Conformational Fluctuations in Deoxy Hemoglobin Revealed as a Major Contributor to Anionic Modulation of Function through Studies of the Oxygenation and Oxidation of Hemoglobins A_0 and Deer Lodge β2(NA2)His → Arg[†]

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ABSTRACT: Organisms rely on regulation at the molecular level, such as the allosteric regulation of hemoglobin (Hb) function by anions, to meet challenges presented by changing environmental and physiological conditions. A comparison of the effects of anions on oxygenation, oxidation, and sulfhydryl reactivity of Hb leads us to suggest that a large and significant part of the shift in oxygen affinity brought about by anion binding occurs as a result of increased conformational rigidity of the T state of deoxy Hb. As conformational rigidity increases, it becomes increasingly difficult for subunits in the deoxygenated T-state tetramer to assume higher oxygen affinity forms (T′, T″, T‴...) with less steric hindrance. The oxygen affinity reflects the average of the rapidly equilibrating conformations within the T state and is correspondingly decreased when anion levels are increased. The initial stage of the oxidation of Hb is relatively insensitive to steric alterations and thus reflects, primarily, the electronic aspects of the quaternary (T, T′, T″, T‴...) ↔ R equilibrium. We show that the reactivity of the sterically obscured sulfhydryl of β93 Cys in deoxy Hb is much greater in chloride-free buffers than in buffers with added chloride. Anion-induced decreases in the extent and frequency of conformational fluctuations of subunits within the T-quaternary state thus reduce sulfhydryl reactivity as well as oxygen affinity. This parallel in anionic control of function allowed us to test, and disprove, the possibility that uncompensated positive charges in the central cavity of Hb Deer Lodge increase the frequency and extent of conformational fluctuations in its deoxy structure. This Hb variant exhibits increased anion sensitivity, increased oxygen affinity, and increased ease of oxidation, but without increased reactivity of its sulfhydryl groups, indicating that active-site alterations in deoxy Hb Deer Lodge are primarily electronic and not associated with increased conformational fluctuations within its T state. The restoration of normal properties in Hb Deer Lodge by addition of anions reinforces our conclusion that anionic control can be exerted through both steric and electronic alterations. The anionic control of fluctuations within the T state of Hb illustrates an important principle of macromolecular structure-function relationships: that functional regulation can be achieved by alterations in conformational rigidity.

It was previously demonstrated that the oxygen affinity of human hemoglobin is decreased when the number of positive charges in the central cavity is reduced. Oxygen-linked effects associated with these positive charges can be reduced either by substitution of uncharged amino acids for the positive ones, or by the binding of anions, which provides a basis for anionic control of function (*1*, *2*). Human hemoglobin variants and hemoglobins of some species that lack specific positive charges in the central cavity are relatively insensitive to anions, while increased positive-charge density generally provides for increased oxygen affinity and increased susceptibility to anionic modulation (*1*, *3*, *4*). Anionic control mechanisms in Hb[1] have been reconsidered in recent publications, and a delocalized chloride effect has been shown by Perutz and co-workers as the mode by which chloride overcomes the T-state destabilization associated with positive charges in the central cavity (*5*).

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[1] Abbreviations: Hb A_0, purified hemoglobin of adult human; His, histidine; Arg, arginine; Hb, hemoglobin; Cys, cysteine; bis-Tris, *N*,*N*-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; Tris, tris(hydroxymethyl)aminomethane; MOPS, 3-Morpholinopropanesulfonic acid; $Ru(NH_3)_6Cl_3$, Hexaamineruthinium (III) chloride; IHP, inositol hexaphosphate; NAD(H), nicotinamide adenine dinucleotide (reduced); NADP(H), nicotinamide adenine dinucleotide phosphate (reduced); 4-PDS, 4,4′-dithiodipyridine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid. Optional abbreviations: T, deoxy-type quaternary structure of Hb; R, oxy-type quaternary structure of Hb; Hb A, normal hemoglobin of adult human; MWC-PSK model, hemoglobin model developed by Monod, Wyman and Changeaux and modified by Perutz, Szabo and Karplus.

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To clarify further the structure/function relationships involved in anionic control of Hb function, we undertook an extended comparison of Hb A_0 and Hb Deer Lodge with regard to the effects of anions on their oxygenation and oxidation. The amino-acid substitution in Hb Deer Lodge, His 2(NA2)$\beta \rightarrow$ Arg, lies in the central cavity between the two β chains (*6*). The β2 position has been implicated in chloride binding by both deoxy and CO-forms of Hb, with a higher Cl^- affinity when deoxy (*7*). The β2 substitution in Hb Deer Lodge alters the positive-charge density in the β-chain anion binding site and is associated with an increased oxygen affinity and increased oxygen-linked anion sensitivity (*8*). The variant Hb also shows increased ease of oxidation, with increased anion sensitivity in its oxidation as documented in this report. We previously reported that significant differences in Hb function can be obscured by the low levels of chloride in bis-Tris/HCl or Tris/HCl buffers (*9*). Consequently, in this study we compare anion effects on oxygenation and oxidation and on sulfhydryl reactivity of Hbs in chloride-free buffers.

Our improved spectroelectrochemical techniques, as previously described (*10*, *11*) and briefly summarized in Methods, enabled us to compare anion effects on the redox potential of the heme iron in Hb A_0 and Hb Deer Lodge (β2 His $\rightarrow$ Arg). The electrochemical mediator utilized in our studies is not itself an anionic effector, a fact that enables us to examine anion effects on redox processes more effectively than with previous electrochemical approaches. Redox curves for both proteins were obtained under anaerobic conditions. The results are presented as Nernst plots, where the transition between the deoxy Fe(II) state and the oxidized Fe(III) state is followed as a function of electrode potential. The Nernst plots for the oxidation process are equivalent to Hill plots for the oxygenation process, where the transition between the deoxy ferrous state and the oxygenated ferrous state is followed as a function of oxygen concentration.

The quaternary conformation of hemoglobin has a large effect on its redox potential. The heme iron in Hb is typically more readily oxidized when in the high affinity R-state conformation than when in the low affinity T-state conformation. This conclusion was drawn by earlier investigators (*12*, *13*) and is confirmed by data presented here and in our previous papers on Hb spectroelectrochemistry (*10*, *11*). When the conformational equilibrium is shifted toward the T state by preferential anion binding, the midpoints of Nernst plots are accordingly shifted to more positive potentials (reflecting less easily oxidized states). Conversely, chemical modifications such as carboxypeptidase digestion that shift the R $\leftrightarrow$ T equilibrium to the left typically shift the Nernst plots toward more negative potentials (reflecting more easily oxidized states). The consequences of cooperative interactions are reflected in hemoglobin's oxidation curves as well as in oxygenation curves and are evidenced by slopes greater than one in the midrange of both Nernst and Hill plots (*10*, *12*, *13*).

Interesting effects of anions on the dynamic equilibrium between conformational states were revealed by comparison of the oxygenation and oxidation curves for Hb A_0 and those of the more anion-sensitive Hb variant, Hb Deer Lodge. As will be shown, when deoxy Hb is the dominant species present, increases in anion levels bring about significantly greater changes in the initial stages of oxygenation curves than in oxidation curves. This finding has led us to reconsider the mechanism of anionic control of active-site reactivity and to carry out additional experiments that probe the anion dependence of reactivity of other groups on the Hb tetramer.

Our review of the data presented here, together with previously published papers, leads us to suggest that the nature and level of anions present in solution have a profound effect on the conformational rigidity of deoxy Hb. The frequency and extent of tertiary-level conformational fluctuations of subunits within the deoxy tetramer are decreased by salt-bridge formation. It is these fluctuations that facilitate O_2 binding by changing steric hindrance. The relative insensitivity of the initial stages of oxidation to changes in anion concentration can be related to the structure of deoxy Hb. Water already in the α chain heme pocket would aid in formation of the aquomet form without requiring exogenous ligand entry, which would make the process relatively insensitive to fluctuations that alter steric hindrance at the active site. Oxygenation, by contrast, is highly sensitive to steric effects at the active site (*14*) and thus is subject to much larger shifts as anions increase the rigidity of the low-affinity conformation.

Our data and our proposed view of anionic control are consistent with the MWC-PSK model that describes the deoxy state in solution as having subunits of variable affinity based on the variable numbers of intact and broken inter-subunit salt bridges (*15*). A significant modification to the MWC-PSK model, as described by Rivetti et al., is our suggestion that the higher-oxygen-affinity subunits in the T-quaternary state (T′, T″, T‴...) are those with decreased steric hindrance, and that these are transiently populated in a dynamic conformational equilibrium. The anion independence of the initial stage of the oxidation process in Hb A_0 precludes the suggestion of Rivetti and co-workers that the higher-affinity subunits within the T-quaternary state have both the steric and electronic features of the R state. Our view, which introduces the concept of a rapid dynamic equilibrium between substates with variable steric hindrance, accommodates the available experimental data by assuming that the deoxy tetramer exhibits the dynamic average of its subunit affinities and that these vary in response to differing pH and anionic conditions. We conclude that Hb, among the best studied of macromolecules, is an excellent example of a multisubunit system in which physiologically significant control is exerted through alteration of conformational rigidity.

MATERIALS AND METHODS

Purified samples of native human hemoglobin (Hb A_0) and Hb Deer Lodge were prepared by using the ammonium sulfate method, stripped of organic phosphate cofactors, and chromatographed as previously described (*8*). The samples were typically dialyzed against 0.05 M MOPS (3-morpholinopropanesulfonic acid) at pH 7.15 with selected anions as desired. Sample concentration and compositions were determined spectrophotometrically. The amounts of oxidized Hb (metHb), oxygenated Hb (oxyHb), and hemichrome were determined by spectral analysis. Samples that contained any detectable hemichrome were discarded. The stock Hb solution, typically 1−2 mM in heme units (Fe porphyrin units), was stored in liquid nitrogen prior to use.

$Ru(NH_3)_6Cl_3$ (Strem Chemical Co. >99%), MOPS (Sigma Chemical Co. 99.5%), Platinum gauze (52 mesh, Fisher Scientific 99.95%), $NaNO_3$ (Fisher Scientific >99%), KCl (Fisher Scientific >99%), IHP (myo-inositol hexakis[dihydrogen phosphate], Sigma Chemical Co. as the 95+% dipotassium salt or the calcium salt of phytic acid) were used as received. NADP and NADP(H) (oxidized and reduced forms of β-nicotinamide adenine dinucleotide phosphate) were purchased from Sigma Chemical Co. and used as received as their tetrasodium salts. A 7 mM stock solution of 4-PDS (4,4′-dithiodipyride), Sigma Chemical Co., was prepared by dissolving the 4-PDS in 0.1 mL 95% ethanol and then bringing the solution to volume (typically 5 mL) with water. Water used in all experiments was doubly distilled.

The electrochemical mediator, $Ru(NH_3)_6Cl_3$, was dissolved to give a concentration of 3−5 mM in the desired buffer/electrolyte solution as described previously (*10*). Final concentrations were typically 0.35−1.0 mM in $Ru(NH_3)_6Cl_3$, and 0.050−0.20 mM in Hb monomer. MOPS buffer was used due to its noncomplexing nature and stability. Trizma buffers are unsatisfactory in that the system is slow to come to equilibrium, and they cause an increase in the rate of hydrolysis of $Ru(NH_3)_6Cl_3$. To evaluate effects of heterotropic ligands critically, anion-concentration-dependence experiments were performed for Hb Deer Lodge and Hb A_0 under the same conditions.

Oxygen Binding and Spectroelectrochemistry. Oxygen equilibria measurements were performed tonometrically, by using a modified spectrophotometric method based on that of Riggs and Wolbach (*16*).

Spectroelectrochemistry was performed with an optically transparent thin-layer-electrode (OTTLE) cell and procedures described previously (*10*, *11*). All potentials are quoted relative to the Ag/AgCl electrode. At the concentrations used $Ru(NH_3)_6Cl_3$ does not influence the Hb redox equilibrium within experimental error. The metHb ↔ deoxyHb equilibrium is reversible with $Ru(NH_3)_6Cl_3$ as a mediator. The absorbances of the Hb Soret band at various potential values were converted to a ratio of [oxidized form]/[reduced form] by using eq 1, where $A_{Tr(430)}$ is the absorbance at 430 nm when the potential of the working electrode is −450 mV and Hb is totally reduced, and A_{E430} is the absorbance at 430 nm at each potential *E*.

$$[\text{oxidized form}]/[\text{reduced form}] = (A_{Tr(430)} - A_{E430})/A_{E430} \quad (1)$$

The log of this ratio plotted as a function of potential constitutes a Nernst plot as expressed in eq 2,

$$\log[\text{oxid}]/[\text{red}] = (n/58.1)E - (n/58.1)E^\circ \quad (2)$$

where E is the potential of the working Platinum gauze electrode controlled by the potentiostat, E° is the midpoint reduction potential (log[oxid]/[red] = 0), n is the slope, and 58.1 is the value of RT/F at 20 °C. For a complex multicentered redox system, the formal redox potential, $E^{\circ\prime}$, is difficult to define. Therefore, we define the E° value as the value of E at half-oxidation, as is done for the case of nonideal Nernstian behavior (*17*). The reproducibility of the various spectroelectrochemical parameters under the conditions of the experiments reported here is $E^\circ = \pm 3$ mV, $n = \pm 0.10$ unit.

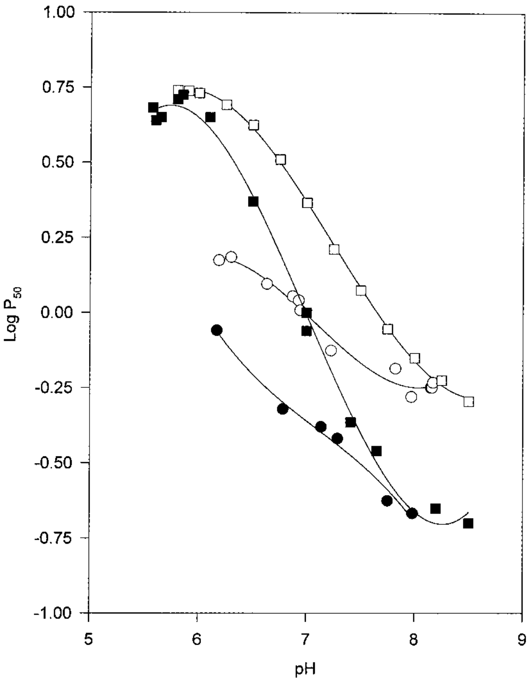

FIGURE 1: Bohr curves showing oxygen-binding characteristics of Hb A_0 (open symbols) and Hb Deer Lodge (closed symbols) in HEPES (circles) and bis-Tris-HCl and Tris-HCl (squares) buffers. Tonometric experiments were done in 0.05 M buffer at 20 °C with Hb at 60 μM (heme). Log P_{50} is the log of the pressure of oxygen required to bring Hb to half saturation.

Determinations of Sulfhydryl Reactivity. The procedures of Grassetti and Murray (*18*) were used to monitor the reaction of 4,4′-dithiodipyridine (4-PDS) with the SH groups of Hb under varied experimental conditions. The reaction progress was monitored spectrophotometrically at 324 nm, the maximum absorbance peak of the reaction product, 4-thiopyridone, with an equimolar ratio of 4-PDS to Hb.

RESULTS

Stereochemistry at the β-Chain Anion Binding Site in Hb Deer Lodge. It was previously determined that Hb A_0 and Hb Deer Lodge differ in primary structure only in the substitution of arginine for histidine at position β2 in the variant Hb (*6*). Although no crystallographic data are available for this variant, molecular modeling shows that the relatively longer arginine side chain can bring positive charge groups into closer proximity in the central cavity. As previously reported (*8*), the destabilization of its T state is attributable to the increased charge density in the central cavity.

Oxygenation at Varied Anion Levels. Chloride, even at low concentrations, can obscure the functional differences intrinsic to Hb variants like Hb Deer Lodge. Use of chloride-free HEPES buffers enables researchers to observe anion-dependent differences in function (*19*, *20*). As previously reported (*9*), the oxygen affinities of both Hb A_0 and Hb Deer Lodge are greatly affected by the presence of chloride in bis-Tris/HCl buffers, with higher affinity exhibited in HEPES. Figure 1, which contrasts the Bohr effect curves for oxygen binding to Hb A_0 and Hb Deer Lodge in HEPES

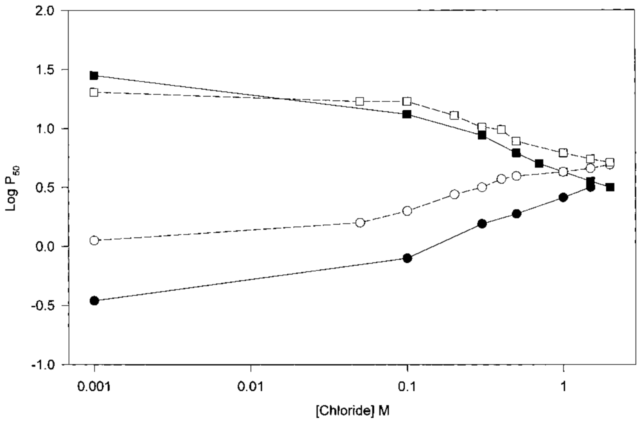

FIGURE 2: Chloride-inositol hexaphosphate competition in oxygen-binding experiments with Hb A_0 (open symbols) and with Hb Deer Lodge (closed symbols). Circles show the chloride titration curve. Experiments represented by squares were done in the presence of 100-fold excess IHP over tetramer, 0.05M HEPES buffer, pH 7.5, at 20 °C with increasing chloride concentrations as indicated on the *x* axis. Other conditions as in Figure 1.

and bis-Tris/HCl, sets the stage for the functional comparison of relative anion effects on these two proteins. As shown in Figure 1, the proteins have about the same affinity at low pH in bis-Tris/HCl, where anionic contributions offset the T-state destabilization in Hb Deer Lodge. The destabilization of the T state of Hb Deer Lodge is most evident at high pH where the β2 position is fully charged only in the variant Hb, resulting in a dramatic increase in the magnitude of its Bohr effect.

Figure 2 shows chloride titrations in HEPES for Hb A_0 and Hb Deer Lodge at pH 7.5 in the presence and absence of 100-fold excess of inositol hexaphosphate (IHP) over Hb tetramer. The chloride titration in the absence of IHP shows that the oxygen affinity of Hb Deer Lodge has enhanced Cl^- sensitivity but is never quite normalized by this effector at this pH. IHP also brings about a relatively larger change in oxygen affinity for Hb Deer Lodge. In the presence of this highly charged anion the two proteins have equal oxygen affinities at pH 7.5. The competitive anion titrations, whose results are presented in Figure 2, show that for both proteins the chloride anion progressively displaces IHP and, at high chloride levels, results in the same oxygen affinity as observed in high chloride in the absence of the organic phosphate effector. At a given chloride-to-IHP ratio, the competitive chloride effect is somewhat more marked for Hb Deer Lodge than for Hb A_0, indicating that Cl^- displaces IHP more readily in Hb Deer Lodge (also seen in NADP/Cl^- competition as reported below).

Some investigations of Cl^- and IHP effects on oxygen binding by Hb A_0 and Hb Deer Lodge were carried out in 0.05 M MOPS buffer to facilitate comparisons with the spectroelectrochemical experiments described in a later section. Oxidation curves obtained in chloride-free HEPES and MOPS buffers are identical (data not shown), while the oxygen affinity in chloride-free MOPS buffer is somewhat lower than in chloride-free HEPES buffer (see Table 1). We interpret this differential buffer effect as an indication of reduced conformational fluctuations in MOPS relative to HEPES (see Discussion).

Table 1: Anion-Induced Changes in log P_{50} and in the Apparent Affinity of the T-State at Half Saturation (indicated by log T_{50}) of 40−80 μM (heme) HbA_0 and Hb Deer Lodge in Oxygen-Binding Experiments Done in 0.05 M MOPS, pH 7.1 ± 0.08 at 20 °C. Data Based on Figure 3, Figure 4, and Similar Experiments (not shown). HEPES Data Are Included for Comparisons. Effector Levels Given Are Relative to Hb Tetramer Concentration

protein	cofactors	log T_{50}	log P_{50}
HbA_0	Cl-free HEPES	0.76	−0.04
	Cl-free MOPS	0.66	0.05
	0.2 M KCl	1.31	0.71
	0.2 M KCl + 0.08 mM NADP	1.15	0.72
	0.2 M KCl + 4-fold IHP	1.72	0.93
	0.2 M KCl + 20-fold IHP	1.93	1.44
Hb Deer Lodge	Cl-free HEPES	−0.03	−0.38
	Cl-free MOPS	0.12	−0.11
	0.2 M KCl	0.90	0.70
	0.2 M KCl + 0.08 mM NADP	0.57	0.54
	0.2 M KCl + 104-fold IHP	1.76	1.43

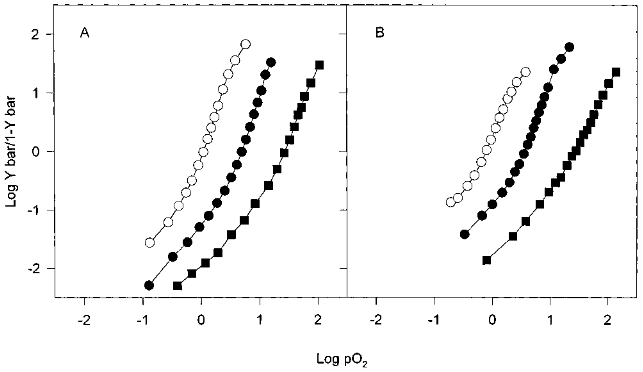

FIGURE 3: Hill Plots of oxygen binding to HbA_0 (A) and Hb Deer Lodge (B) in 0.05 M MOPS, pH 7.16, at 20 °C. Open circles are in chloride-free buffer; closed circles show oxygen binding in the presence of 0.2 M KCl; closed squares represent oxygen binding with both 0.2 M KCl and saturating amounts of IHP (20-fold excess for HbA_0, 104-fold excess for Hb Deer Lodge per Hb tetramer). Other conditions as in Figure 1.

Representative Hill plots are shown in Figure 3 for Hb A_0 and Hb Deer Lodge in MOPS at pH 7.16 with no added Cl^-, with 0.2M Cl^-, and with 0.2M Cl^- with saturating amounts of IHP. As shown, the initial stages of oxygen binding to deoxy Hb, and the apparent K_T, are significantly shifted by addition of anions. The relative magnitude of anion-induced shifts of the deoxy state, indicated in Table 1 as log T_{50}, is relevant to points raised later in this paper.

Titrations (not shown) revealed that for both Hb A_0 and Hb Deer Lodge the IHP effect on oxygen binding or oxidation in the presence of 0.2 M Cl^- reaches its maximum at IHP:tetramer ratios of about 20:1. The requirement for such high levels of IHP is a reflection of the IHP/chloride competition. Hb Deer Lodge exhibits significantly reduced cooperativity in oxygen binding at high IHP levels, with *n* close to 3 in the absence of IHP and about 2 at 26-fold IHP over tetramer, in contrast to the unchanged cooperativity (n_{50} about 3) observed in experiments with Hb A_0. It appears that IHP shifts the oxygenated form of Hb Deer Lodge toward the T state more effectively than that of Hb A_0.

The nicotinamide adenine dinucleotides (NAD, NAD(H), NADP, and NADP(H)) all contain a pair of phosphates in their structures that makes it possible for these materials to bind to Hb's β-chain anion binding site and act as allosteric effectors (*20*). We conducted experiments to determine if these effectors have different binding affinities in Hb Deer Lodge relative to Hb A_0 as a consequence of the altered anion

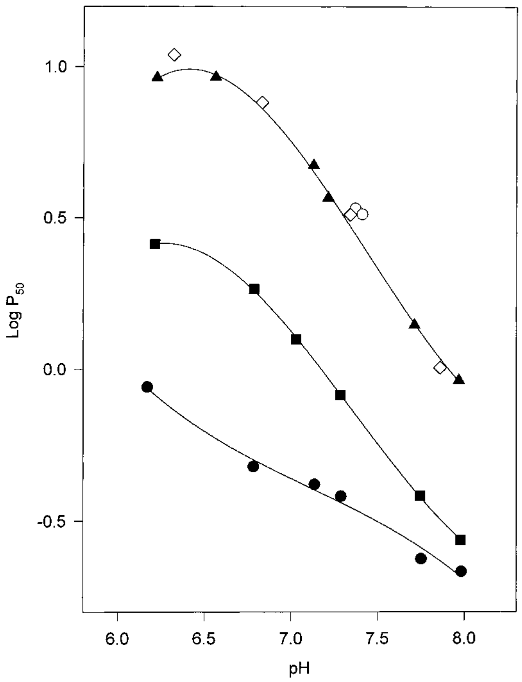

FIGURE 4: Effects of 8-fold excess over tetramer of NAD(H) and NADP(H) on oxygen binding of Hb Deer Lodge (closed symbols) and Hb A_0 (open symbols). Closed circles represent Hb Deer Lodge in 0.05 M HEPES buffer at 20 °C. Closed squares are Hb Deer Lodge with 0.5 mM NAD(H); closed triangles, Hb Deer Lodge with 0.5 mM NADP(H). Open circles are Hb A_0 in HEPES with NADP(H); open diamonds, Hb A_0 in bis-Tris or Tris with NADP-(H). Other conditions as in Figure 1.

binding site in the Hb variant. Figure 4 shows Bohr effects obtained for Hb Deer Lodge in HEPES and with added NAD(H) and NADP(H) and comparable data for Hb A_0. These dinucleotides show increasing oxygen-affinity shifts for NADP(H) over NAD(H) as effectors, indicating the functional significance of the additional phosphorylation of NADP(H). As was found for IHP, the NADP(H) effect is greater for Hb Deer Lodge than for Hb A_0. A rather surprising feature, evident in Figure 4, pertains to the binding site for this class of effectors. The affinity shift brought about by addition of NADP(H) to Hb Deer Lodge is most marked at high pH, where NADP(H) but not NAD(H) is able to maintain the variant Hb at an affinity like that of Hb A_0. This suggests that interactions between the phosphate group of NADP(H) and the arginines at the $\beta 2$ position in the anion binding site offset the positive-charge effects that normally destabilize the T state in the variant Hb. We regard this as a clue to how positive charges in the central cavity destabilize the T state and how the destabilization can be overcome.

Some experiments with the dinucleotide effectors were carried out in 0.05 M MOPS with 0.2 M Cl$^-$ to enable us to compare directly our spectroelectrochemical and oxygen binding data. When NADP is added to Hb A_0 under these conditions, no alteration in oxygen binding is observed, while NADP offsets some of the chloride effect in Hb Deer Lodge as evidenced by a slightly *increased* affinity (log P_{50} decreases from 0.70 to 0.54). This result indicates competition between the effectors and is reminiscent of the IHP/Cl$^-$ competition results presented in Figure 2.

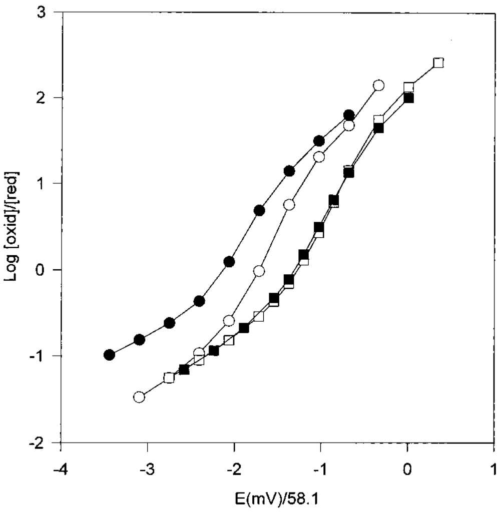

FIGURE 5: Influence of chloride on oxidation of Hb Deer Lodge and Hb A_0. Nernst plots are shown for Hb A_0 (open symbols) and Hb Deer Lodge (closed symbols) in 0.05 M MOPS, pH 7.1, at 20 °C (circles) and 0.2 M KCl (squares), vs Ag/AgCl. [heme] = 80 μM to 120 μM. The mediator, Ru(NH$_3$)$_6$Cl$_3$, is in a 10-fold excess over [heme].

Oxidation at Varied Anion Levels. Oxidation data obtained by our spectroelectrochemical methods complement and extend the oxygenation data presented above. Figure 5 presents oxidation curves for Hb A_0 and Hb Deer Lodge in 0.05 M MOPS at pH 7.1 in the presence and absence of 0.2 M Cl$^-$. In the absence of Cl$^-$, the oxidation curves (Nernst plots) for Hb Deer Lodge and Hb A_0 are quite different in the initial stages of oxidation. Decreased cooperativity, with lower values of n and n_{max}, and a shift toward greater ease of oxidation signal the destabilization of the T state of Hb Deer Lodge. There is much less difference between Hb Deer Lodge and Hb A_0 in the final stages of the curves where their R states are dominant. Figure 5 shows that the two proteins have equivalent oxidation curves at this pH in the presence of 0.2 M Cl$^-$.

Significant differences in anionic control of oxidation and oxygenation processes are very much in evidence when one contrasts the initial stages of Nernst plots and Hill plots where the deoxy protein is the dominant species present. This is readily apparent when one compares Figure 3 (oxygenation) and Figure 5 (oxidation). Additions of IHP or other anions have much smaller effects on the apparent affinity of the T state for the oxidation process than for the oxygenation process. These differences are not due to effects of subunit dissociation. Under all conditions examined, the T state is highly associated at the concentrations used in these experiments. Experimental results equivalent to those shown here are observed at ten-fold lower or higher protein concentrations.

Further insight into the differences in anion controls of oxygenation and oxidation were obtained by adding progressive amounts of IHP to Hb A_0 and Hb Deer Lodge in the presence of 0.2 M Cl$^-$. Representative results are shown in Figure 6. We are able to probe differential IHP effects under these conditions since this chloride concentration makes the redox binding curves equivalent for the two proteins as shown in Figure 5. By maintaining a constant pH of 7.1, we ensure that the charges on the heterotropic ligand stay the same, that the T ↔ R equilibrium is not responding to pH shifts, and that the amount of OH$^-$ bound to metHb is negligible. Figure 6 shows that increasing the ratio of IHP:

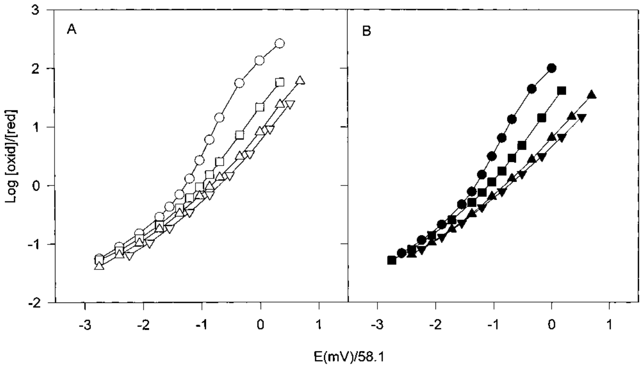

FIGURE 6: Nernst Plots of Hb A_0 and Hb Deer Lodge in 0.05 M MOPS, pH 7.15, 0.2 M KCl, at 20 °C with with varying amounts of IHP. (A) Hb A_0: circles, no IHP; squares 1-fold excess IHP over tetramer; up triangles, 10.4-fold excess IHP over tetramer; down triangles, 18-fold excess over tetramer. (B) Hb Deer Lodge: circles, no IHP; squares, 1-fold IHP; up triangles, 10.8-fold IHP; down triangles, 22-fold IHP.

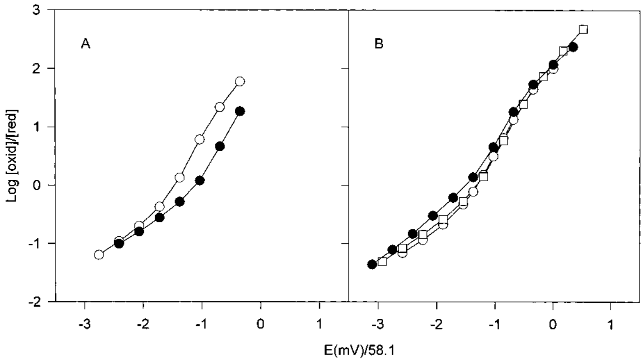

FIGURE 7: Effects of Cl^- and NADP on the oxidation of Hb Deer Lodge. (A) Nernst Plot of Hb Deer Lodge in 0.05M MOPS, pH 7.1, with 0.02 M KCl (open circles), and with 0.02 M KCl and 8-fold excess NADP (closed circles). (B) Nernst Plot of Hb Deer Lodge in 0.05 M MOPS, pH 7.1, with 0.2 M KCl (open circles), with 0.2 M KCl and 4-fold (open squares) and 8-fold (closed circles) NADP over Hb tetramer.

Hb progressively shifts the Nernst plots to more positive potentials for Hb Deer Lodge and Hb A_0; and, as in the oxygenation studies, relatively high ratios of IHP to tetramer are required to bring about a maximal response. Cooperativity in the oxidation process decreases as IHP is increased for both proteins, with n_{50} values of unity for Hb Deer Lodge and 1.2 for Hb A_0 at high IHP levels. IHP thus appears to stabilize the T state of the fully oxidized form of both proteins. As in the oxygenation process, the IHP effect on the upper asymptote of the curves is most pronounced with Hb Deer Lodge.

The nicotinamide adenine dinucleotides, like IHP, shift the oxidation curves of Hb A_0 and Hb Deer Lodge, with variable results depending on the chloride concentration. Figure 7 shows results obtained with Hb Deer Lodge in the presence of various concentrations of NADP and Cl^-. Redox shifts in response to NADP(H) are not shown because the reduced cofactor is oxidized to NADP by the oxidizing potential applied by the electrode, with the consequence that experiments initiated with NADP(H) give approximately the same results as those initiated with NADP present. When NADP is added to Hb Deer Lodge in the near absence of chloride (0.02 M), the effector shifts the Nernst plots to the right (toward the T state) as shown in Figure 7A. In chloride-free conditions Hb Deer Lodge is more strongly shifted than Hb A_0. When the experiment is carried out in the presence of 0.2 M Cl^-, NADP causes no appreciable shift in the Hb A_0 oxidation curves ($\Delta E° = 0$) but causes a significant shift toward the more easily oxidized and oxygenated (R-state) condition in Hb Deer Lodge as its concentration is increased. This shift in Deer Lodge is shown in Figure 7B. This result is in accord with the oxygenation data and further supports the conclusion that NADP binding displaces Cl^- or interferes with its allosteric effect in both redox and oxygen-binding curves of Hb Deer Lodge.

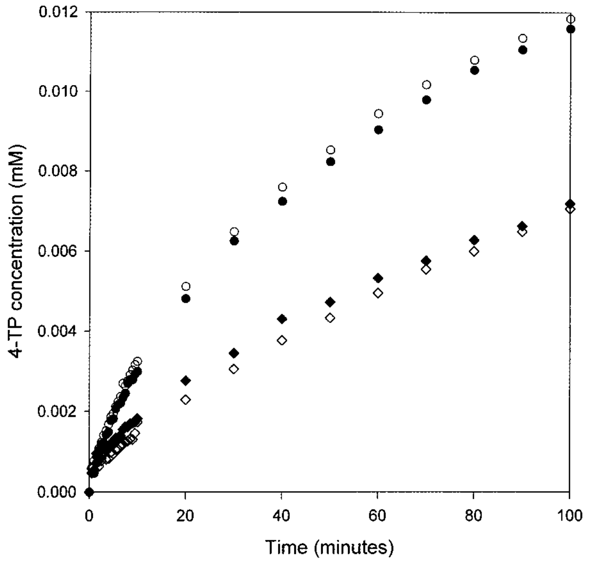

FIGURE 8: Time course of formation of 4-thiopyridone from 4,4′-dithiodipyridine (*22*) when reacted with deoxygenated Hb A_0 and Hb Deer Lodge with and without 0.2 M Cl^-. Samples were in 0.05 M Hepes pH 7.1 at 20 °C. Initial concentraion of 4-PDS was 0.03 mM; heme concentration was 0.03 mM. Open circle, control Hb A_0; open diamond, Hb A_0 + 0.2 M NaCl; closed circle, Hb Deer Lodge; closed diamond, Hb Deer Lodge + 0.2 M NaCl.

Sulfhydryl Modifications of Hb A_0 and Hb Deer Lodge. In their studies, Grassetti and Murray (*18*) found that the sulfhydryl-selective reagent 4,4′-dithiodipyridine (4-PDS) reacts readily with thiols to form 4-thiopyridone (4-TP). The pyridine and the pyridone have characteristic spectra, allowing for facile monitoring of the change. To test the hypothesis that conformational fluctuations are enhanced in chloride-free buffers, we examined the reaction of 4-PDS with deoxy Hb A_0 and Hb Deer Lodge under varied conditions. As shown in Figure 8, the reaction is much faster in chloride-free HEPES buffers than in the presence of 0.2 M Cl^-. The reaction in chloride-free buffers is slower in Hb, where the SH groups are less accessible than in free Cys, where the reaction is complete in less than 2 min under comparable reaction conditions (data not shown). The effect of Cl^- on the reaction rate of SH groups in deoxy Hb has not been previously reported. However, phosphate anions typically slow the rate of sulfhydryl modifications (*21*, *18*, *22*). As pointed out by Ampulski et al. (*23*), sulfhydryl reactivity toward 4-PDS mirrors the effects of phosphates observed in oxygen-binding studies. A particularly significant finding in our work is that although Hb Deer Lodge has higher oxygen affinity and is more easily oxidized than Hb A_0 in the absence of anions, the two proteins show equivalent sulfhydryl reactivity.

DISCUSSION

The data obtained for Hb Deer Lodge relative to Hb A_0 show that effects of its primary structure substitution (*β*2His → Arg) are most evident in chloride-free buffers. Destabilization of the T state of Hb Deer Lodge relative to Hb A_0 is evident in increased ease of oxidation and oxygenation, but not in increased rates of sulfhydryl modification. In oxidation and oxygenation studies both singly charged anions like chloride and polyvalent phosphate anions bring about larger functional changes in Hb Deer Lodge than in Hb A_0. Competitive and antagonistic allosteric effects are evident when chloride is present with polyphosphate anions or sulfhydryl-reactive reagents, respectively. Comparison of the anion effects on redox and oxygen-binding curves of Hb Deer Lodge and Hb A_0 allows us to distinguish between electronic and steric components of anionic control and has led us to a new view of Hb function in which anionic control of a dynamic conformational equilibrium within the T state plays a large part.

Previous studies on Hb Deer Lodge showed its T state to be destabilized relative to Hb A_0 (*8*). The differences between the two proteins are most pronounced at high pH where only the variant Hb has charged residues at the *β*2 position. This is in accord with the hypothesis put forward by Bonaventura and Bonaventura (*1*, *2)* that excess positive charges in the central cavity of Hb destabilize its T structure by electrostatic repulsion and thereby shift the allosteric equilibrium toward the R state. This allosteric mechanism of chloride interactions was recently expanded in a paper by Perutz et al. (*5*) in which the repulsion between positively charged groups in the central cavity was shown to be diminished by delocalized binding of chloride ions.

The feature we now add to this increasingly detailed picture is that anions restrict the frequency and extent of conformational fluctuations that transiently populate higher affinity structures within the T-quaternary state of Hb. This is consistent with crystallographic studies that show that anion binding adds stabilizing salt bridges but does not change the T-state structure (*3*, *24*, *25*). Our view, which is a modification of that put forward to explain the differences between the T state observed in crystals and that observed in solution (*15*) is that the ligation and redox equilibria are best described by presuming a rapid fluctuation between a variety of T-state structures (T, T′, T″, T‴ etc.) of variable steric hindrance and consequently variable oxygen affinity, the ensemble of which is in equilibrium on a longer time scale with the higher affinity, more-easily-oxidized R state. Since the (T, T′, T″, T‴...) ↔ R equilibrium is on a slower time scale than the fluctuations between substates within the T structure, the R state sees an "average" T state, the nature of which depends on experimental conditions.

The existence of a (T, T′, T″, T‴...) ↔ R equilibrium and fluctuations between these states can accommodate the anion and pH dependence of the "average" T state, proton release upon ligation of T-state molecules, and the noncooperative ($n = 1$) asymptotes observed for the initial phases of Hill plots. These experimentally determined features of hemoglobin function are extensively discussed by Lee et al. (*26*), in their analysis of proton release in oxygen binding by Hb. Anion-dependent alterations of the T-state tetramer are also shown by NMR studies that probe the environment of surface histidine residues (*27*−*29*).

In our view, the Hill-plot slopes for time-averaged T states would be expected to be unity under conditions where ligation of one T-state monomer does not alter the reactivity of its neighbors and the fluctuations are rapid with respect to the phenomena measured. Conditions may exist where these two requirements for unit slope are not met, with cooperative interactions contributing to slopes greater than one and heterogeneity between chains contributing to slopes less than one. There have, in fact, been reports of cooperative oxygen binding within the T-quaternary structure (*15*, *30*−*32*).

The salt bridges formed in T-state Hb between *β*146His and α94Asp make *β*93Cys in deoxy Hb less reactive toward SH reagents than that in oxy Hb (*21*, *33*). Data presented in this report on the anion sensitivity of the 4-PDS reaction with Hb supports our concept of a rapid T-state (T,T′,T″, T‴...) dynamic equilibrium. Decreased steric hindrance of the substates is reflected in faster reactivity toward 4-PDS in chloride free buffers, without the biphasic behavior one would expect if the substates were not in rapid equilibrium. Under all conditions examined, the reaction goes to completion in deoxy Hb as His-Asp salt bridges are progressively broken.

If the extent and frequency of conformational transitions in deoxy Hb Deer Lodge exceed those in deoxy Hb A_0, an expected consequence would be considerably faster reactivity toward 4-PDS. Strikingly, the reaction of 4-PDS with the *β*93 sulfhydryl in deoxy Hb Deer Lodge was found to be equivalent to that of Hb A_0 in the absence of anions, and both proteins show equally slowed rates in the presence of 0.2 M Cl^-. This indicates that the T-state destabilization in Hb Deer Lodge is primarily electronic rather than steric, possibly resulting from alterations in iron−proximal histidine interactions at the active site that favor the more-easily-oxidized R state.

The oxidation curves reported here provide clear evidence for electronic changes in Hb Deer Lodge relative to Hb A_0 (Figure 5). Destabilization of the T state of Hb Deer Lodge results in a decreased cooperativity for both its oxygenation and oxidation curves when the protein is examined in chloride-free buffers. Addition of Cl^- largely overcomes the T-state destabilization, but cooperativity of oxygen binding to Hb Deer Lodge remains somewhat depressed since the upper parts of the Hb Deer Lodge curves are also shifted toward the T state. These results indicate that chloride interacts with both the oxidized and oxygenated forms of the variant Hb more strongly than with the comparable forms of Hb A_0. We refer to the change in $E°$ in the presence and absence of Cl^- as $\Delta E°$. As listed in Table 2, we find for Hb A_0 a value of $\Delta E° = 26 \pm 4$ mV. For Hb Deer Lodge, the value of $\Delta E° = 48 \pm 4$ mV. The larger shift in the $E°$ values for Hb Deer Lodge is consistent with the interaction of heterotropic ligands with Hbs as found in our previous work (*10*). The chloride effects on the oxidation-curve differences between Hb Deer Lodge and Hb A_0 can thus be described in general terms by the two-state allosteric model of Monod, Wyman, and Changeux (*34*).

The two-state MWC model can also be used to describe the central features of the IHP and NADP effects on the oxidation curves of Hb Deer Lodge relative to Hb A_0. As

Table 2: Effects of Chloride and Inositol Hexaphosphate (IHP) on Oxidation of Hb Deer Lodge and Hb A_0[a]

protein	effector[b]	$E°$ (mV)[c]	n_{max}[c]	ΔE_n (mV)[d]
Hb A_0	none	−100	2.10	0
Hb Deer Lodge	none	−125	1.70	<10
Hb A_0	0.2 M Cl^-	−74	2.10	30
Hb Deer Lodge	0.2 M Cl^-	−77	1.85	25
Hb A_0	0.2 M Cl^- + IHP:Hb = 1:1	−63	1.40	45
Hb Deer Lodge	0.2 M Cl^- + IHP:Hb = 1:1	−63	1.40	50
Hb A_0	0.2 M Cl^- + IHP:Hb = 4:1	−53	1.35	50
Hb Deer Lodge	0.2 M Cl^- + IHP:Hb = 4:1	−46	1.30	>50
Hb A_0	0.2 M Cl^- + IHP″Hb = 10:1	−49	1.40	50
Hb Deer Lodge	0.2 M Cl^- + IHP″Hb = 10:1	−49	1.10	>50
Hb A_0	0.2 M Cl^- + IHP:Hb = 20:1	−43	1.20	80
Hb Deer Lodge	0.2 M Cl^- + IHP:Hb = 20:1	−43	1.00	>100

[a] All data taken at 0.05 M MOPS, pH 7.15, + 20 °C vs Ag/AgCl. [b] The effectors listed relate to both Hb A_0 and Hb Deer Lodge grouped together. [heme] = 0.080 mM to 0.12 mM; $[Ru(NH_3)_6Cl_3]$ = 1.04 mM to 1.117 mM. The IHP:Hb ratios are based on the Hb tetramer. [c] The uncertainties in the parameters are: $E° = \pm 2$ mV, $n_{max} = \pm 0.1$ unit. [d] $\Delta E_n = E_{n\max} - E_{n50}$ and is dependent on experimental conditions and is used to describe the asymmetry of the Nernst plot induced by the interaction of Hb with the heterotropic effector.

previously reported, the polyvalent anions are bound more strongly to deoxy Hb than to oxy or met Hb and shift the conformational equilibrium toward the T state (*3*, *35−37*). The Nernst plots are shifted in a positive direction in a nonuniform manner by addition of organic phosphates, just as in the Cl^- effect. There are large shifts in the E values at log [oxid]/[red] = +1, where metHb predominates. When the starting point for comparison is 0.2M Cl^-, the largest effect of IHP is on the oxidized Hb form, where IHP appears to shift the equilibrium strongly toward the less-easily oxidized T state (Figure 6). This confirms previous studies of IHP effects on metHb (*38*), anion-induced redox shifts (*37*), and decreased cooperativity in the oxidation of Hb A_0 in the presence of IHP (*13*). The oxidation curves for Hb Deer Lodge are noncooperative at high IHP levels (n_{max} = 1.0), while those for Hb A_0 maintain some cooperativity (n_{max} = 1.2), indicating pronounced IHP-stabilization of the T state of the oxidized forms of both proteins (Table 2).

We find that at high ratios of IHP:Hb, the initial stages of the oxidation process have n values considerably less than one that extend well above the 50% level of oxidation. This heterogeneity is attributable to appreciable differences in the redox potential of the α and β chains. Chain differences in oxidation potential under other conditions were reported previously and are believed to contribute to depressed levels of cooperativity in oxidation curves relative to oxygenation curves (*39*, *40*).

At high pH where the differences between Hb A_0 and Hb Deer Lodge are most striking, we find that NADP(H), but not NAD(H), can equalize their oxygen affinities, suggesting that the phosphorylated nucleotide interacts at or near β2 Arg. The effects of the dinucleotides on both oxygenation and oxidation of Hb Deer Lodge relative to that of Hb A_0 are most evident at low chloride levels. This is an expected result, in light of the foregoing chloride and IHP results. An unexpected observation was the apparent R-state stabilization of both oxidation (shown in Figure 7) and oxygenation curves observed when NADP is added at higher (0.2 M) levels of chloride. This shift is attributable to NADP interference with the T-state stabilization brought about by chloride. A comparable reduction of the chloride effect by NADP addition was not observed in oxygenation or oxidation curves with Hb A_0, consistent with more facile destabilization of the T state of Hb Deer Lodge. We previously reported that oxygen-linked interactions of Hb with NADP(H) can arise from electrostatic interactions with the linking phosphates of NADP(H) or its phosphorylated nucleotide, and from hydrophobic interactions between Hb and the aromatic rings (*20*). The interaction of two fluorophores, namely adenine and an amino acid such as tryptophan, would explain the quenching of NADP(H) fluorescence observed in our previous studies (*41*). The hydrophobic interactions of NADP(H) with Hb may oppose the electrostatic interactions. As Cl^- levels are increased, the ionic bonds between the phosphates and positive residues of the Hb will weaken, and the hydrophobic interactions will become more significant.

The data reported here are consistent with a mechanism of anionic control that involves anion-linked shifts of a (T, T′, T″, T‴...) ↔ R equilibrium. This model retains many of the conceptual features of the two-state model and is in accord with the experimental observation of two primary quaternary structures, the R and T states (*24*, *25*, *3*). The two-state model in its simplest form does not, however, accommodate the pH and anion-dependent variation of the calculated affinity of the T state (*42−46*). As pointed out by Poyart and co-workers, there is a problem with calling a state R or T since the apparent R- and T-state affinities vary by over a factor of 10 in the presence of allosteric effectors (*47*). Minton and Imai (*43*) presented arguments for a three-state model, showing that the unmodified two-state model could only describe the curves obtained under any one condition.

Perutz (*48*) put forward a mechanism derived from the two-state theory which differed in that it allowed for tertiary level changes of the α and β chains within the alternative quaternary states. This mechanism takes into consideration X-ray data showing that while addition of either IHP or DPG to deoxy Hb A shifts the oxygen affinity observed in the initial stages of binding, these effectors do not alter the quaternary conformation or markedly change the active-site region of deoxy Hb from that seen in the absence of these effectors (*49*, *50*). The crystallographic studies, along with functional studies (*36*, *42*, *51*), showed that salt bridges formed by anionic effectors contribute to stability of the T conformation. Evidence of tertiary-level changes that affect the oxygen affinity of deoxy Hb has accumulated since the early studies of Desbois and Banerjee (*37*) who reported that several polyanions change Hb's oxygen affinity without causing the spectral changes typically associated with quaternary structural changes. Striking pH and anion-dependent affinity shifts are also observed in noncooperative Hb systems that are presumably devoid of quaternary shifts, such as the half-oxidized Hb valency hybrids (*52*) and chemically modified *Amphiuma* Hb (*53*). Perutz, drawing on crystallographic data, postulated that the effectors which bring about shifts in the oxygen affinity of the apparent T state, as evident in shifts in the apparent K_T, act by fortifying the salt bridges of the T structure, thereby inducing a continuum of thermodynamic states associated with subtle changes in tertiary structure (*54*).

To explain the differences between Hb in solution and that in crystals, Rivetti et al. (*15*) made use of a synthesis of the MWC and Perutz models, with aspects adopted from the statistical mechanical formulation of Szabo and Karplus (*26*, *55*). The extended model, referred to as a MWC-PSK model, continues to stimulate research, controversy, and speculation.

It is within this historical and conceptual context that we have sought to understand our comparative data on the oxidation and oxygenation curves of Hb A_0 and Hb Deer Lodge. Unlike oxygenation curves, the oxidation curves do *not* show pronounced anion-dependent changes in behavior in the initial stages of oxidation when the deoxy condition is dominant, and those of Hb Deer Lodge resemble those of Hb A_0 once its T-state destabilization is overcome by addition of 0.2 M Cl. As shown in Figure 5, there is no change in the apparent T state for oxidation of Hb A_0 as chloride levels are increased from 0 to 0.2 M, although the system is cooperative and the Nernst plot shows changes in the midrange consistent with the a shift of allosteric-equilibrium constant toward the T state. In the same buffer system the oxygen-binding curves show a large chloride-induced shift in apparent K_T, with a shift of log T_{50} from 0.66 to 1.31. Subsequent addition of IHP to a level of 4 IHP per tetramer causes a further shift in the apparent T-state affinity of the oxygen-binding curves, to a log T_{50} of 1.72. At IHP levels of 20 per tetramer, the apparent T-state affinity of the oxygenation curves is still further decreased, giving a log T_{50} of 1.93 (see Table 1), while the lower asymptote of the oxidation curves is only slightly shifted (Figure 5). Small IHP-induced changes in the heme region responsible for these changes in the redox potential may be associated with larger structural modifications elsewhere, such as the IHP-induced movement of the A helices of the β chains toward the center of the molecule (*50*).

Why is the oxidation process relatively insensitive to anions in its initial stages? Crystallographic data on oxy and metHb in the T structure show that the heme iron moves less upon oxidation than upon oxygenation (*56*) thereby explaining a relative decrease in free-energy changes for the T-to-R shift in the oxidation process. Given that redox changes associated with the full T-to-R shift are readily detectable in our system, as are the extensive redox changes in T-state destabilized Hb Deer Lodge, we have to search elsewhere for the explanation of anion insensitivity in the initial stages of the process. We know that electron withdrawal or addition can occur through the heme edge, so that this process is governed by electronic rather than steric considerations. In oxidation of the β hemes a water molecule enters and forms the sixth ligand, while in the α hemes there is a partially occupied water site in deoxy Hb, so less water has to enter to form the aquomet derivative. Therefore, in the initial stages of oxidation there are no major steric obstacles to overcome, since the α chains already have a water molecule poised for occupancy of the sixth position. Previous studies have shown that the α chains are, in fact, more easily oxidized (*57*, *58*). These considerations, taken together, provide a satisfactory structural explanation for why the oxidation process in its initial stages is primarily responsive to electronic aspects of the quaternary equilibrium, and relatively insensitive to anion-induced increases in steric hindrance. Due to the need for water entry into the central cavity, both electronic and steric effects are operable in the full oxidation of both types of chains, and anion effects are correspondingly evident in the later stages of the Nernst plots. We are currently carrying forward a more detailed modeling of these differences between Nernst and Hill plots as part of an investigation of the comparative oxidation and oxygenation properties of active-site metal hybrids.

The differences between the oxidation and oxygenation processes (Tables 1 and 2; Figures 3, 5, and 6) show that the sensitivity of oxygenation to conformational rigidity within the T state is not a minor effect, but has the consequence of greatly extending the impact of anionic control of function. As described above, we are able to reconcile the differences in anion effects on the apparent K_T as observed in Hb's oxygenation and oxidation curves by considering that anion modulation of function operates through two components, one electronic and one largely steric. The electronic and steric components are not generally appreciated as being distinct since both are clearly involved in the full T-to-R conformational shift (*24*, *25*, *3*). It was shown in early NMR studies that anions affect the stereochemistry of the active site, bringing E11 valines closer to the iron atom in carbonmonoxy Hb (*59*). The electronic component of anion modulation operates through changes in structure that alter electron affinity at the active-site heme, such as alterations in the distance between the iron and porphyrin nitrogens and the proximal histidine residues as seen in crystallographic (*24*, *25*) , resonance Raman (*60*), and NMR (*32*, *61*) investigations.

Our view is that the oxygen affinity of deoxy Hb decreases as anion binding forms salt bridges that restrict the frequency and extent of subunit fluctuations to higher-affinity, less-sterically-hindered states in a multi-T-state (T, T', T'', T'''...) ensemble. The T- and R-state quaternary structures have clearly distinct redox properties, as shown by studies of fully R-like carboxypeptidase-digested Hb (*11*) and as reported here for Hb Deer Lodge where the T state is partially destabilized. Since the initial stages of oxygenation, but not oxidation, are very sensitive to anions, we have concluded that the higher-oxygen-affinity conformers in deoxy Hb are ones with less steric hindrance but with the electronic nature characteristic of the T state. This conclusion is a departure from the MWC-PSK model as described by Rivetti et al. (*15*), which also puts forward the hypothesis that deoxy Hb maintains the T-quaternary conformation but contains subunits in higher-oxygen-affinity states. Rivetti et al. (*15*) hypothesized that the higher-affinity states are those with broken salt bridges and R-state tertiary structures. Our data indicate that the subunits in the T-state (T, T', T'', T'''...) ensemble vary primarily in degree of steric hindrance and do not exhibit the electronic characteristics of the R state.

Successes in genetically altering Hb so that its active-site steric barriers are diminished or removed, such as replacement of the distal histidine by glycine or replacement of the distal valine by alanine, are leading to an increased understanding of how barriers at the active site of Hb affect active-site reactivity toward various ligands (*14*, *62*). These studies have provided conclusive evidence that steric hindrance plays an important role in establishing Hb's oxygen affinity. By controlling the frequency and extent of conformational fluctuations involving critical residues, anions can magnify the effect of steric hindrance at Hb's active site. Reactivity of groups at places other than the active site can clearly also be subject to anion-controlled conformational fluctuations.

In general, alterations in the conformational rigidity of a macromolecule can operate at the tertiary or quaternary structural level via any change that alters the ease of conformational transitions. Alterations in conformational rigidity can alter the kinetics of a system and will shift equilibria when the changes preferentially alter the free energy of reactants or products. As more fully discussed by Friend et al. (*63*), electrostatic interactions can stabilize specific conformations without changing them, either as a result of formation of salt bridges or by changing the solvent accessibility of given sites. The available crystallographic data suggest that anion-dependent increases in rigidity of the T state of Hb in solution result from formation of additional salt bridges that alter the dynamic flexibility of the molecule although anion-induced changes in solvent accessibility are not ruled out. Thus, at a structural level, we propose that additional salt bridges that form as anions are bound to deoxy Hb increase the effect of steric hindrance of critical residues at the active site, such as the distal histidine and valine, and thereby alter the affinity of the deoxygenated Hb molecule. This is in accord with the significance consistently ascribed to intrinsic and extrinsic salt bridges by Perutz in his many publications. As noted by Perutz (*24*), “One of the strangest features of both the R and T structures is the absence of any entrance to the heme pocket wide enough to allow an oxygen molecule to pass through.” His analysis of the barriers hindering ligand binding and release (*54*) stimulated many subsequent studies on the dynamic nature of the hemoglobin molecule and on the thermodynamic barriers that are now known to exist to variable degrees for different heme ligands (*60*, *64*, *65*). Following this lead, we are presently engaged in collaborative studies with the aim of quantifying the anion dependence of the conformational rigidity of normal and genetically engineered Hbs in their liganded and unliganded states.

Hemoglobin continues to be a prototype of those macromolecules that change their functional properties in response to alterations in physiological conditions, such as the levels of anionic effectors. Through use of improved spectroelectrochemical methods for measuring oxidation curves, we have shown that there are significant differences in the anionic control of the initial stages of oxidation and oxygenation processes in Hb. These differences support our view that anionic control involves two separate and distinct components: one component is associated with electronic changes at the active site, and the other is due to anion-dependent alterations in conformational rigidity. This viewpoint provides increased insight into the controversial two-state model and the experimental factors that have been cited to support or discredit it as a portrayer of Hb function. Many prior objections to the two-state model fall away when one considers the apparent K_T of ligation curves as a variable that gives an indication of the extent of structural rigidity of the deoxygenated molecule. Variations in K_T can thus be considered as indicative of the dynamic equilibrium between the truly rigid T state observed in studies of crystals of Hb and the higher-affinity tertiary states that the molecule can assume while preserving the quaternary T conformation. As we have now shown, some probes of the T-state conformation, like the initial stages of oxidation curves, reflect primarily the two-state (T-to-R) allosteric switch associated with electronic active-site alterations; and some, like the initial stages of oxygenation curves, show a strong dependence upon steric factors.

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