Novel Allosteric Conformation of Human HB Revealed by the Hydration and Anion Effects on O_2 Binding†

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ABSTRACT: The effect of anions on the stability of different functional conformations of Hb is examined through the determination of the dependence of O_2 affinity on water activity (a_w). The control of a_w is effected by varying the sucrose osmolal concentration in the bathing solution according to the "osmotic stress" method. Thus, the hydration change following Hb oxygenation is determined as a function of Cl^- and of DPG concentration. We find that only ∼25 additional water molecules bind to human Hb during the deoxy-to-oxy conformation transition in the absence of anions, in contrast with ∼72 that bind in the presence of more than 50 mM Cl^- or more than 15 μM DPG. We demonstrate that the increase in the hydration change linked with oxygenation is coupled with anion binding to the deoxy-Hb. Hence, we propose that the deoxy-Hb coexists in two allosteric conformations which depend on whether anion is bound or not: the tense T-state, with low oxygen affinity and anion bound, or a new allosteric P-state, with intermediate oxygen affinity and free of bound anions. The intrinsic oxygen affinity of this unforeseen P-state and the differential binding of Cl^-, DPG, and H_2O between states P and T and P and R are characteristics which are consistent with those expected for a putative intermediate allosteric state of Hb. These findings represent a new opportunity to explore the structure−function relationships of hemoglobin regulation.

INTRODUCTION

The stepwise oxygen binding to hemoglobin induces tertiary and quaternary structural changes in the protein, resulting in a steady increase in O_2 affinity with saturation (*1*). The structural and physicochemical characteristics of the two end states of oxygenation, the deoxy-T and oxy-R, have been clearly established, and although many characteristics of Hb[1] oxygenation can be explained with a two-state allosteric model (*1*−*4*), functional studies have clearly demonstrated the existence of energetic intermediates in this process (*5*−*7*). Likewise, recent X-ray diffraction studies have shown that changes in crystallization conditions (*8*), or chemical (*9*−*11*) and mutational modifications (*12*), yield Hb structures distinct from the canonical R and T. These functional and structural studies indicate that the control of the Hb function is achieved through a more complex structural pathway than that which is suggested by a concerted transition between two end states. Albeit that the need for considering a third functional state to describe the Hb properties has been a subject of considerable debate (*13*−*15*) mainly because the putative intermediate states of Hb oxygenation still require clear characterization. However, the solution conditions that would unambiguously stabilize any of these species have not yet been discovered.

It is generally accepted that the isolation of fully functional Hb intermediates is obstructed by the cooperativety of the O_2 binding, which strongly drives the equilibrium to the end states. Despite the recent discovery that carbon monoxide-Hb crystallizes in a new R_2 structure in "low", near to physiological, salt conditions at pH 5.8 (*8*), the possibility of using different anion concentrations or pH as a strategy to stabilize Hb intermediates in solution has not been thoroughly explored. The reason is that it is difficult to detect, and even more difficult to gauge, the advent of global conformational changes associated with functional allosteric events in solution. In this work, we address this long-standing shortcoming by using the "osmotic stress" method to measure the hydration change linked with oxygen binding to Hb. We show that chloride and DPG modulate the hydration change, Δn_w, between the fully oxygenated and the fully deoxygenated Hb structures. In the absence of these anions, the number of water molecules that binds to Hb on the deoxy-to-oxy transition is ∼65% smaller than that measured when Hb is saturated by either Cl^- or DPG. It is shown that the experimental dependence of Δn_w on anion concentration can be fully described assuming that deoxy-Hb coexists in two alternative allosteric conformations in solution, T and P, depending on whether it is bound or unbound with anions. This new P-state of Hb, that reaches maximum thermodynamic stability in salt-free and O_2-free solutions, binds oxygen with intermediate intrinsic affinity and, can be a

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[1] Abbreviations: DPG, 2,3-biphosphoglyceric acid; Hb, hemoglobin; HbAo, purified major component of human adult hemoglobin; a_w, solution water activity; Hepes, *N*-[2-hydroxyethyl]piperazine-*N*′-(2-ethanesulfonic acid).

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rationale for both the higher Hb-affinity and for the diminished release of Bohr protons upon oxygenation observed in distilled water and in salt-free Hepes buffer (*16*, *17*).

The energetic consequences of the change in protein hydration to O_2 affinity have been recently elucidated (*18*) using the osmotic stress method (*18*, *19*). This method consists of to use osmolytes, solutes that are preferentially excluded from the protein surface (*20*), to adjust the activity of water (a_w) in a macromolecular solution. In the case of ideal exclusion, an osmolyte is only soluble into the bulk water, not into the macromolecular hydration water. The phase separation of water brought about by solute exclusion is alike to a dialysis experiment, but without a physical membrane. Via this conceptual experiment of dialyses, or of *isobaric* "osmotic stress" in solution, the activity of water sensed by the macromolecule is adjusted to that of the reservoir, the bulk with added solute. Since varying solute concentration ineluctably changes a_w, the differential binding of water molecules between states of different hydration, e.g., between the T and R structures of Hb, can be measured from the shift of the equilibrium of the reaction using traditional linkage equations (*18*). This possibility was clearly demonstrated for the first time measuring the O_2-binding curves in the presence of different osmolytes, that allowed us to show that the O_2 affinity of Hb correlates with water activity (*18*). Using equations of chemical linkage, originally proposed by Wyman (*21*), or a formalism based on the Gibbs−Duhen equation (*18*, *22*), we determined that ∼70 water molecules bind to Hb with oxygenation. We also found that this hydration change is in agreement with differences in the water-accessible surface area computed by Chotia et al. for the R and T canonical structures (*23*). Thus, we suggest that water molecules act thermodynamically the same as any other allosteric ligand. This original proposal was next examined in many different biochemical reactions, ranging from macromolecular stability to drug and protein DNA interactions (*19*, *24*, *25*). The energetic consequences of changing a_w were found to be associated with the Cl^- effect on O_2 affinity of Hb (*26*) and also on the energetic coupling between the free energies of Hb assembly and of oxygen binding (*27*). More recently, it was shown that the hydration change linked with oxygenation of clam Hb in solution, determined by applying OS, agrees quite well with that which is inferred counting the number of water molecules, found by X-ray crystallography, in the structures of the deoxy and oxy forms of this protein (*28*). These discoveries have clearly indicated that the hydration change probed by osmotic stress is intrinsically correlated with the global conformational changes associated with allosteric binding in solution. Thus, we can take advantage of the OS method to explore the effect of chloride and DPG binding on Hb structure in solution.

MATERIAL AND METHODS

HbAo was purified from human blood collected from healthy nonsmoking adults as previously described (*22*) and checked by nondenaturating PAGE. The purified Hb and the neutral solutions were extensively deionized by several passages through an Amberlite MB-1 column. Stock solutions of Hepes buffer, NaCl, and DPG were prepared with Milli-Q water (Millipore). All of these chemicals were from Sigma. The oxygen-binding curves for Hb at 60 or 180 μM Hb/heme were determined either by the tonometric-spectrophotometric method (*22*), or using an Imai cell (*17*) modified to improve mixing (*29*). The Hb oxygen affinity, p_{50}, and Hill coefficient were determined from Hill plots. The water activity of the solution was adjusted with the addition of Sucrose, ultraPure grade from Gibco-BRL, at several osmolar concentrations (Osm). The sample osmolality was determined after binding experiments with an Osmomette A (Precision Systems Inc.). The Osm was converted to $\ln(a_w)$ with $\ln(a_w) = -\mathrm{Osm}/55.5$ (*22*).

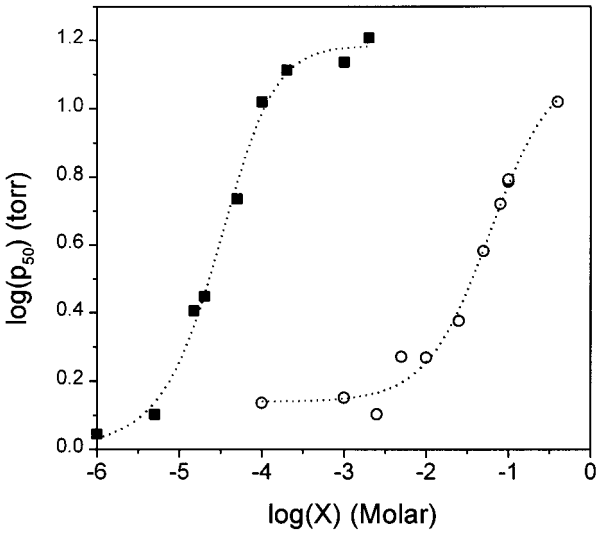


FIGURE 1: The effect of DPG (■) and Cl (○) concentration on the oxygen partial pressure at half saturation of HbA_o, p_{50}, determined in 10 mM Hepes, pH 7.2, 25 °C, 80 μM/heme.

RESULTS

The Hb oxygen affinity, measured by the oxygen pressure at half saturation, p_{50}, is highly dependent on anion concentration. For example, while $p_{50} = 1.7$ mm Hg for Hb in salt-free Hepes buffer at 25 °C, it raises to 4.8 mm Hg with the addition of 100 mM NaCl or to 5.0 mm Hg in the presence of 50 μM DPG. In the absence of anions, Hb binds oxygen cooperatively with the highest affinity. The addition of either DPG or Cl^- lower Hb oxygen affinity as shown in Figure 1 by the plots of $\log(p_{50})$ versus log([anion]). Concerning the interactions of anions with Hb, these linkage plots can be divided into three concentration regions. First, at $[Cl^-] < 1$ mM or [DPG] < 1 μM, p_{50} is insensitive to anion concentration because the binding of anions to the protein is insignificant; second, the p_{50} suddenly becomes sensitive to salt concentration between ∼ 1 and 50 mM Cl^-, or ∼1 and 10 μM DPG. Within these regions, these anions bind to the deoxy state of Hb lowering the oxygen affinity because the relative fraction of deoxy-Hb associated with Cl^-, or with DPG, increase with salt concentration. Therefore, the fraction of the Hb molecules stabilized in the T state, due to extra salt bridges involving the anions bound, changes (*1*). Above about 50 mM Cl^- and 20 μM DPG, $\log(p_{50})$ increases linearly with the increase of anion concentration. The prevailing analysis of such effects is made within the scope of the MWC, two-state, allosteric model (*30*). In this model, cooperative binding arises from the concerted conformation transition of Hb between a low oxygen affinity-T and a high oxygen affinity-R state, induced by O_2 binding. The lowering

of O_2 affinity of Hb by anions is attributed to the displacement of the allosteric equilibrium toward the T structure, due to the extra stabilization of the T-state bound with anions (*1*−*4*, *15*). Conversely, the positive slope of the plots of log(p_{50}) on log([Cl^- or DPG]) (Figure 1) reflect the anion release due to O_2 binding. Furthermore, it is intrinsically assumed that the conformation and the intrinsic O_2 affinity of the T and R states of Hb are the same whether the anion is bound or unbound. To investigate this hypothesis, we measured the influence of anion concentration on the apparent difference between the number of water molecules bound to the fully oxygenated and the fully deoxygenated conformations of Hb, $\Delta n_w = n_w^{oxy} - n_w^{deoxy}$. Particularly, we are seeking differences in Δn_w that could give us some insight about the influence of anion on the structure of Hb in solution.

The dependence of O_2 affinity on water activity was measured using sucrose to change a_w. Previously, we used sucrose, as well as other sugars, polyols, and an amino acid, to show that different osmolytes lower O_2 affinity through an indirect osmotic effect (*18*, *22*, *26*). We found that this solute concentration-dependent effect correlates with water binding through a_w solution, rather than direct solute-Hb interaction or changes in the bulk dielectric constant (*22*). We arrived at this conclusion using osmolytes of different sizes and chemical nature (*18*) and by examining the possibility of a direct solute interaction with Hb via an unbiased thermodynamic analyzes (*22*). On the strength of these previous analyses, the difference in hydration between the fully unligated and the fully oxygenated form of Hb tetramer, $\Delta n_w = n_w^{oxy} - n_w^{deoxy}$, is given by the following linkage equation (*18*),

$$\left.\frac{d \ln(p_{50})}{d \ln(a_w)}\right|_{ai} = -\frac{\Delta n_w}{4} \qquad (1)$$

where n_w^{oxy} and n_w^{deoxy} are the apparent number of water molecules bound to the oxy and deoxy forms of Hb, respectively.

Figure 2 shows the plots of ln(p_{50}) versus ln(a_w) determined in 0.1, 5, and 100 mM NaCl and in 50 μM DPG, in 10 mM Hepes buffer, pH 7.2. In all these examples, the logarithm of O_2 affinity decreases linearly with the decrease of the logarithm of a_w but with different slopes. According to eq 1, upon oxygenation, fewer water molecules bind to Hb at lower than at higher Cl^- or DPG concentration. The same behavior emerges when we compare the water effect on Hb oxygenation measured with and without DPG in salt-free Hepes buffer. These results seem to be in qualitative accord with Jiang and Ferrone's observations (*31*). They showed the isotherm of oxygenation of Hb measured in salt-free buffer was unaltered by the addition of 2 M sucrose.

The full characterization of the effect of Cl^- and DPG concentration on Δn_w is presented in Figures 3 and 4, respectively. The data show that, below ~1 mM and above ~50 mM NaCl, Δn_w remains unchanged with averaged values of 25.1 ± 1.8 and 72.0 ± 2.0, respectively. Within 1 and 50 mM NaCl, Δn_w steadily increases from 25 to 72 water molecules. Because the chloride dissociation constant to the deoxy-Hb is about 10 mM (*32*), the relative fraction of Hb molecules in the T state with Cl^- bound must vary within this range of salt concentration. Therefore, it is evident that

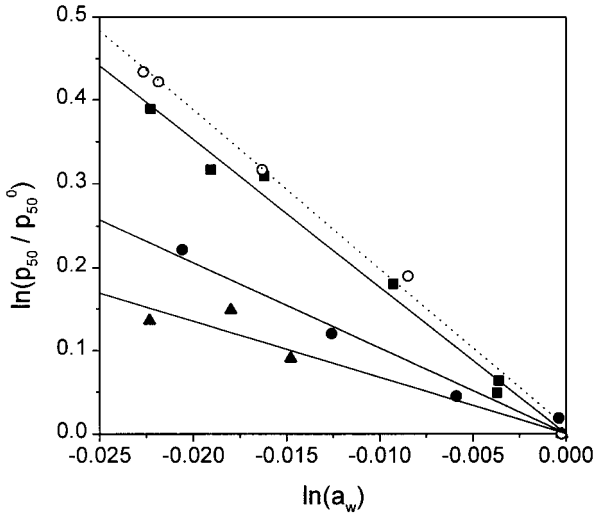


FIGURE 2: Dependence of p_{50} on water activity, measured in the presence of 0.1 (▲), 5 (●), and 100 mM NaCl(■) and in the presence of 50 μM DPG (○). Other conditions as in Figure 2. The different slopes yields different hydration changes associated with the transition from deoxy to oxy HbAo. The rate of change of p_{50} on a_w were found to be insensitive to protein concentration (80 and 180 μM heme), suggesting that if there is a change in the Hb dimer-to-tetramer equilibrium constant in salt free conditions it does not alter the hydration change measured.

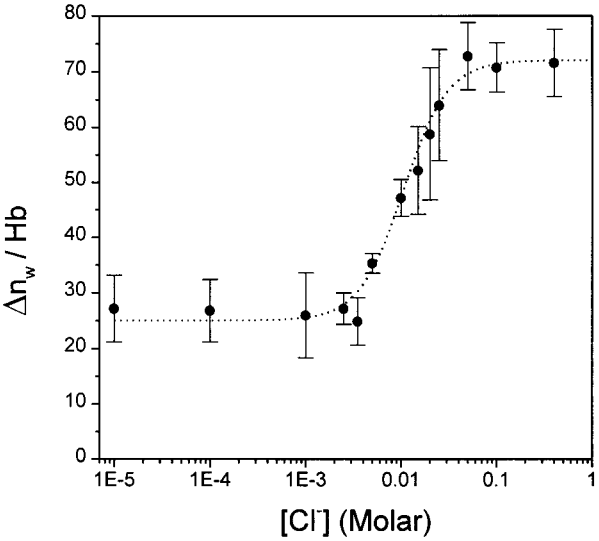


FIGURE 3: Plot of the hydration change linked with HbAo oxygenation in 10 mM Hepes, pH 7.2, versus the logarithm of chloride concentration. The dotted line represents the best fit of eq 3 to the data with parameters: $\Delta n_w^{initial} = 25.1 \pm 1.8$, $\Delta n_w^{final} = 72.0 \pm 2.0$, $K_{obs} = 10.6 \pm 1.0$ mM, and $n = 1.9 \pm 0.3$.

the observed change in Δn_w is linked with anion binding to the deoxy-T state of the protein. The mean value and the standard deviation of the mean of the Hill coefficient (n_H) measured below 1 mM NaCl (24 data points) and above 50 mM NaCl (22 data points) is 2.56 ± 0.06 and 2.69 ± 0.06, respectively. These mean values are not significant different, nor are they significantly different from the mean value of n_H computed from 87 oxygenation curves measured from 0 to 0.4 M NaCl at different a_w, which is 2.66 ± 0.03. Thus, it is not possible to decide if the change in Δn_w brought about by Cl^- binding to the deoxy-Hb is accompanied by a change in cooperativity. The dependence of Δn_w on DPG concentration follows the same pattern as that observed with Cl^-. However, a much lower concentration of DPG is needed to

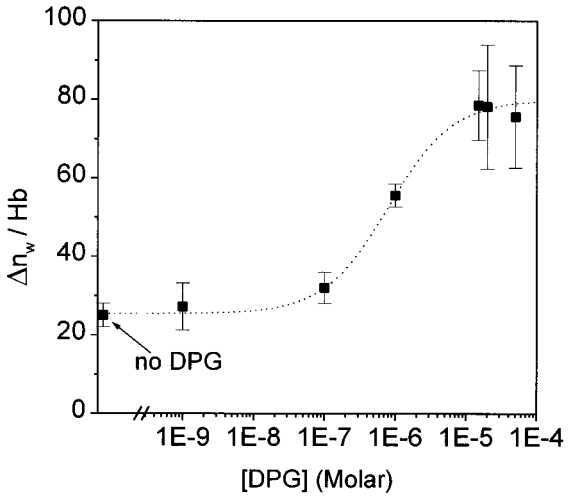


FIGURE 4: Plot of the hydration change linked with the HbAo oxygenation in 10 mM Hepes, pH 7.2, versus the logarithm of DPG concentration. The dotted line represent the best fit of eq 3 to the data with parameters: $\Delta n_w^{initial} = 25.4 \pm 2.7$, $\Delta n_w^{final} = 79.9 \pm 8.7$, $K_{DPG} = 0.8 \pm 0.3$ μM, and $n = 0.96 \pm 0.38$.

change Δn_w, reflecting the much higher affinity of this anion to the deoxy-Hb. These results support the interpretation that the change in Δn_w is directly linked with anion binding to the deoxy-T state of Hb. The line through the experimental points shown in Figure 3 and in Figure 4 is a nonlinear fit of a proposed mechanistic model to the data, which is elaborated in the Discussion. In this model, we assume deoxy-Hb coexists in two different allosteric conformations, T and P, whose relative equilibrium distribution varies with anion concentration. Additionally, we assume that the conformation of the oxy form of Hb is insensitive to the presence of anion within the range of Cl^- and DPG concentrations investigated in this work. The description of the dependence of Δn_w on log(anion) with this model gives the binding parameters of Cl^- and of DPG to deoxy-Hb. The values of these parameters are in very good agreement with reported values determined by other methods. This agreement is evidence supporting our hypothesis that deoxy-Hb free of anions assumes a new allosteric conformation.

DISCUSSION

The effect of Cl^- or DPG on O_2 affinity (Figure 1) and on Δn_w (Figure 3 or 4), are closely related. The comparison of the plots shown in Figures 1, 3, and 4 demonstrate that the increase in Δn_w is coupled with the binding of Cl^- or DPG to Hb, since Δn_w changes within the same range of anion concentration in which log(p_{50}) starts to become sensitive to these anions. Aside from the transition region, which is evidenced in the plots shown in Figures 3 and 4, both the Δn_w and the number of Cl^- or DPG released with O_2 binding are constant. Upon oxygenation, no Cl^- or DPG molecules are released below the transition region and a constant number of 1−2 Cl^- (*26*, *32*) or 1 DPG (*32*, *33*) is released at the anion concentrations where Δn_w is leveled off at its maximum. These interrelationships demonstrate that the change in Δn_w is brought about by anion binding to the Hb molecule, since Δn_w is constant either when no anion is bound or when all anion-binding sites of Hb are fully saturated.

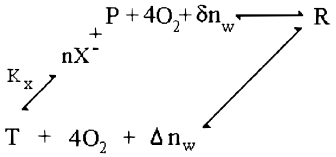
Although Δn_w is a differential measurement, between the apparent number of water molecules associated with the fully oxygenated and the fully deoxygenated states of Hb, it is possible to establish that it is the deoxy end state of Hb, not the oxy form, that changes hydration upon anion binding. This can be done noting that the increase in Δn_w occurs at a DPG concentration range where it binds exclusively to the T structure (*32*, *33*). When DPG binds to a specific site formed by an optimal arrangement of six cationic groups located at the entrance of the central cavity of Hb, the T structure is stabilized (*34*). Chloride binding (or condensation) to the charges in the central cavity, alike DPG, also stabilizes the T-state (*15*, *17*). Hence, we conclude that the intrinsic hydration of the deoxy-Hb state is anion dependent.

In addition, it is possible that the intrinsic hydration of oxy-Hb changes due to anion binding or release. For example, Silva et al. suggested that low concentrations of inorganic anions may favor the stabilization of the new R2 quaternary state of liganded human Hb (*8*). The arising question is if the structure of oxy-Hb in solution would undergo a further conformational change at lower salt concentrations and, therefore, contribute to the observed influence of anions on Δn_w. First, if the R structure changed within the range of salt concentration investigated, a change in its intrinsic O_2 affinity, K_R, would also be expected. However, it has been demonstrated that K_R is quite insensitive to Cl^- and DPG (*32*). Second, as both chloride and DPG binding induces equivalent changes in Δn_w and DPG does not bind to the oxy-Hb structure, at the concentrations investigated in this work, it is safe to conclude that the hydration and, therefore, the conformation of the oxy-Hb are insensitive to anions. Computational (*35*) and NMR experiments (*36*), that suggest that the oxy-Hb is already in the R2 state at moderate salt conditions, support this conclusion. However, we must emphasize that, at higher salt concentrations than those used in this work, both anions may bind to oxy-Hb and cause a further shift in Δn_w. Indeed, we have already observed that Δn_w steadily decreases from ~78 to ~45 H_2O with the increase in [DPG] above 0.1 mM in chloride-free Hepes buffer, while it causes a parallel decrease in Hb oxygen affinity (unpublished results). This is extra evidence that the steep increase in Δn_w, demonstrated in Figures 3 and 4, reflects the induced change in the intrinsic hydration of the deoxy-Hb due to anion binding.

Since $\Delta n_w = n_w^{oxy} - n_w^{deoxy}$ is always positive, the binding of Cl^- or DPG to the deoxy-Hb is linked to the release of water molecules. From the values of Δn_w of oxygenation, measured in the presence (~72 H_2O) and in the absence (~25 H_2O) of salts, we estimate that the deoxy-Hb bound with anion is less hydrated than the deoxy-Hb free of anions by about 45−50 water molecules. This dehydration may be ascribed either to the local dehydration of the anion-binding site upon complex formation or to a global change in the protein conformation induced by anion binding. A release of 45−50 water molecules appears, however, to be rather large to be attributed only to the reorganization of bound water molecules at the target anion-binding sites. Moreover, similar to DPG and Cl^-, many other anions bind preferentially to the deoxy-Hb and stabilize the more hydrated state of the protein (*37*). Indeed, we found that Δn_w stays at ~70 water molecules in chloride-free solutions of several buffers,

such as citrate, phosphate, glycine, and bis-tris (*results not shown*). Apparently, the stabilization of deoxy-Hb in its least hydrated state is quite unspecific in relation to the structure of the anion bound. Consequently, global conformational changes, besides a specific reorganization of water molecules at the anion-binding site, must contribute to the release of hydration water molecules linked with anion binding to deoxy-Hb. Therefore, we conclude that deoxy-Hb switches conformation upon anion binding. Since a deoxy-Hb complex to anions is in the T structure, the release of the anions bound switches its conformation to a novel allosteric state. This proposition is in accord with the suggestion that chloride and other anions affect the p*K*s of the histidyl residues on the surface of deoxy-Hb via long-range electrostatics and/or global conformational changes of the protein (*38*).

Quantitative Model of the Effect of Anion Binding on the Release of Water Molecules Linked with O_2 Binding to Hb. Operationally, we shall call the new allosteric state assumed by the deoxy-Hb free of anions as the "P-state". So the binding of different negative heterotropic ligands to the deoxygenated state of Hb, such as Cl^-, DPG, phosphate, and citrate, seems to be a sufficient requisite to switch the conformation of Hb from a P to a T state in a concerted way. A direct test for this hypothesis is achieved by modeling the observed dependence of Δn_w^{obs} on anion concentration, [X] (Figures 3 and 4), through the following scheme.



This describes the coexistence of deoxy-Hb in two conformations, T and P, in equilibrium with anion concentration and, also, that the oxy-Hb is in the R state regardless of anion concentration; δn_w and Δn_w are the differences in hydration between the states P and R and T and R, respectively. K^x is the apparent dissociation constant of X^- to the T state of Hb and n_X is the apparent number of anions bound to the T in relation to the P state of Hb. At any anion concentration, the apparent hydration change measured upon oxygenation, Δn_w^{obs}, is given by

$$\Delta n_w^{obs} = f_P\, \delta n_w + f_T\, \Delta n_w \qquad (2)$$

where f_P and f_T are the fractions of the Hb following the O_2-binding reaction pathway from P → R and from T → R, respectively. With these definitions Δn_w^{obs} is given by eq 3.

$$\Delta n_w^{obs} = \frac{(\delta n_w - \Delta n_w)}{[1 + (X/K_{obs})^n]} + \Delta n_w \qquad (3)$$

where K_{obs} is the apparent overall dissociation constant for the anion in the transition from P → T, and n is the apparent stoichiometry of binding.

The continuous lines drawn through the experimental points, shown in Figures 3 and 4, represent the best fit of eq 3 to the data, with the parameters shown in captions. As seen, the description of the data with a three-state allosteric model is quite remarkable. Moreover, quantitative support for this model is found by comparing the Cl^- and DPG apparent dissociation constants, determined via eq 3, with earlier published values, determined by other methods. The values of the binding constant of chloride to deoxy-Hb, previously determined by $^{35}Cl^-$ NMR Quadrupole Relaxation (*39*), by proton uptake data (*40*), and by oxygenation (*32*, *41*), are 100, 63, and 130 M^{-1}, respectively. The averaged Cl^- dissociation constant among these independent evaluations is 11 ± 3 mM. This value is in good agreement with the value determined by modeling the change in Δn_w on log(Cl^-) via eq 3 (Figure 3), which is 10.6 ± 1.0 mM. The comparison between the value of the DPG dissociation constant to deoxy-Hb, as determined in this work, with published values is not as straightforward as that for chloride. Most of the DPG-binding constants to deoxy-Hb reported in the literature were measured in the presence of 100 mM Cl^- at pH between 7.0 and 7.5 (*42*, *43*). At these conditions, the averaged DPG dissociation constant is on the order of 10 μM, which is 1 order of magnitude higher than 0.8 ± 0.3 μM, as determined in this work in the absence of chloride. This difference, however, may be attributed to the weakening of the strength of DPG binding to deoxy-Hb with the increase in chloride concentration (*17*, *32*, *42*). Nonetheless, the fit of eq 3 to the data shown in Figure 4 gives the correct stoichiometry of 1 mol of DPG bound/mol of Hb tetramer. These agreements provide experimental verification of our model to account for the effect of anion binding on the conformation of the fully deoxygenated state of Hb.

Giving the experimental evidence that deoxy-Hb is in a concerted equilibrium between two allosteric states, P and T, regulated by anion interactions, the question arises as to whether the P-state serves any particular functional role. Although the full stabilization of this eventual new allosteric state of Hb is achieved by the unligated protein at a very low salt concentration, other combinations of anion concentration and O_2 saturation could act together to stabilize the P-state. This is possible giving the allosteric behavior of Hb, which allows different distributions of cross interactions, among different distant binding sites, to act cooperatively in order to stabilize alternative thermodynamic accessible conformations of the protein. For example, the increase in O_2 saturation might be attained either by decreasing bulk anion concentration at a constant O_2 activity, or by the increase in O_2 activity at a constant anion concentration. In these two instances, anions are displaced from the Hb molecule and its O_2 saturation increases, because the two events are energetically linked via tertiary and/or quaternary changes in protein conformation. We have shown in this work that the transition from the low O_2-affinity T-state to the intermediate O_2-affinity P-state is followed by the apparent release of ~2 Cl^- (or one molecule of DPG) with a dissociation constant of about 10 mM (about 1 μM for DPG). Both the number of Cl^- ions (or of DPG) released on the T−P transition and its dissociation constant determined in this process correspond to those determined via measurements of oxygen binding in solutions with NaCl varying from 0.1 to 0.7 M (*32*, *41*). Consequently, the differential binding characteristics of Cl^- (or DPG) between states P and T appear to be indistinguishable from those measured on the transition from the low-T to the high-R affinity states of Hb by oxygenation. These quantitative agreements suggest that the P-state may be involved on the mechanism of anion regulation of Hb function.

The need for considering a third allosteric state to fully describe the influence of heterotropic ligands, such as anions and protons, on Hb oxygenation had been carefully considered by Minton and Imai (MI model) (*44*). Within the MI three state allosteric model, the ligation of DPG and Cl to the Hb displace the equilibrium from a non-R structure, the S-state, to the canonical T structure. The dependence of Δn_w of oxygenation on DPG and on Cl^- concentration (Figures 3 and 4) appears to reflect the stabilization of such third state of Hb since the binding of both anions to the deoxy-Hb lead to an equivalent increase on the intrinsic hydration of the protein.

Other mechanisms of Hb regulation, besides the three-state model, has been more recently suggested on the basis of functional studies of different Hbs carried out in the absence and in the presence of anions (*45*, *46*). In one of such studies, Perutz et al. proposed that the regulation of the Hb-O_2 affinity is achieved by Cl^- condensation, rather than by Cl^- specific binding, to the net excess of positive charges created by three anionic and eight cationic residues located in the central cavity of the quaternary deoxy-T structure (*45*). Accordingly, many Cl^- ions must be sequestered from the bulk solution by deoxy-Hb in order to stabilize the T-form. Our results appear to support this view. Unlike DPG, the stoichiometry of Cl^- binding defined in eq 3, which equals ~2, represents a minimal number of Cl^- bound to the T state, since it is a Hill coefficient. This implies that multiple Cl^- ions must bind to Hb in the transition from the deoxy-P to the deoxy-T states. Perutz and others also suggested that the condensation of additional chloride ions on the T-state occurs due to the widening of the central cavity on the transition from the R to the T structure (*45*). According to this interpretation, the release of chloride would displace the allosteric equilibrium straight to the R state. Our results show, however, that the release of chloride ions (or of DPG) bound to deoxy-Hb is linked with the binding of a smaller number of water molecules than that one linked with the transition from the T to the R state. This suggests, again, that the anionic regulation of Hb-O_2 affinity may be exercised via the effect of anion binding on the equilibrium between the states T and P, rather than on the equilibrium between the states T and R.

Bonaventura and colabs have proposed another alternative model of chloride regulation of Hb function (*46*). On the basis of Hb-O_2 binding and Hb-redox potential measurements in the absence and presence of anions, they suggested that the T state of Hb exist as a collection of many T forms (T, T′, T″,T‴, ...), with different rigidity, whose equilibrium fluctuation is controlled by anion binding (*46*). The meticulous determination of the dependence of Δn_w of oxygenation on Cl^- or DPG concentration (Figures 3 and 4) is evidence, however, that the binding of either of these anions to deoxy-Hb induces a concerted transition between two deoxygenated states of the protein, as described by eq 3. This result would only be consistent with the suggestion of many T forms of Hb if these species fluctuate between two classes of conformational/hydration states of the deoxygenated protein.

The question whether a third allosteric state of Hb is necessary to explain Hb function, and particularly, the effect of Cl^-, DPG, and H^+ on the stepwise O_2 binding affinity of the Hb molecule as been a matter of considerable debate (*2*−*6*, *13*−*15*). Nonetheless, there is an apparent agreement on the need for considering the coupling of tertiary structural changes within the T-state induced by homotropic (O_2) and/or heterotropic binding with changes in the O_2 intrinsic affinity of this state. This cognition has been dictated, for instance, by (a) the variability of the binding affinity to the first heme site with solution conditions (*4*, *17*, *32*); (b) the unequal distribution of Bohr protons released along stepwise oxygenation (*1*, *47*); and (c) the site-specific contributions to free energies of O_2 binding cooperativity by the eight partially ligated intermediates of Hb (*48*). Our finding that the hydration of the deoxy-Hb increases steeply upon anion release could reflect such tertiary structural changes. To examine this possibility, we measure the dependence of K_1, the binding constant for the first O_2 molecule to Hb, on water activity, since this step of the reaction involves the rupture of some of the eight salt bridges within the T structure (*1*, *15*). From the slope of the plots of $\log(K_1)$, determined in 100 mM NaCl buffered solution, versus $\log(a_w)$, we found that some 40−50 water molecules binds to Hb along the binding of the first oxygen (*unpublished results*). Indeed, this change in hydration agrees with that predicted from the difference in accessible surface area of the salt bridges in the deoxy-T and in the met-R Hb crystallographic structures, as computed by Chothia et al. (*23*). More striking is that the 40−50 H_2O, that binds to Hb in 100 mM NaCl along the binding of the first O_2, agrees with the differential hydration between P and T states, computed from the difference between the two characteristic values of the Δn_w of oxygenation measured in the absence (~25 H_2O) and at saturating concentrations of anions (~72 H_2O). Although no detailed structural information can be safely drawn from these measured hydration changes, these numbers appears to be consistent with the increase in Hb hydration expected to occur with the rupture of the salt bridges within the deoxy-Hb, suggesting that the P-state may be an alternative, unconstrained T-state.

The association of DPG, chloride, or other different anions with Hb leads to the stabilization of the T state in relation to the P and R states and, consequently, to the decrease of the overall Hb O_2 affinity. Although these events take place at different anion concentration for different anions, reflecting distinct binding affinities to the T-state, the magnitude of the hydration change connected with the transition from T to R does not change significantly for different anions. For example, while Δn_w measured in the presence of Cl^- is 72 ± 2 H_2O per tetramer, in the presence of DPG, it is 79.9 ± 8.7, a little higher than that found in Cl^-, perhaps within the experimental error. These results suggest that once an anion-binding site(s) of deoxy-Hb is fully saturated, the protein assumes the same overall T structure whatever the kind of anion bound or the binding mode (specific or charge condensation). This anion unspecificity implies that the stabilization of the T in relation to the P structure is primarily determined by allosteric changes induced by charge neutralization. Bonaventura and Bonaventura were the first to recognize that the increase in the net positive charge lining the central cavity of the T structure leads to the increase on O_2 binding affinity (*49*). This was later confirmed by Perutz et al. correlating the O_2 affinity of several mutant Hbs with the net charge within their central cavity (*45*). Our findings that anion release induces a concerted change on the global hydration of the structure of deoxy-Hb with a concomitant

increase on its O_2 affinity follows the same correlation between binding affinity and electrostatic observed with mutants of Hb. This is evidence that similar dependence of Δn_w of oxygenation with anion binding should be observed for other Hbs.

We have tested the latter hypothesis for bovine Hb (bov-Hb). Although the end values of Δn_w of oxygenation for bov-Hb, corresponding to those measured in the absence and in the presence of saturating concentrations of Cl^- (or DPG), differ from those measured for human Hb, its dependence on anion concentration is similar to that measured for human Hb (*unpublished results*). As expected, the dissociation constant of Cl^- and of DPG from the T state of bov-Hb, determined via eq 3, is higher for bov-Hb than for HbAo, which can be attributed to the deletion of two charged groups in the N-terminal of the bovine β-chain (*50*, *51*). Nonetheless, the number of Cl^- (and DPG) bound to the T state in the transitions from P to T structure is, within error, the same for both Hbs. If the anions displaced in the course of T to R transition by oxygenation are those mainly bound in the induced P to T transition, the prediction would be that both hemoglobin should display similar oxygen-linked chloride effects. Indeed, this is the case (*50*).

Yet, the stabilization of the P state offers a rationale for the influence of anions on the Bohr effect. Alike the O_2-linked water binding, the number of alkaline Bohr protons released upon Hb oxygenation depends on anion concentration. In salt-free Hepes buffer, it is half of that released in the presence of anions (*15*, *52*). In human Hb, the two His146β have been proposed to be the only residues to contribute to the chloride independent part of the Bohr effect (*15*). However, Bush and Ho and Ho and colabs have shown by high-resolution ^{1}H NMR that, although this residue does not bind anions, its contribution to the Bohr effect is largely affected by their presence in solution, suggesting long-range electrostatic and/or conformational changes induced by anions (*38*, *53*). Taking together, our experimental findings and the NMR data suggest that the water and the alkaline Bohr effects might be closely related. We have also tested this hypothesis through the determination of the dependence of the Bohr effect on NaCl concentration (*54*). We have found that the dependence of Δn_H, the number of protons released upon oxygenation, on log([NaCl]) is very similar to the dependence of Δn_w on log([NaCl]) (*manuscript in preparation*). These unpublished results demonstrate that the water and the Bohr effect on Hb function are in parallel, probably reflecting simultaneously the allosteric changes on the protein structure induced by oxygen binding.

Whether the allosteric state of reduced Hb fully stabilized in salt and oxygen-free solutions represent an intermediate state on Hb function remains to be determined. However, the oxygen affinity of this P state and the differential binding of chloride, DPG, and water between T and P, P and R, and T and R states linked with oxygenation are all attributes one would expect to find in an intermediate state in Hb function.

A Remark on the Practice of the Osmotic Stress Method. The quantitative practice of osmotic stress, as earlier proposed by us (*18*, *19*) and as applied in the present study, was recently criticized by Timasheff (*55*). He argued that the possibility of direct interaction between the solute, used to set the activity of water in solution, and the protein is ignored on the linkage equations used to analyze the thermodynamic reaction equilibrium. First we want to stress that this criticism is unjustified for the Hb case, since the thermodynamic analyses of the possibility of concomitant binding of solutes and water to Hb during oxygenation was published previously (*22*). Second, we want to point out that the concomitant analysis of the effect of anion and water binding to Hb, via eq 1 (previously proposed) and eq 3 (originally presented in this work), allowed us to determine the correct values for the binding constants of Cl^- and of DPG to deoxy-Hb. If the fundamental linkage equation between $\log(p_{50})$ and $\log(a_w)$, that gives Δn_w (eq 1), is erroneous as suggested by Timasheff, then the excellent agreement among the anion binding parameters determined in this work, via the analysis of Δn_w versus log([X]), (X = Cl^- or DPG), with those published in the literature would be rather unlike to occur. In our view, this agreement supports the practice of the Osmotic Stress Method. Additionally, and contrary to Timasheff's view (*55*), the contribution of water and solute binding to a macromolecule can be simultaneously analyzed by using, for instance, the extended Wyman linkage equation, as originally proposed by Tanford, which includes the effect of macromolecular hydration to the chemical equilibrium (*56*). Particularly elucidative examples of how to perform such kind of examination are given in studies of protein to DNA binding (*57*), salt effects on DNA conformational stability (*58*), and the additive effect of NaCl and osmolyte on Hb oxygen binding (*22*, *26*). Among others, these examples also support the use of linkage equations on the analysis of the controlled perturbation of a reaction equilibrium by the "osmotic stress" generated with the addition of osmolytes. Thus, it is possible to follow macromolecular conformational changes, via hydration changes, induced by binding of either regulatory or functional ligands, and, as originally demonstrated in this work, to determine ligand binding parameters associated with these events.

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