Effects of Heterotropic Allosteric Effectors on the Equilibrium and Kinetics of the Reaction of a Single Ligand Molecule with an α or β Subunit of Deoxygenated HbA†

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ABSTRACT: Symmetrical FeZn hybrids of human HbA have been used to measure $K_1(\alpha)$ and $K_1(\beta)$, the dissociation constants for the binding of a single molecule of oxygen to unliganded HbA at an α subunit and at a β subunit, respectively. The kinetic constants, $l_1'(\alpha)$ and $l_1'(\beta)$, for the combination of the first CO molecule to unliganded HbA at an α or a β subunit, respectively, were also measured. Measurements were carried out between pH 6 and pH 8 in the presence and absence of inositol hexaphosphate (IHP). Both equilibrium constants exhibit a significant Bohr effect in the absence of IHP. The addition of IHP to a concentration of 0.1 mM increases both dissociation constants in a pH-dependent manner with the result that both Bohr effects are greatly reduced. These results require a negative thermodynamic linkage between the binding of a single oxygen at either an α or a β subunit and the binding of IHP to the T quaternary structure of HbA. Although the β hemes are relatively near the IHP binding site, a linkage between that site and the α hemes, such that the binding of a single oxygen molecule to the heme of one α subunit reduces the affinity of the T state for IHP, requires communication across the molecule. $l_1'(\alpha)$ exhibits a very slight pH dependence, with a maximum variation of 20%, while $l_1'(\beta)$ varies with pH three times as much. IHP has no effect on the pH dependence of either rate constant but reduces $l_1'(\alpha)$ marginally, 20%, and $l_1'(\beta)$ by 2-fold at all pH values.

The properties of the T quaternary structure of hemoglobin continue to be a topic of interest and controversy. In the classic formulation of the two state model (*1*), the ligand affinities of the R and T states of hemoglobin were fixed, and the ligand affinity of the hemoglobin molecule was modulated by variation of *L*, the equilibrium between the T and the R states in the absence of ligand. This modulation could be affected by a variety of heterotropic allosteric effectors such as hydrogen ions and organic polyanions such as DPG[1] or IHP, which bind to one quaternary structure better than to the other. This picture became untenable when it was found that oxygen equilibrium curves obtained under different solution conditions could be fitted to the two state model only if the oxygen affinity of the T state was permitted to vary with pH and to be affected by the additions of other heterotropic effectors. These effects were accommodated within the two state model by permitting K_T, the oxygen affinity of the T state, to be a function of these parameters (*2*). Still, it was argued that these were all tertiary effects on the individual subunits, and because they did not involve communication or energy transfer between the subunits, the basic formulation of the two state model was still sound. For this to be true in the case of organic polyanions, it would be necessary to suppose that the effects of these heterotropic effectors, which bind in the pocket between the two β subunits, were limited to modification of the properties of the β subunits. To get around the latter limitation, Minton and Imai proposed a three state model (*3*).

Symmetrical FeZn hybrids of HbA permit a more detailed examination of the reaction of a single ligand with deoxygenated HbA than is easily accomplished when the protein contains four heme groups. It is possible to replace some, or all, of the heme groups of HbA with zinc protoporphyrin IX. This porphyrin mimics closely the deoxygenated heme group, as shown by the thermodynamic analysis of Ackers (*4*) but is unable to bind ligand. Thus, a subunit containing Zn porphyrin is permanently unliganded. In symmetrical FeZn hybrids, the hemes of two identical subunits, either α or β, are replaced by Zn porphyrin. Measurements of the equilibria of oxygen binding to the two symmetrical FeZn hybrids permit the separate determination of the affinities of the α and the β subunits within the unliganded HbA tetramer for the first oxygen molecule, $K_1(\alpha)$ and $K_1(\beta)$. The availability of only two ligand binding sites also permits the

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[1] Abbreviations: IHP, inositol hexaphosphate; DPG, diphosphoglycerate; HbA, human adult hemoglobin A; $K_1\alpha$, the equilibrium dissociation constant for the reaction of a single oxygen molecule to an otherwise unliganded HbA molecule at an α subunit; $K_1\beta$, the dissociation equilibrium for the reaction of a single oxygen molecule at a β subunit; K_T, the equilibrium dissociation constant for the reaction of oxygen with the T state of HbA (two state model); $l_1'\alpha$, the rate constant for the combination of a single CO molecule with an α subunit of an otherwise unliganded HbA molecule; $l_1'\beta$, the rate constant for the combination of a single CO molecule with a β subunit of an unliganded HbA molecule.

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determination of the separate rate constants for the combination of the first CO molecule to an otherwise unliganded HbA molecule at an α or β subunit, $l_1'(\alpha)$ and $l_1'(\beta)$, respectively.

In the accompanying article (*5*), the oxygen equilibria and CO combination kinetics of the symmetrical FeZn hybrids of a series of variants of HbA in the presence of IHP at pH 7 are reported. Log K_1 is observed to be linearly related to log l_1' for both α and β subunits. Although there is ample evidence in the literature of a relationship between l'_1 and K_1, the precision of the observed relationships seems surprising in view of the fact that the binding of the first ligand to hemoglobin is associated with a Bohr effect (*6*). The presence of a Bohr effect indicates a ligand-linked conformational change of sufficient magnitude to alter the p*K* of one or more acid groups. Because the structural change is ligand-linked, the new monoliganded structure must have a higher ligand affinity than that of the deoxygenated tetramer. One would not expect that the effect of a mutation on the difference between the energies of two different structures of hemoglobin would always be proportional to the change it causes in the activation energy of CO combination. The above relationship seems more in keeping with changes in the constraints on ligand binding at the heme within a fixed protein matrix. Oxygen binding to the T state crystal of HbA is without a Bohr effect (*7*). It has been suggested that crystallization selects for the lowest affinity conformers of the T state of hemoglobin (*8*). The presence of IHP results in a similar selection by virtue of the preferential binding of IHP to low affinity conformers. Because in the crystal a similar conformational selection results in a loss of pH dependence, it is reasonable to expect that IHP might similarly attenuate the Bohr effect associated with the binding of the first ligand to unliganded hemoglobin. Therefore, the effects of IHP on the pH dependencies of the oxygen affinities and CO combination kinetics of the two hybrids, $(\alpha[Fe])_2(\beta[Zn])_2$ and $(\alpha[Zn])_2(\beta[Fe])_2$, have been examined.

EXPERIMENTAL PROCEDURES

Symmetrical FeZn hybrids of HbA were prepared as previously described by combining a Zn protoporphyrin IX containing chain with its corresponding, heme containing, partner chain, Zn α chains with heme containing β chains and vice versa (*9*).

Oxygen equilibria were measured tonometrically as previously described using a 500 mL tonometer with a 2 mm cuvette attached (*10*). The HbA concentration used for these measurements was 160 μM in porphyrins equivalents. The buffers used were 0.1 M HCl bisTris at pH 7 or below, with or without the addition of 100 μM IHP, and 0.1 mM HCl Tris above pH 7, with or without the addition of 100 μM IHP. As described in the accompanying article (*5*), titration curves were fitted to the two step Adair equation solving not only for K_1 and K_2 but also for the absorbance change associated with complete saturation with oxygen.

Kinetics of CO combination with unliganded FeZn hybrids were measured by stopped-flow procedures as previously described using an OLIS (On Line Instrument Systems, Inc., Bogart, GA) stopped-flow apparatus (*9*). The concentrations of CO and HbA (in porphyrin equivalents) were 20 and 2 μM, respectively, after mixing. Each data point is the average of the data from measurements at minimally two wavelengths, at each of which multiple kinetic traces were averaged and then fitted to a two step sequential mechanism as described in the accompanying article (*5*).

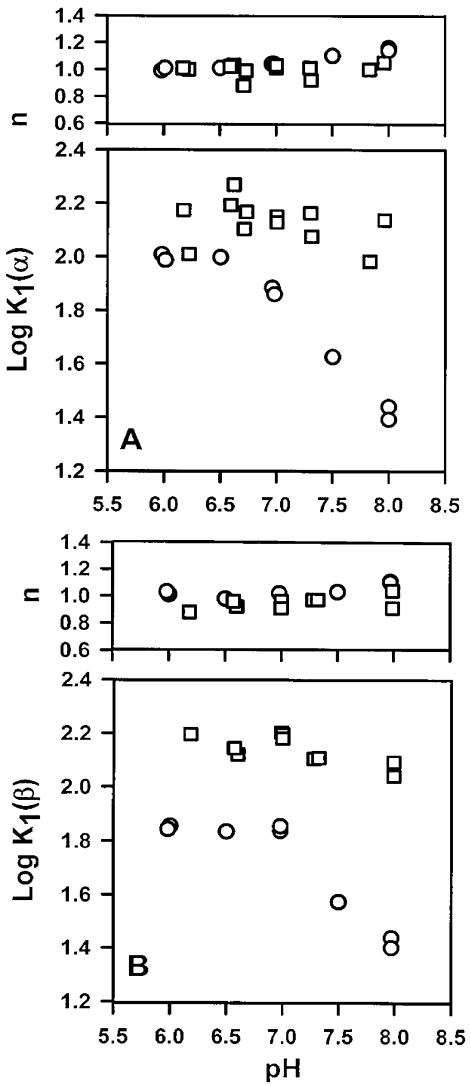

FIGURE 1: pH dependence of log K_1, the intrinsic dissociation constant, and the Hill coefficient, *n*, for the equilibrium of binding of the first oxygen molecule to the otherwise unliganded hybrids of HbA, α[Fe]β[Zn] (A) and α[Zn]β[Fe] (B), in the absence (○) and presence (□) of 100 μM IHP.

The kinetics of CO recombination following flash photolysis were used to estimate the degree of dissociation of the diliganded FeZn hybrid of HbA into $\alpha\beta$ dimers. The measurements were performed as previously described by Doyle el al. (*10*). Equilibrium and kinetic measurements were carried out in the pH range from 6 to 8. Above pH 8, the affinity of the HbA tetramer for IHP is progressively reduced, presumably due to electrostatic effects. To assess the effects of IHP binding on K_1 values, it is essential that the IHP be bound even in the presence of a ligand on the HbA tetramer, and above pH 8, it is not certain that this is the case.

RESULTS

Figures 1 and 2 present the pH dependencies of the equilibria of oxygen binding to and kinetics of CO combina-

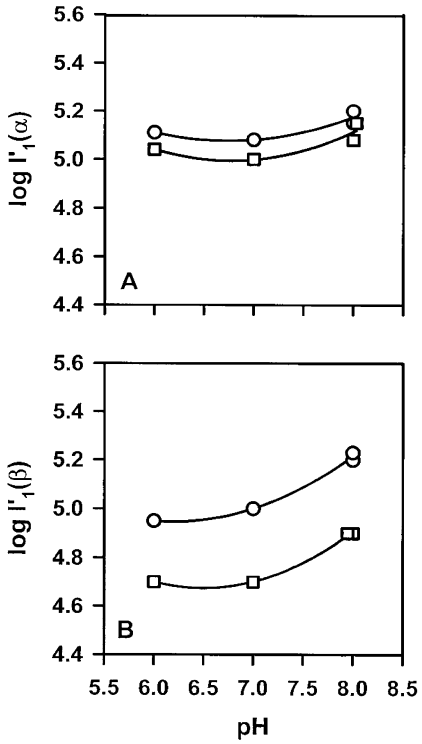

FIGURE 2: pH dependence of log l_1', the rate constant for the combination of the first CO molecule with the otherwise unliganded hybrids of HbA, α[Fe]β[Zn] (A) and α[Zn]β[Fe] (B), in the absence (○) and presence (□) of 100 μM IHP. At pH 6 and 8, each complete experiment is plotted as a data point. At pH 7, the plotted values are the averages of a large number of independent experiments on different samples of the two FeZn hybrids. The standard errors of these latter data are contained within the data points as they appear in the figure.

tion with the two symmetric FeZn hybrids of HbA. In Figure 1, the intrinsic equilibrium dissociation constants (Torr) for the interaction of the first oxygen molecule with unliganded HbA are presented for the α subunits (panel A) and the β subunits (panel B). In the absence of IHP, the β subunits exhibit a significant Bohr effect in K_1. The dissociation constant decreases by a factor of 2.7 between pH 6 or 7 and pH 8. When IHP is added, the dissociation constant is increased at all pH values but to a greater extent at higher pH with the result that the pH dependence is greatly reduced. Between pH 6 and pH 8 in the presence of IHP, the dissociation constant decreases by less than 25%. In the absence of IHP, the α subunits exhibit a 4-fold decrease in the first dissociation constant, $K_1(\alpha)$, between pH 6 or 6.5 and pH 8. Again, the addition of IHP increases the dissociation constant more at a high pH than at a low pH. Despite the scatter of the data, it is clear that little pH dependence persists after the addition of IHP.

Before it can be concluded that IHP alters $K_1(\alpha)$ and $K_1(\beta)$ and reduces the Bohr effects of these parameters, the possibility that dissociation of the HbA tetramer into αβ dimers contributes to the observed pH dependencies must be evaluated. The binding of ligands to HbA progressively destabilizes the tetramer as a result of the high ligand affinity of the αβ dimers. Because the dimer−tetramer equilibrium has been reported to be pH-dependent (*11*), the significant formation of dimers as a result of oxygen binding could result in an apparent Bohr effect. It is known that IHP stabilizes the T state tetramer with respect to both the transition to the R quaternary structure and the dissociation into αβ dimers. Therefore, IHP would be expected to decrease or eliminate an apparent Bohr effect resulting from the formation of dimers. It could be argued that just like the transition from the T to the R quaternary state, the ligand-linked dissociation of the Hb tetramer into αβ dimers would result in cooperative ligand binding, which is not observed. However, at pH 7.4 in the absence of organic phosphates, Ackers (*4*) finds negative cooperativity in the binding of two ligands to the same type of subunits in an otherwise unliganded tetramer, and such negative cooperativity could conceivably mask positive cooperativity resulting from dimer formation.

To determine the extent to which dissociation of the HbA tetramer into αβ dimers might affect measurements of K_1, the kinetics of recombination of CO following flash photolysis were examined as a function of pH and the absence and presence of IHP at a hemoglobin concentration of 2 μM in porphyrin equivalents. In the absence of IHP, the percentage of the recombination occurring at the rapid rate indicative of dimers varied slightly with pH: it was 65% at pH 6, 60% at pH 7, and 56% at pH 8. In the presence of 100 μM IHP, no rapid kinetic phase was observed at pH 6 or 7, but the rapid kinetic phase made up 50% of the total at pH 8.

From the measurements of the degree of dissociation of diliganded HbA into dimers at low concentrations (2 μM porphyrin), it is possible to calculate the fractional dissociation into dimers at 160 μM porphyrin, the concentration at which equilibrium measurements were carried out, not only for the diliganded but also for the monoliganded and unliganded HbA molecule. The amount of tetramer reassembly associated with ligand removal is directly related to the ratio of the affinity of the dimer to that of the tetramer. Because this ratio decreases with increasing pH, dimer formation should present the greatest problem for the results of measurements at pH 8. From the measurements reported here and the oxygen affinity of the αβ dimer reported by Ackers et al. (*12*), the affinity ratio at pH 8, in the absence of IHP, is estimated to be 55. A degree of dimer formation of 56% at 2 μM porphyrin results in 9% of the porphyrins being in dimers at 160 μM in porphyrin equivalents for the diliganded hybrid. From this, one computes a degree of dissociation into dimers to be 1.25% for the monoliganded hybrid and 0.17% for the unliganded tetramer. The effect of these amounts of dimer on the value of K_1 is much smaller than the scatter of the data in Figure 2. Furthermore, at pH 8, dimers are also apparent in the presence of IHP, accounting for 50% of the heme groups at 2 μM. If dimers were the cause of the observed pH dependence in the absence of IHP, then they should also result in a Bohr effect in its presence. The appearance of dimers at pH 8 in the presence of IHP is clear evidence of the reduction in the effectiveness of this allosteric effector in dimer assembly with increasing pH.

The pH dependencies of the kinetic constants present a different picture than those of the equilibrium constants. In Figure 2, the second-order rate constants for the combination of a single CO with an α subunit (panel A) or a β subunit (panel B) in an otherwise unliganded HbA molecule in the absence (○) and presence (□) of IHP are plotted on logarithmic axes as functions of pH. The α subunits exhibit a modest kinetic Bohr effect with the maximum variation in $l_1'(\alpha)$ between pH 7 and pH 8 of only about 30%. The addition of IHP has no significant effect on the observed

pH dependence and decreases the values of $l_1'(\alpha)$ at all pH values by only 20%. The β subunits exhibit a 60% increase in $l_1'(\beta)$ between pH 7 and pH 8. Again, IHP has no significant effect on the pH dependence but decreases $l_1'(\beta)$ by a factor of 2.

DISCUSSION

In the absence of IHP, both $K_1(\alpha)$ and $K_1(\beta)$ exhibit a Bohr effect, consistent with the results of Imai and Yonetani (*6*) and more recently of Miyazaki et al. (*13*). This indicates that the binding of a single oxygen molecule at either an α or a β heme alters the pK of one or more acid groups, presumably as a result of a ligand-linked conformational change. However, the presence of IHP severely attenuates the pH dependencies in both cases. This suggests that IHP constrains the protein matrix, inhibiting the ligand-linked conformational changes, which occur in its absence upon binding a single ligand molecule. The addition of IHP results in a pH-dependent reduction of the affinity of both types of subunit for the binding of the first oxygen molecule. The linkage relationships between binding a single oxygen molecule and binding a molecule of IHP are shown by the follow diagram:

$$\begin{array}{ccc} \mathrm{HbA + IHP + O_2} & \overset{K_1}{\longleftrightarrow} & \mathrm{HbAO_2 + IHP} \\ \Big\updownarrow {}^{0}K_{\mathrm{IHP}} & & \Big\updownarrow {}^{1}K_{\mathrm{IHP}} \\ \mathrm{IHP\text{-}HbA + O_2} & \overset{{}^{\mathrm{IHP}}K_1}{\longleftrightarrow} & \mathrm{IHP\text{-}HbAO_2} \end{array}$$

where K_1 is the dissociation constant for the reaction of the first oxygen with HbA when no IHP is bound, ${}^{\mathrm{IHP}}K_1$ is the dissociation constant for the binding of the first oxygen when IHP is bound, ${}^{0}K_{\mathrm{IHP}}$ is the dissociation constant for the binding of IHP to HbA when no oxygen is bound, and ${}^{1}K_{\mathrm{IHP}}$ is the dissociation constant for the binding of IHP when a single oxygen is bound to HbA. Because the equilibrium constant of a state with itself must equal unity and be independent of the pathway, it is necessary that

$$(K_1 \times {}^{1}K_{\mathrm{IHP}})/({}^{\mathrm{IHP}}K_1 \times {}^{0}K_{\mathrm{IHP}}) = 1$$

or

$${}^{\mathrm{IHP}}K_1/K_1 = {}^{1}K_{\mathrm{IHP}}/{}^{0}K_{\mathrm{IHP}}$$

This means that the effect of binding IHP on the dissociation constant for the first oxygen must be precisely equal to the reciprocal effect of the binding of a single oxygen molecule on the dissociation constant for the reaction of HbA with IHP. Therefore, the binding of a single oxygen molecule at either an α or a β heme has the effect of reducing the affinity of the HbA molecule for the allosteric effector, IHP. At pH 8, the binding of an oxygen molecule at either an α or a β subunit reduces the affinity for IHP by approximately 5-fold while at pH 7 the reduction in affinity for IHP is some 2-fold. At pH 6, the situation differs for the two types of subunits. Binding an oxygen molecule at the β subunit again reduces the affinity for IHP by 2-fold while binding at the α subunit has a smaller effect.

Miyazaki et al. (*13*) reported the equilibria of oxygen binding to FeZn hybrids of HbA at pH 6.5, 7.5, and 8.5 in the presence and absence of IHP. There are several reasons for carrying out additional measurements. First, a single experiment was carried out under each solution condition, and there was no indication of the variation to be expected in duplicate experiments. More importantly, there are reasons to be concerned about the validity of the procedure by which the oxygen binding data of Miyazaki et al. were analyzed. In their article, it is stated that the data were fitted to the two step Adair equation. If one examines closely the data points and the fitted curves in the Hill plots of the hybrids that appear in their article, one observes that in general neither the data points nor the fitted curves display the appropriate symmetry. As is derived in the Appendix, any ligand binding curve that is represented by the two step Adair equation must yield a Hill plot, which is symmetrical with respect to a 180° rotation about the point at which log [Y/(1 − Y)] = 0, the point at which fractional saturation, Y, is equal to 0.5. The fact that the fitted curves do not display such symmetry means that they are not the results of correct fittings to the two step Adair equation. That the data lack such symmetry could have numerous sources. The fact that the Hill coefficients appear to frequently increase as the saturation increases suggests the possibility that a consistent error occurs in the assignment of the multiplying factor for the conversion from absorbance change to fractional saturation, i.e., the absorbance change associated with complete saturation of the binding sites may be consistently underestimated. The interpretation of the asymmetric curves by which these data are fitted is more problematic. Suffice it to say, they do not represent plots of the two step Adair equation. Although they report Hill coefficients, which are consistently slightly higher than those reported in Figure 1, the K_1 values reported by Miyazaki et al. (*13*) in the presence of IHP are reasonably consistent with those reported here. However, the K_1 values that they report in the absence of IHP deviate significantly from the values in Figure 1. They indicate higher dissociation constants and smaller Bohr effects than found in the present work. The Hill plots that display the greatest asymmetry are associated with these data obtained in the absence of IHP. The data of Miyazaki et al. do indicate Bohr effects in $K_1(\alpha)$ and $K_1(\beta)$, which are reduced by the addition of IHP. However, no discussion is presented of the significance of this finding in terms of linked function theory or communication within the T quaternary state of the HbA molecule.

The increase in the dissociation constants, $K_1(\alpha)$ and $K_1(\beta)$, and the inhibition of the K_1 Bohr effects by IHP are reminiscent of the effects of crystallization on deoxygenated HbA. The affinity of HbA in solution in the absence of IHP for the binding of the first oxygen molecule is greater than the oxygen affinity of T state crystals (*8*). However, in the presence of IHP, the affinity for the first oxygen in solution approaches the affinity of the crystals (*7*, *14*), which is insensitive to pH. Noble et al. (*15*) reported a linear relationship between the log p50 of T state crystals of a series of variants of HbA and the log l_{init}', the initial rate constant for the reaction of CO with the unliganded variants. The slope of the line, d log $\mathrm{p50_{crystal}}$/d log l_{init}', was 1.3. In the accompanying article (*5*), d log $K_1(\alpha)$/d log $l_1'(\alpha)$ is reported to be 1.76, and d log $K_1(\beta)$/d log $l_1'(\beta)$ is reported to be 1.07.

The average of these latter two values is 1.4, in close agreement with the slope of the line for the relationship between solution kinetics in the presence of IHP and crystal affinity. It appears likely that the constraints placed by IHP on the conformational flexibility of the protein matrix during the binding of the first ligand in solution are similar to the constraints imposed by crystallization.

The rate constants for the combination of CO with α and β subunits of an unliganded HbA molecule are also pH-dependent. Although the pH dependence of $l_1'(\alpha)$ is small, that of $l_1'(\beta)$ is quite significant. If only the data obtained in the absence of IHP were available, it might be concluded that these pH dependencies are the kinetic reflection of the Bohr effects in $K_1(\alpha)$ and $K_1(\beta)$. However, this conclusion would be in error. In contrast to the equilibrium Bohr effects, the pH dependencies of both $l_1'(\alpha)$ and $l_1'(\beta)$ persist unchanged in the presence of IHP. They are probably related to ionizations, which are unique to the formation of the transition states of the combination reactions and are unrelated to either equilibrium Bohr effect. It seems that the structural transitions involved in the Bohr effects of $K_1(\alpha)$ and $K_1(\beta)$, which are inhibited by the presence of IHP, occur after ligand binding and not as part of the formation of the transition states of the combination reactions. IHP does affect the rate of binding of the first CO molecule, but unlike its global effect on equilibrium, its kinetic effect is largely local, affecting primarily the β subunits with which it interacts directly. Its effect on the α subunits is very modest. It appears that the relationship between log K_1 and log l_1' would be far less perfect than that reported in the accompanying article in the presence of IHP (*5*) if data, obtained at different pH values and in the absence of IHP, were included. The hypothesis that the precise correlations observed at pH 7 in the presence of IHP result from the prevention of significant ligand-linked structural transitions upon binding a single ligand appears reasonable.

There is an abundance of evidence for ligand-linked conformational transitions within the T state quaternary structure, beginning with the demonstration of the T state or K_1 Bohr effect (*6*) and the modification of the two state model to accommodate this observation (*2*). Yonetani et al. (*16*) have now reported that both the T and the R states change functional properties in response to a large variety of solution variables. Recently, Samuni et al. (*17*) examined the structural and functional effects of binding two ligands to the symmetric FeZn hybrids of HbA. To do this, they used a variety of probes, including UV and visible resonance Raman spectroscopy and kinetics of geminate and bimolecular CO recombination following photodissociation. The binding of the two ligands was carried out in a number of different environments, which placed different constraints on the conformational flexibility of the protein. They report evidence of conformation and functional plasticity in the T state with properties ranging from that of the lowest affinity, deoxygenated T state conformation to T state conformations with high ligand reactivity and reduced T state constraints within the globin.

It has often been theorized that the variations in T state properties are local effects resulting from changes in the tertiary structure of the subunit to which the ligand has bound. In this framework, the K_1 Bohr effects would be the results of changes of the pK values of some residues of the subunit to which the single ligand is bound. However, the fact that IHP nearly eliminates the K_1 Bohr effect by reducing the affinity of both the α and the β subunits of unliganded HbA for the first oxygen means that the change in conformation, which occurs in the absence of IHP upon binding the first O_2 molecule, reduces the protein's affinity for both protons and IHP. It is generally agreed that monoliganded HbA in solution is in the T quaternary structure. Therefore, it appears that in the absence of organic phosphates, the binding of a single ligand to the unliganded HbA is associated with a transition to a T state structure, which differs substantially from that seen in crystals of the deoxygenated protein or from that which occurs in solution in the presence of IHP when a single ligand is bound. Because the β146 His residues have been implicated in the T state Bohr effect (*18*), presumably that of $K_1(\beta)$, it is imaginable that the dissociation of protons from one of these residues, as the result of binding a ligand to a single β subunit, might result in a significant electrostatic reduction of the affinity of the T quaternary state for IHP. However, the data indicate that the binding of ligand at a single α subunit also induces a conformational change, which lowers the affinity of the protein for IHP. It seems unlikely that the K_1 Bohr effect in only one α subunit could alter the electrostatic potential of the IHP binding site sufficiently to result in a substantial reduction in IHP affinity. It is even more unlikely that the effects would be nearly the same as that resulting from the Bohr effect of the β subunit. Some other mode of communication between or among the subunits of the T quaternary structure is necessary to produce the observed effect. There are other examples of communication between the components of the T quaternary structure of HbA in the absence of IHP. The cooperativity observed by Ackers and co-workers (*4*), between the subunits of an αβ dimer within the T quaternary structure in the absence of IHP, is certainly one and could be related to the same ligand-linked changes in protein structure associated with the binding of the first ligand as discussed here. Miyazaki et al. (*13*) clearly demonstrated that in unliganded, symmetric, Fe−metal hybrids of HbA the properties of the heme groups are sensitive to the nature of the metal ion in the nonheme porphyrins, even when the nonheme porphyrins are unliganded. The existence of communication between and among the subunits of HbA within the T quaternary structure, with the potential for long-range cooperativity and heterotropic effects, seems clear. However, such linkage effects clearly depend on the nature and composition of the medium in which the protein resides.

APPENDIX

Symmetry of the Hill Plot for a Two Step Adair Equation. Consider the reaction

$$\mathrm{Hb} + 2\mathrm{L} \overset{K_1}{\longleftrightarrow} \mathrm{HbL} + \mathrm{L} \overset{K_2}{\longleftrightarrow} \mathrm{HbL_2}$$

where K_1 and K_2 are dissociation constants. Fractional saturation is given by the Adair equation:

$$Y = \frac{\frac{[L]}{2K_1} + \frac{[L]^2}{K_1K_2}}{1 + \frac{[L]}{K_1} + \frac{[L]^2}{K_1K_2}}$$

and it follows that

$$1 - Y = \frac{1 + \frac{[L]}{2K_1}}{1 + \frac{[L]}{K_1} + \frac{[L]^2}{K_1K_2}}$$

Therefore,

$$\frac{Y}{1 - Y} = \frac{\frac{[L]}{2K_1} + \frac{[L]^2}{K_1K_2}}{1 + \frac{[L]}{2K_1}},$$

which simplifies to

$$\frac{Y}{1 - Y} = \frac{\frac{1}{2} + \frac{[L]}{K_2}}{\frac{1}{2} + \frac{K_1}{[L]}}$$

At half saturation, $Y/(1 - Y) = 1$ and $[L]^2 = K_1K_2$.

For any ligand concentration, [L], with fractional saturation, Y, one can define another ligand concentration, [Q], such that $[Q] = K_1K_2/[L]$ and the associated fractional saturation is Y'. From the equation for $Y/(1 - Y)$, it can be seen that

$$\frac{Y'}{1 - Y'} = \frac{\frac{1}{2} + \frac{[Q]}{K_2}}{\frac{1}{2} + \frac{K_1}{[Q]}} = \frac{\frac{1}{2} + \frac{K_1}{[L]}}{\frac{1}{2} + \frac{[L]}{K_2}} = \frac{1 - Y}{Y}$$

and

$$\log \frac{Y'}{1 - Y'} = -\log \frac{Y}{1 - Y}$$

when $\log[Q] = \log(K_1K_2) - \log[L]$. Therefore, the plot of $\log Y/(1 - Y)$ as a function of $\log[L]$ has 2-fold rotational symmetry about its point of intersection with the line at $\log\{Y/(1 - Y)\} = 0$. Clearly, at half saturation, $[L] = [Q]$.

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