BHC—The number of determinations performed with this hemoglobin were very limited due to the small amount of protein available. Only three determinations were possible (pH 7.0, 7.5, and 8.0). The interaction at pH 7.0 gave an absorption of protons comparable to that of normal hemoglobin (1.96 eq/tetramer), and the reaction was essentially stoichiometric. At pH 7.5 the absorption of protons was less than in normal hemoglobin (1.50 eq/tetramer). At pH 8.0 the titration with BHC failed to give a distinct plateau at the end of the titration and $H_{\text{max}}$ could not be measured. This was due to the low affinity of the mutant deoxyhemoglobin for BHC (probably below $10^{-3}\text{ M}^{-1}$) at this pH.

Measurements of Circular Dichroism—The circular dichroism of carbonmonoxyhemoglobin A in the Soret region is sensitive to the presence of $10^{-3}\text{ M}$ BHC and IHP as shown in Fig. 8. The same is not true for the CD spectra of the carbonmonoxy derivatives of hemoglobins Deer Lodge and Providence-Asp, which otherwise were not distinguishable from that of normal hemoglobin. Addition of sodium dithionite (0.1 mg/ml) to the various solutions did not modify these results, nor did the method of hemoglobin preparation. This experiment was repeated several times to ensure that the effect observed with Hb A was not related to the presence of methemoglobin or to a particular preparation of the protein.

The circular dichroism spectra in the Soret region of the deoxy derivatives of the three hemoglobins investigated were very similar to each other and were not sensitive to the addition of effectors.

**DISCUSSION**

Groups That Interact with BHC in Deoxyhemoglobin—In the attempt to determine the ionization characteristics of the groups in deoxyhemoglobin that interact with BHC, rather than searching for a unique set of parameters in the various simulations, we searched for the best numerical solution possible within the framework of existing models for the binding of effectors to deoxyhemoglobin. The assumptions derived from the models of Arnone (1) and Arnone and Perutz (2) not only produced a very good correspondence between experimental and simulated data, but the fitting parameters are very close to data obtained from entirely different approaches. According to the crystallographic models there are
three pairs of groups in deoxy hemoglobin A that produce proton absorption in our experiments. We have assigned pK values near proton absorption in our experiments. We have assigned pK values near 5.59, 6.78 and 7.66 to these groups. Results obtained by Garner et al. (19) would assign a pK of 6.85 to the β1 valines. Results of Fung et al. (20) assign a pK of about 7.5 to the β2 histidines. We regard these values as virtually identical to those assigned to the neutral groups in our simulations. This suggests that the acid groups with pK = 5.59 are the β143 histidines. This value might appear somewhat low for a histidyl residue, but it is within the reported variation range of the pK values of these side chains in globular proteins (21, 22). The proximity of lysyl residues at β2 and at β144 to the β143 histidines might explain the relatively low apparent pK of these histidyl residues.

The apparent pK values so obtained for the ionization of the acid and neutral groups include electrostatic factors which we have not been able to evaluate quantitatively and the averaging factor introduced by assuming the same pK shift for all of the interacting residues. Indeed the pK shift is related to the distance between the opposite charges, and probably it is not identical in all of the salt bridges which are formed.

It must be stressed that these apparent values are regulated by the protein conformation. Thus variations in these values may reflect structural modifications at least in the area where the binding of effectors occurs.

In hemoglobin Deer Lodge the apparent pK of the β1 valines increases from 6.78 to 7.18 and the ΔpK also increases from 1.25 to 1.49. This indicates that the average length of the salt bridges is shorter in this hemoglobin than in hemoglobin A, suggesting at least a slight rearrangement of the groups in the binding site. This rearrangement, in turn, might increase the distance between the positive charges of residues β1 and β2, thereby allowing a higher apparent pK for the β1 valines.

Affinity of BHC for Deoxyhemoglobin A and Deer Lodge - De Bruin et al. (23) and Brygier et al. (24) have reported that the interaction of polyphosphates with liganded human or avian hemoglobin causes absorption of protons at lower pH values than for the unliganded derivatives. Our experiments with BHC are consistent with those of De Bruin et al. (23) and Brygier et al. (24) in the sense that above pH 7.5 proton absorption by the liganded form becomes measurably small. The absorption of protons decreases rapidly with increasing pH, changing from about 2.3 protons/mol at pH 7.0 to nearly none with an increase of less than 1 pH unit. This suggests that there may be a cooperative protonation of the side chains which participate in the interaction. Similar cooperativity has been reported for the interaction of BHC with liganded β chains (10). Additionally, it is clear that the absorption of protons by the neutral groups in deoxyhemoglobin is absent in liganded hemoglobin. De Bruin et al. (23) and Brygier et al. (24) suggested that these groups have lower pK values in liganded hemoglobin than in deoxyhemoglobin. Our data on hemoglobin Deer Lodge are relevant to this hypothesis. Comparison of the interaction of the liganded derivatives of hemoglobin A and Deer Lodge with BHC reveals no differences. The substitution of arginine for histidine at the β2 position might be expected to produce a lower absorption of protons and a higher affinity for the effector in liganded hemoglobin Deer Lodge (as for its unliganded derivative) if the β2 residues were involved in the interaction. It seems more probable that the binding sites are different in liganded and unliganded hemoglobin, as proposed by Kilmartin (25), or that the binding sites are topologically similar (between the adjacent β subunits), but the different conformations of the protein make different groups available to the interaction. The latter has been proposed by Salahuddin and Bucci in connection with experiments on isolated β chains (10).

Optical Activity Measurements - It is relevant to stress that in the Soret region of the spectrum the optical activity of the unliganded derivatives of the three hemoglobins investigated were indistinguishable. This supports the idea that the potentiometric interaction of deoxyhemoglobins Deer Lodge and Providence-Asp with BHC is different from that of normal hemoglobin because of changes in the electrostatic environments of the mutant hemoglobins, not because of special protein conformations produced by the mutations.

It is important to note that the CD spectra of the unliganded derivatives are not sensitive to either BHC or IHP. This suggests that the small conformational change detected by Arnone (1) and Arnone and Perutz (2), in the deoxyhemoglobin A crystals, in the presence of polyphosphate, did not indicate that such treatment does not significantly alter the functional behavior of normal hemoglobin. The crystallographic model of Arnone (1) and Arnone and Perutz (2) suggests that the presence of the aspartate in position β288 decreases the affinity of hemoglobin Providence-Asp for BHC not only because one salt bridge becomes impossible, but also because of active repulsion between the negative charge of the aspartyl carboxyl group and the negative charges of the effector. It appears that the interaction of deoxyhemoglobin Providence-Asp with BHC is accompanied by absorption of protons from acid groups, while the absorption of protons from neutral groups could not be measured because of the low affinity of this hemoglobin for BHC above pH 7.5. This suggests that the interaction of BHC with the β1 and β2 residues is greatly reduced by the presence of aspartate at the β28 position.

Interaction of BHC with Liganded Hemoglobins A and Deer Lodge - De Bruin et al. (23) and Brygier et al. (24) have reported that the interaction of polyphosphates with liganded human or avian hemoglobin causes absorption of protons at lower pH values than for the unliganded derivatives. Our experiments with BHC are consistent with those of De Bruin et al. (23) and Brygier et al. (24) in the sense that above pH 7.5 proton absorption by the liganded form becomes measurably small. The absorption of protons decreases rapidly with increasing pH, changing from about 2.3 protons/mol at pH 7.0 to nearly none with an increase of less than 1 pH unit. This suggests that there may be a cooperative protonation of the side chains which participate in the interaction. Similar cooperativity has been reported for the interaction of BHC with liganded β chains (10). Additionally, it is clear that the absorption of protons by the neutral groups in deoxyhemoglobin is absent in liganded hemoglobin. De Bruin et al. (23) and Brygier et al. (24) suggested that these groups have lower pK values in liganded hemoglobin than in deoxyhemoglobin. Our data on hemoglobin Deer Lodge are relevant to this hypothesis. Comparison of the interaction of the liganded derivatives of hemoglobin A and Deer Lodge with BHC reveals no differences. The substitution of arginine for histidine at the β2 position might be expected to produce a lower absorption of protons and a higher affinity for the effector in liganded hemoglobin Deer Lodge (as for its unliganded derivative) if the β2 residues were involved in the interaction. It seems more probable that the binding sites are different in liganded and unliganded hemoglobin, as proposed by Kilmartin (25), or that the binding sites are topologically similar (between the adjacent β subunits), but the different conformations of the protein make different groups available to the interaction. The latter has been proposed by Salahuddin and Bucci in connection with experiments on isolated β chains (10).

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It is important to note that the CD spectra of the unliganded derivatives are not sensitive to either BHC or IHP. This suggests that the small conformational change detected by Arnone (1) and Arnone and Perutz (2), in the deoxyhemoglobin A crystals, in the presence of polyphosphate, did not
affect the conformation of the heme pocket. Additionally, this supports the hypothesis that the modification of the oxygen affinity produced by anionic effectors is due primarily to their preferential binding to deoxyhemoglobin (26).

The CD spectra of the carbonmonoxy derivative of hemoglobin A is sensitive to both BHC and IHP. This indicates modifications in subunits tertiary structure, and is consistent with observations of Lindstrom and Ho (27), Adams and Schuster (28), and Perutz et al. (29). We do not ascribe these results to a major shift in the equilibrium between R and T conformations since the proton absorption of liganded Hb A results to a major shift in the equilibrium between R and T with observations of Lindstrom and Ho (27), Adams and Schuster (28), and Perutz et al. (29).

The interaction of deoxyhemoglobin with polyphosphates is also valid for the interaction of deoxyhemoglobin with BHC.

It is surprising that the CD spectra of the carbonmonoxy derivatives of hemoglobins Deer Lodge and Providence Asp are nearly insensitive to both BHC and IHP. The interaction was not tested potentiometrically for hemoglobin Providence Asp, while in the case of hemoglobin Deer Lodge the interaction produced an absorption of protons similar to that obtained with normal liganded hemoglobin. We do not have a simple explanation for this phenomenon.

Functional Implications – In the alkaline range, near pH 8, the affinity of hemoglobin Deer Lodge for BHC was higher than that of normal hemoglobin. In the same pH range, the affinity of hemoglobin Providence-Asp for BHC was much lower than that of normal hemoglobin. From these observations we would predict that, in this pH range, the effects of polyamions would be enhanced in hemoglobin Deer Lodge, and greatly reduced in hemoglobin Providence-Asp. These predictions, based on the potentiometric study of the interaction of BHC with these deoxyhemoglobins, are consistent with functional studies conducted with IHP on hemoglobins Deer Lodge and Providence-Asp (6, 7).

These studies show the utility of abnormal human hemoglobins as probes of hemoglobin's anion binding site. From the results obtained, it appears that the model proposed by Arnone (1) and Arnone and Perutz (2) for the interaction of deoxyhemoglobin with polyphosphates is also valid for the interaction of deoxyhemoglobin with BHC.

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Characterization of the ionizable groups interacting with anionic allosteric effectors of human hemoglobin.
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Additions and Corrections

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Enrico Bucci, Ahmad Salahuddin, Joseph Bonaventura, and Celia Bonaventura

Page 822, Column 2, Equation 1:

The denominator of the equation should have a plus sign. The correct equation is shown below:

\[ \frac{[c]}{[E]+[C]} \]

Page 824, Legend to Fig. 5:

In the legend of Fig. 5 “Equation 1” should read “Equation 3.”

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Incorporation of radioiodotyrosines into proteins formed during cell free translation.

Neal H. Scherberg, Hisao Seo, and Richard Hynes

Page 1773, Summary, Line 22

Due to a printer’s error, 66-fold was written as 668-fold. The line should read:

was increased 66-fold by pretreatment of the lysate with

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