Ligand Binding to Symmetrical FeZn Hybrids of Variants of Human HbA with Modifications in the α1−β2 Interface†

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ABSTRACT: The equilibria of oxygen binding to and kinetics of CO combination with the symmetrical iron−zinc hybrids of a series of variants of human adult hemoglobin A have been measured at pH 7 in the presence of inositol hexaphosphate (IHP). In addition, the kinetics of CO combination have also been measured in the absence of IHP. The hybrids have the heme groups of either the α or the β subunits replaced by zinc protoporphyrin IX, which is unable to bind a ligand and is a good model for permanently deoxygenated heme. The variants examined involve residues located in the α1β2 interface of the hemoglobin tetramer. Alterations of residues located in the hinge region of the interface are found to affect the properties of both the α and the β subunits of the protein. In contrast, alterations of residues in the switch region of the interface have substantial effects only on the mutant subunit and are poorly communicated to the normal partner subunit. When the logarithms of the rate constants for the combination of the first CO molecule with a single subunit in the presence of IHP are analyzed as functions of the logarithms of the dissociation equilibrium constants for the binding of the first oxygen under the same conditions, a linear relationship is found. The relationship is somewhat different for the α and β subunits, consistent with the well-known differences in the geometries of their ligand binding sites.

The parameters controlling the reaction of oxygen with hemoglobin remain a matter of some debate. According to the two state model (*1*), three parameters should be sufficient: K_T,[1] the equilibrium constant for the reaction of oxygen with the T quaternary state of hemoglobin; K_R, the equilibrium constant for the reaction of oxygen with the R quaternary state of hemoglobin; and L, the equilibrium between the T and the R state when the hemoglobin molecule is unliganded. With each refinement of this model, new parameters have been added (*2*, *3*). The studies of HbA by Ackers and co-workers (*4*) indicate a far more complex situation, with the fundamental assumption of symmetry being violated once the first ligand binds to the HbA tetramer. Irrespective of the model used to describe the properties of hemoglobin, the low ligand affinity of the deoxygenated molecule in its T quaternary structure is a fundamental variable, which is crucial if ligand binding is to be cooperative.

The low ligand affinity of deoxy HbA is a unique property of the intact tetrameric molecule. The tetramer is composed of two dimeric units, each composed in turn of one α and one β subunit. The isolated subunits display high affinity for oxygen, as do the αβ dimers, and all bind oxygen without cooperativity. However, when two deoxygenated dimers, which we will denote as α1β1 and α2β2, associate with one another to form the deoxygenated Hb tetramer in its T quaternary structure, there is a dramatic reduction in oxygen affinity.

The combination of the two dimers results in the formation of identical α1β2 and α2β1 interfaces along with the α1α2 interface where ionic interactions occur involving COOH-terminal residues. There are no direct interactions between the two β subunits. However, in the T quaternary state, the cavity between the β subunits forms a cationic binding pocket for polyanionic allosteric effectors such as diphosphoglycerate and IHP. The details of the structures of the interfaces, which form when the dimers interact, must hold much of the secret to the uniquely low ligand affinity of deoxygenated HbA.

Noble et al. (*5*) examined the kinetic properties of a large set of HbA variants with single amino acid substitutions at sequence positions at or near the subunit interfaces. Most of the variants reported were substitutions of Gly or Ala for the naturally occurring residue and, as a result, were effectively deletions of the normal side chain. Because the rate of the reaction of carbon monoxide with unliganded HbA

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[1] Abbreviations: IHP, inositol hexaphosphate; HbA, human adult hemoglobin A; desArg, HbA from which the C-terminal arginine residues of the α subunits (α141) have been removed enzymatically; desHis, HbA from which the C-terminal histidine residues of the β subunits (β146) have been removed enzymatically; HCl Tris, buffer that is prepared by titrating a known amount of HCl to the desired pH with solid Tris base and whose concentration refers to that of the chloride ion; HCl bisTris, same as above but with the pH adjusted by titration with solid bisTris base; α1α2, β1β2, α1β1, α2β2, α1β2, and α2β1, refer to the six subunit−subunit pairings within the $\alpha_2\beta_2$ hemoglobin tetramer; βW37A and other notations of single amino acid replacement, used to refer to an HbA molecule containing the indicated mutation; K, an intrinsic dissociation equilibrium constant for a reaction of hemoglobin with oxygen; K', a dissociation constant from the Adair equation.

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is limited by the rate of the initial combination of the ligand with the deoxygenated T quaternary state of the protein, these authors used the rate constant for CO combination as a rapid and convenient indicator of the effect of a mutation on the ligand affinity of the unliganded T state. However, in the absence of IHP, a number of the variants being examined were significantly dissociated into $\alpha\beta$ dimers, even when unliganded. Dissociation was indicated by the presence of a rapid kinetic phase in the reactions of these hemoglobins with CO and the elimination of the rapid phase by either cross-linking the hemoglobin or addition of IHP (*5*). The presence of rapidly reacting $\alpha\beta$ dimers complicates the estimation of the initial rate of CO binding to the slowly reacting HbA tetramers. For this reason, rate constants measured in the presence of IHP were used for comparison as this allosteric effector preferentially binds to the T state tetramer and effectively assembles unliganded dimers into tetrameric molecules. Of the residues examined, those with the largest effects on T state properties are located in the hinge region of the $\alpha1\beta2$ interface. This is the region lying closest to the rotation axis defining the conformational transition from the deoxy T quaternary structure to what we now know to be the ensemble of R quaternary structures, R^e, of HbA (*6*, *7*). The hinge region is located between the FG corner and C terminus of the α subunit and the C helix of the β subunit. The other portion of the $\alpha1\beta2$ interface, lying between the C helix of the α subunit and the FG corner and C terminus of the β subunit, is termed the switch region and is the region where the greatest movement of the two dimers relative to one another occurs in the T to R^e structural transition.

The deletion of the side chain of an amino acid might have only a local effect on the properties of the subunit to which the residue belongs. Alternatively, it could produce a global effect, changing the properties of both types of subunits. The reactivity of a subset of subunits in an unliganded HbA molecule can be examined by rendering the other subunits unreactive with ligand and therefore permanently unliganded. This can be accomplished by replacing the normal protoporphyrin IX heme group with zinc protoporphyrin IX. The zinc porphyrin is an excellent mimic of deoxygenated heme (*8*) but is permanently unliganded, being unable to bind a sixth ligand (*9*). In symmetrical FeZn hybrids of HbA, the hemes of either the α subunits or the β subunits are replaced by zinc protoporphyrin IX. With such hybrids, one can examine separately the reactions of ligands with the two different types of subunits. Despite early indications that Zn porphyrin disrupts the normal structure of myoglobin and hemoglobin subunits (*10*, *11*), it is now widely accepted that this is not the case (*4*, *8*, *12*). Specifically, Acker's data clearly show that Zn porphyrin has nearly the same effect on the stability of the HbA tetramer as an unliganded heme group (*4*). Crystallographic analysis of the deoxygenated derivative of the symmetrical α[Zn]β[Fe] hybrid of the βW37E variant of HbA indicates a completely normal T state structure, the Zn containing subunit exhibiting no significant rearrangement of its helical elements (Arnone, A., and Kavanaugh, J. Manuscript in preparation). Furthermore, in solution in the presence of IHP, the affinity of an α subunit of the α[Fe]β[Zn] hybrid of HbA for the first oxygen molecule, $K_1(\alpha)$, and that of a β subunit of the α[Zn]β[Fe] hybrid, $K_1(\beta)$, reflects the oxygen affinity of the crystalline

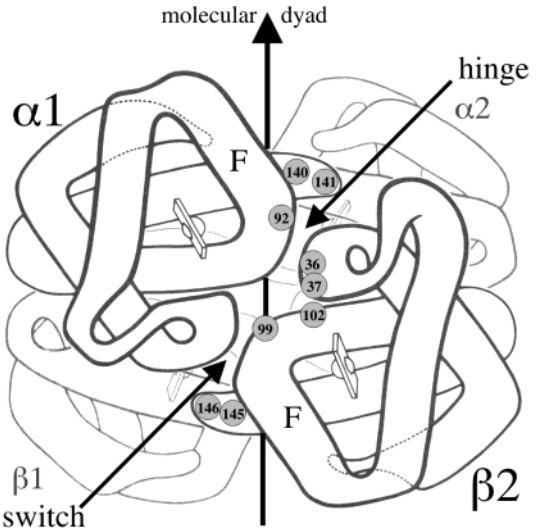

FIGURE 1: Using one of the Irving Geis diagrams of the hemoglobin molecule, the positions of the α carbons of the amino acid residues referred to in this article are indicated. [Adapted from Figures 1.15 and 2.17 of Dickerson, R. E., and Geis, I. (1983) *Hemoglobin: Structure, Function, Evolution, and Pathology*. The Benjamin/Cummings Co., Inc., Menlo Park, CA.] Note that the rights to the illustration by Irving Geis are owned by the Howard Hughes Medical Institute. It is not to be used without permission.

T state of HbA as well as the oxygen affinities of these same subunits in the T state crystals of these hybrids, indicating a similarity of function in solution and crystal (*13*). Noble et al. (*5*) carried out a preliminary study of the distribution of mutational effects on the properties of the two types of subunits within the T quaternary state. They prepared the symmetrical FeZn hybrids of variants with side chain deletion mutations at β37, α140, and β145, all of which result in markedly abnormal CO combination kinetics. The kinetics of CO combination with the hybrids in the presence of IHP permitted the estimation of $l_1'(\alpha)$ and $l_1'(\beta)$, the rate constants for the combination of the first CO molecule with unliganded HbA at an α or at a β subunit, respectively. It was found that in the presence of IHP, mutations at α140 and at β37, both of which are located in the hinge region, resulted in large changes in the kinetic properties of both the α and the β subunits. In contrast, side chain deletion at β145, which is a site in the switch region with substantial effects on T state properties, resulted in a large change in the properties of the β subunit while having only a modest effect on the α subunit.

The above study has now been expanded to include measurements of CO combination with the FeZn hybrids of additional variants, three at the β102 position, βN102A, βN102Q, and βN102T, as well as desArg HbA and desHis HbA, which are HbA from which the C-terminal arginines of the α subunits or the C-terminal histidines of the β subunits, respectively, have been removed enzymatically. In addition, measurements of equilibria of oxygen binding in the presence of IHP have been carried out on the complete set of FeZn hybrids. The equilibrium measurements make possible the estimation of $K_1(\alpha)$ and $K_1(\beta)$, the equilibrium dissociation constants for the binding of the first oxygen molecule to unliganded hemoglobin at an α or at a β subunit, respectively. In combination, these data permit a direct evaluation of the precision with which changes in oxygen affinity correlate with changes in CO combination kinetics

for hemoglobin variants. Figure 1 illustrates the locations of these amino acid residues in the hemoglobin molecule.

EXPERIMENTAL PROCEDURES

Human HbA was prepared from freshly drawn blood and was stripped of endogenous organic phosphates as previously described (*14*). HbA_0 was isolated from HbA chromatographically on DEAE cellulose (DE52) essentially by the method of Williams and Tsay (*15*). Isolated α and *β* chains were prepared from HbA_0 by a modification of the method of Bucci and Fronticelli (*16*) as described by Hernan et al. (*17*).

Des(Arg 141) α chains and des(His 146) *β* chains were prepared by enzymatic hydrolysis of α chains and *β* chains, respectively, with carboxypeptidase B according to the methods of Kilmartin, Hewitt, and Wootton (*18*). Purification of desArg α chains was achieved with a DE52 ion exchange column equilibrated with CO saturated 0.01 M HCl Tris, pH 8.6, and eluted with a linear salt and pH gradient from 0.01 M HCl Tris, pH 8.6, to 0.04 M HCl Tris, pH 8.05, according to the method of Blough and Hoffman (*19*). Purification of desHis *β* chains was carried out by chromatography on a CM-52 column as previously described (*18*).

The globins of α chains, *β* chains, and desHis *β* chains were prepared by the acid acetone method of Rossi-Fanelli et al. (*20*) as modified and described by Hui et al. (*21*). The zinc protoporphyrin IX containing α chains, *β* chains, and desHis *β* chains were prepared as described by Noble et al. (*5*). The Zn containing derivative of desArg α chains was prepared by the direct displacement of heme by zinc porphyrin as described by Noble et al. (*5*). The FeZn hybrids of HbA, desArg, and desHis were prepared by simply combining the desired Zn containing chain with the appropriate, heme containing, partner chain.

Variant α and *β* globins were prepared as previously described (*5*). Variant *β* globin genes were generated by cassette or polymerase chain reaction (PCR) mutagenesis. As described by Hernan et al. (*17*) and Kwiatkowski et al. (*22*), the T7 expression plasmid, containing a synthetic *β* globin gene, was used. The *β* globins were expressed in *Escherichia coli* BL21(DE3), extracted from the bacteria into 8 M urea, and purified (*17*, *22*). Variant α globin genes were created by PCR mutagenesis using the QuikChange Kit and a pET17b-based expression vector containing an operon for the coexpression of α and *β* globins (*17*, *23*). The expressed hemoglobin was purified, the hemes were removed, and the α globin was purified in 8 M urea as first described by Hui et al. (*21*).

The FeZn hybrids of hemoglobin variants were assembled and purified either by chromatography on DE52 columns or by preparative high-performance liquid chromatography (HPLC) on a TosoHaas DEAE column as described by Noble et al. (*5*).

Oxygen equilibrium measurements were carried out tonometrically at 20° essentially by the method of Allen et al. (*24*) as modified by Nagel et al. (*25*). A 500 mL tonometer with an attached 2 mm path length cuvette was used. The buffer used was 0.1 M HCl bisTris, pH 7, with or without the addition of 100 *μ*M IHP. The hemoglobin concentration was 160 *μ*M in porphyrin equivalents. The enzyme system of Hayashi et al. (*26*) was used to maintain the heme groups in their fully reduced state. Spectral measurements were carried out between 500 and 650 nm with a Cary 14 spectrophotometer modified by OLIS (On Line Instrument Systems, Bogart, GA) for computer control and on-line data acquisition.

A number of the FeZn hybrids described here have oxygen affinities so low that even when equilibrated with an atmosphere of pure oxygen they are only partially saturated with this ligand. For a tetrameric hemoglobin molecule with four ligand binding sites, this situation would create a serious problem in the analysis of equilibrium data, since without the spectrum of the fully saturated species, fractional saturation cannot be calculated. However, for FeZn hybrids with two intrinsically identical ligand binding sites, the equations can be solved easily. Ligand binding to such a system must be described by a two step Adair equation in which fractional saturation, *Y*, as a result of the reaction:

$$\mathrm{Hb} + 2\mathrm{O_2} \overset{K_1'}{\rightleftharpoons} \mathrm{HbO_2} + \mathrm{O_2} \overset{K_2'}{\rightleftharpoons} \mathrm{Hb(O_2)_2} \qquad (1)$$

is given by the equation:

$$Y = \frac{\dfrac{\mathrm{pO_2}}{2K_1'} + \dfrac{(\mathrm{pO_2})^2}{K_1'K_2'}}{1 + \dfrac{\mathrm{pO_2}}{K_1'} + \dfrac{(\mathrm{pO_2})^2}{K_1'K_2'}} \qquad (2)$$

The change in optical density or absorbance observed in going from a solution of deoxygenated hemoglobin to a partially oxygenated solution at oxygen partial pressure pO_2 is linearly related to fractional oxygenation by a factor that is the absorbance difference between the fully deoxygenated and fully oxygenated sample, $(\Delta\mathrm{Abs})_{sat}$. Therefore,

$$\Delta\mathrm{Abs} = \left(\frac{\dfrac{\mathrm{pO_2}}{2K_1'} + \dfrac{(\mathrm{pO_2})^2}{K_1'K_2'}}{1 + \dfrac{\mathrm{pO_2}}{K_1'} + \dfrac{(\mathrm{pO_2})^2}{K_1'K_2'}}\right)(\Delta\mathrm{Abs})_{sat} \qquad (3)$$

After measuring the absorbance change, ΔAbs, as a function of oxygen partial pressure, the data were fitted to the above equation, using the Levenberg−Marquart routine found in Sigma Plot, to solve for the three variables: K_1, K_2, and $(\Delta\mathrm{Abs})_{sat}$, the absorbance change that would be observed in going from unliganded to fully saturated hemoglobin. Because of the identity of the two ligand binding sites, the values of K_1' and K_2' in the above equations are related to the intrinsic dissociation constants of the individual heme groups for the two binding steps, K_1 and K_2, simply by statistical factors related to the number of binding sites: $K_1 = 2K_1'$ and $K_2 = K_2'/2$. It should be obvious that the presence of high affinity α*β* dimers would invalidate the above analysis.

The kinetics of CO combination were measured by rapid mixing, stopped-flow procedures using an OLIS (On Line Instrument Systems, Inc.) stopped-flow apparatus, as described by Doyle et al. (*14*). Measurements were carried out at 20°, in the same pH 7 buffer used in the equilibrium measurements. The concentrations of CO and hemoglobin

Table 1: Oxygen Equilibria of FeZn Hybrids in the Presence of IHP[a]

sample	$K_1(\alpha)$ (Torr)	$n(\alpha)$	$K_1(\beta)$ (Torr)	$n(\beta)$	$\Delta\Delta G_1°(\alpha)$ (kcal/mol)	$\Delta\Delta G_1°(\beta)$ (kcal/mol)
HbA	138	1.02	156	0.93		
βW37A	4.0	1.1	8.8	0.96	−2.08	−1.69
βW37E	1.5	1.04	3.4	0.9	−2.65	−2.24
βN102A	112	0.96	154	1.06	−0.12	−0.01
βN102Q	113	1.02	16	0.94	−0.12	−1.34
βN102T	175	1.03	36	0.95	+0.14	−0.86
βY145G	74	1.0	7.1	0.96	−0.37	−1.81
desHis146β	101	1.01	98	0.97	−0.18	−0.27
αY140A	2.7	0.98	43	0.94	−2.31	−0.75
αY140G	1.1	0.95	14	1.0	−2.83	−1.41
desArg141α	13.4	1.0	18	1.08	−1.36	−1.27

[a] $K_1(\alpha)$ and $K_1(\beta)$ are the intrinsic dissociation equilibrium constants for the reaction of a single oxygen molecule with an otherwise unliganded hemoglobin molecule, as measured using the symmetrical hybrids, α[Fe]β[Zn] and α[Zn]β[Fe], respectively. $n(\alpha)$ and $n(\beta)$ are the Hill coefficients for the binding of oxygen to the same pair of hybrids.

were generally 20 and 2 μM, respectively. When possible, the kinetic transients were fitted to a two step sequential mechanism as described by Noble et al. (*5*). Otherwise, kinetic transients were fitted to a sum of two exponential functions.

RESULTS

Equilibria of Oxygen Binding. In Table 1, the values of $K_1(\alpha)$ and $K_1(\beta)$, in the presence of IHP, are reported for the series of genetically and enzymatically modified derivatives of HbA. The values for normal HbA are included for comparison. Along with the dissociation constants, the differences between the Gibbs free energies for the binding of the first O_2 to each type of subunit of HbA and the variant hemoglobins are listed. Finally, the Hill coefficients associated with oxygen binding to the two identical α or β subunits, $n(\alpha)$ and $n(\beta)$, are reported. As will be described, there is independent evidence that the estimates of K_1 are of reasonable precision. No such independent verification of the estimates of K_2 exists, and for this reason, these estimates are not being reported at this time.

Mutation-induced changes in the free energy of binding of the first O_2 in the presence of IHP vary from −0.01 to −2.8 kcal/mol, a 100-fold increase in oxygen affinity. In general, the subunit on which the mutation is found is the subunit exhibiting the greatest change in oxygen affinity. The exceptions are the modifications at two of the residues in the hinge region of the $\alpha1\beta2$ interface, βW37 and αR141 (desArg). In the case of these variants, the magnitudes of the effects on the two types of subunits are quite similar. For both of the βW37 variants, the oxygen affinities of the α subunits are actually increased somewhat more than are those of the β subunits. None of the other mutations produce such global effects. Modifications of a third residue of the hinge region, αY140, were also examined. The two variants at this site cause significant changes to both the α and the β subunits. However, for both variants, the change in the binding free energy of the β subunits is less than that for the α subunits by more than 1.4 kcal/mol.

The effects of modifications of two of the residues of the switch region of the $\alpha1\beta2$ interface have been examined, deletion of the side chain of β145 and enzymatic removal of the C-terminal histidine, β146. The removal of the tyrosine side chain at β145 results in primarily a local effect, increasing the oxygen affinity of the β subunit by more than 20-fold while increasing that of the α subunit by less than a factor of 2. This is in agreement with the kinetic findings of Noble et al. (*5*). The removal of His 146β has only a marginal effect on the oxygen affinity of either subunit.

The FeZn hybrids of the βN102 variants have been examined because mutations of this asparagine residue result in the largest reductions in the overall oxygen affinity of HbA that have been observed. This is the site of the mutations in Hb Kansas (βN102T), Hb Beth Israel (βN102S), and Hb St. Mande (βN102Y), all of which have greatly reduced oxygen affinities. Yanase et al. (*27*) first reported that the simple removal of the asparagine side chain by means of an alanine substitution resulted in a greater decrease in overall oxygen affinity than observed with any of the naturally occurring mutants listed. Noble et al. (*5*) confirmed the very low oxygen affinity of this variant. The results in Table 1 show a normal value of $K_1(\beta)$ and a nearly normal $K_1(\alpha)$ for this variant, indicating that the low overall oxygen affinity of the βN102A variant of HbA is not associated with a decrease in the oxygen affinity of the deoxygenated T quaternary structure of hemoglobin in the presence of IHP. This mutation actually results in a slight increase in the oxygen affinity of the deoxygenated T state. Hence, the reduction in the overall oxygen affinity caused by this mutation must result from alterations in the transitions in functional properties that occur later in the saturation process.

The βN102Q and βN102T variants present a rather different picture. Again, the overall oxygen affinity is decreased as compared to HbA (data not shown). The equilibrium properties of the FeZn hybrids indicate that the βN102Q and βN102T substitutions have only modest effects on the properties of the α subunits in the deoxygenated T state. On the other hand, these substitutions increase the affinity of the first oxygen molecule for the β subunit by about 10-fold and 4-fold, respectively. The combination of a reduced overall oxygen affinity with an increase in the ligand affinity of only the β subunit within the deoxygenated T state suggests the presence of two separate effects. The removal of the side chain of βN102 reduces overall affinity, presumably, at least in part, as a result of destabilizing the R state structure of hemoglobin through the loss of the stabilizing interaction between βN102 and αD94. Interestingly, the insertion of a Gln at β102 does not substitute for the missing Asn. Instead, its presence or that of a Thr produces a large, local effect on the β subunit, increasing its affinity in the low affinity T state. These data represent a part of an extensive examination of the effects of β102 substitutions on the properties of HbA, the results of which are described in a manuscript in preparation.

CO Combination Kinetics. The kinetics of CO combination with the deoxygenated FeZn hybrids of the β37, α140, and β145 variants have been reported by Noble et al. (*5*). The rate constants for the same reaction with the FeZn hybrids of desArg, desHis, βN102A, βN102T, and βN102Q are presented in Table 2. Data obtained in the absence and presence of IHP are listed. The properties of the FeZn hybrids of HbA, α[Fe]β[Zn] and α[Zn]β[Fe], are included for comparison. In the absence of IHP, both hybrids of desArg

Table 2: CO Combination Kinetics for Symmetrical FeZn Hybrids of HbA, desArg, desHis, and βN102A

	second-order rate constants (μM^{-1} s^{-1})			
	no IHP		+IHP	
	$l_1'(\alpha)$	$l_2'(\alpha)$	$l_1'(\alpha)$	$l_2'(\alpha)$
	FeZn hybrid			
α[Fe]β[Zn]	0.13	0.10	0.10	0.10
α[Fe]βN102A[Zn]	0.12	0.11	0.11	0.09
α[Fe]βN102Q[Zn]	0.12	0.11	0.11	0.10
α[Fe]βN102T[Zn]	0.1	0.07	0.09	0.08
α[Fe]β(des His146)[Zn]	0.20	0.16	0.12	0.11
α(des 141Arg)[Fe]β[Zn]	[2.0 (30%), 0.5 (70%)][a]		0.34	0.33
	$l_1'(\beta)$	$l_2'(\beta)$	$l_1'(\beta)$	$l_2'(\beta)$
α[Zn]β[Fe]	0.11	0.09	0.046	0.036
α[Zn]βN102A[Fe]	0.053	0.036	0.04	0.03
α[Zn]βN102Q[Fe]	0.65	0.49	0.44	0.27
α[Zn]βN102T[Fe]	0.24	0.15	0.15	0.10
α[Zn]β(des His146)[Fe]	0.33	0.26	0.05	0.035
α(des 141Arg)[Zn]β[Fe]	[4.0 (30%), 0.9 (70%)][a]		0.28	0.18

[a] These rate constants result from fitting the kinetic data to the sum of two exponential functions. Except as noted, all rate constants are the result of fitting the kinetic data to a two step sequential kinetic mechanism.

HbA exhibit complex CO combination kinetics, which cannot be fitted by a two step sequential reaction. In both cases, two rapid kinetic components are observed, one being roughly 4-fold more rapid than the other and contributing 30% of the total reaction. The rate constants for the most rapid kinetic components are consistent with $\alpha\beta$ dimers, while the slower kinetic components of each hybrid are much more rapid than the comparable reactions for HbA. Addition of 100 μM IHP changes the kinetic properties of both hybrids, with the result that they can be well-fitted by the sequential mechanism. When compared to the hybrids of HbA in the presence of IHP, this chemical modification can be seen to increase l_1' for the α subunit by a factor of 3.4 and that of the β subunit by a factor of 6.

The kinetics of the hybrids of desHis HbA and the βN102A and βN102Q variants fit well to the sequential mechanism in the absence and presence of IHP. In the absence of IHP, the removal of the COOH-terminal histidine of the β subunits increases l_1' for the α subunits by 50% and that for the β subunit by 200%. The effect of this modification is virtually eliminated by the addition of IHP, with differences between l_1' values of desHis and HbA being at most 20%. In the presence of IHP, the βN102A mutation also has a negligible effect on the kinetic properties of the two types of subunits. However, the properties of this variant are far less sensitive to the presence of IHP than are those of HbA. As a result, in the absence of allosteric effectors, the mutant β subunit reacts with CO at less than half the rate of the β subunit of HbA. The kinetic properties of the α subunit of the βN102Q and βN102T variants are similar to those of the α subunit of βN102A. However, just as was found with the equilibrium measurements, the βN102Q and βN102T substitutions have substantial local effects on the β subunits, greatly increasing the rate constants for their combination with CO.

As previously stated, Noble et al. (*5*) made use of the rate constants for CO combination as indicators of the ligand affinity of the deoxygenated T state of HbA and the effects of mutations on this ligand affinity. The validity of this correlation was argued from the parallelism between oxygen and CO affinities and the association between changes in CO affinity and changes in CO combination kinetics. It was reinforced by the recent demonstration by Noble et al. (*28*) of a correlation between the initial rate constants for CO combination with deoxygenated hemoglobin variants in solution in the presence of IHP and the oxygen affinities of T state crystals of the variants. However, until now, the correlation between the rate of CO combination with a particular heme group on an unliganded Hb tetramer and the oxygen affinity of that heme group has not been carefully examined. In Figure 2, the values of log l_1' for the FeZn hybrids are plotted as a function of log K_1 as reported in Table 1. The values of l_1' for the previously reported FeZn hybrids in the presence of IHP are taken from Noble et al. (*5*). The values for desArg, desHis, βN102A, and βN102Q are from Table 2. In this graph, the properties of the α subunits are indicated by open circles, and those for the β subunits are indicated by open squares. Although the entire set of data points indicates a good correlation between the equilibrium and the kinetic parameters, with the data plotted in this manner, it is evident that the correlations obtained with the two types of subunits are not identical. Therefore, least-squares fits of the two data sets have been computed. The qualities of the two correlations are clear.

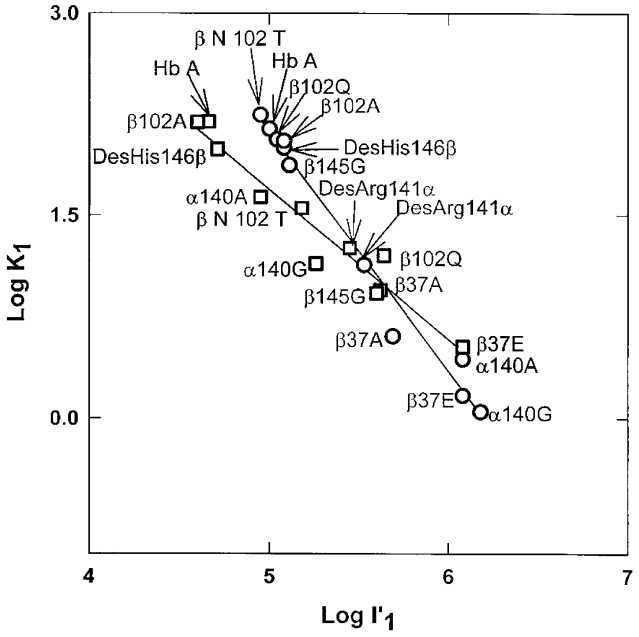

FIGURE 2: Values of log K_1, the intrinsic dissociation constant for the equilibrium of binding of oxygen to the α (○) or β (□) subunits of the indicated variants of HbA, are plotted as a function of log l_1'. Both thermodynamic parameters have been measured in the presence of 100 μM IHP at 20° and pH 7.

DISCUSSION

The analysis of the kinetics and equilibria of ligand binding to an FeZn hybrid of hemoglobin is far easier than the analysis of these processes for the normal hemoglobin tetramer. This simplicity results from the fact that the hybrids contain only two ligand binding sites, and prior to the binding of the first ligand, these sites are equivalent. Ligand binding kinetics can be treated as a two step sequential process with

initially equivalent binding sites, for which there is an analytical expression as described by Noble et al. (*5*). Binding equilibria are described by the two step Adair equation with the intrinsic equilibrium constants being related to the overall constants simply by the obvious statistical factors as described under the Experimental Procedures. This ease of analysis permits determination of the equilibrium and kinetics constants for these processes with good precision with a modest number of accurate experimental measurements. However, this simplicity is available only if all of the hemoglobin present in solution is in the tetrameric state.

In the absence of IHP, many of the variants being examined are significantly dissociated into $\alpha\beta$ dimers, even when deoxygenated. This was first shown to be the case for a number of substitutions of the β37 residue, specifically βW37E, βW37G, and βW37A (*29*). Because the $\alpha\beta$ dimers have high ligand affinity and react rapidly with CO, their presence results in a rapid kinetic phase in the reaction of these variants with CO (*22*). Similar kinetic properties were reported for the αY140 variants and βY145G (*5*). The rate constants associated with the rapid kinetic phases of the reactions of CO with the hybrids of desArg hemoglobin (Table 2) are indicative of the presence of $\alpha\beta$ dimers in this derivative as well. In all cases, the addition of IHP eliminates the rapid kinetic phase and appears to result in the complete assembly of the hemoglobin variants into stable tetramers. This assembly is the result of the preferential binding of the allosteric effector to the T quaternary state of the tetrameric protein. For this reason, all measurements of oxygen equilibria of FeZn hybrids have been carried out in the presence of 100 μM IHP. There are other advantages associated with using these solution conditions. The functional properties of deoxygenated hemoglobins in solution in the presence of IHP are of particular interest since it is under these conditions that the properties of deoxy HbA most closely approximate those of HbA in T state crystals (*30*, *31*). In addition, it has been demonstrated that in the presence of IHP the rates of CO combination to a set of variants of HbA are correlated with the oxygen affinities of the same variants when crystallized in the deoxygenated T quaternary state (*28*). However, it should not be forgotten that IHP has profound effects on the properties of the deoxygenated T state of HbA. As illustrated by the kinetic properties of desHis hemoglobin, IHP can also alter the effect that a chemical modification has on the deoxygenated T state. The removal of His 146β has a substantial effect on the kinetic properties of the T state in the absence of IHP, but in the presence of this allosteric effector, the kinetic effects all but disappear.

The correlation between the rate constants for CO combination and the oxygen affinity of the subunits of deoxygenated variants of HbA in the presence of IHP is remarkable and clearly validates the use of CO combination kinetic to assess changes in ligand affinity and, more specifically, oxygen affinity. The discovery of different relationships between log l_1' and log K_1 for the α and β subunits is not surprising. The geometries of the ligand binding sites of the two subunits are not identical. It is well-known that it is possible to differentially modulate the affinities for CO and oxygen. This is evidenced by the well-known difference between the CO/O_2 partition coefficients of human hemoglobin and myoglobin (*32*). It appears that the modulations of the ligand affinity of the deoxygenated T quaternary structure in the presence of IHP, resulting from the structural modification examined in this study, do not involve any parameters that have significantly differential effects on the affinities for the two ligands.

The close correlation between the values of K_1 and the values of l_1', in the presence of IHP, offers an independent assessment of the precision of the estimates of K_1. There are important requirements for such correlations between two different parameters. First, the correlation itself must exist. Then, the data for both sets of parameters must be sufficiently precise that the correlation can be discerned. The precision of the observed correlation indicates that the values of both K_1 and l_1' have been estimated with good precision.

It is the lack of an independent assessment of precision that has prompted the decision not to report the values of K_2 that are obtained from fitting the data to eq 3. Because of the procedure by which the oxygen binding measurements are carried out, the first halves of the saturation curves are consistently better defined than the latter halves. As a result, it is suspected that the estimates of the K_2 values may be associated with greater uncertainty than those of K_1. Instead, the Hill coefficients are reported in order to define the binding curves with reasonable precision. Although there is some variation in the apparent Hill coefficients for the different hybrids, a general finding is an absence of significant cooperativity, either positive or negative, between identical subunits in the presence of IHP.

Given the close correlation between CO combination kinetics and oxygen equilibria, it is not surprising that the equilibrium results reported here are in accord with the conclusion reached earlier on the basis of kinetic measurements (*5*). As previously mentioned, Noble et al. (*5*) demonstrated that the residues most critical for the normally low ligand affinity of the deoxygenated T state of HbA are located in the hinge region of the $\alpha1\beta2$ interface. The members of this set of critical residues are αR92, αY140, αR141, βP36, and βW37. The side chains of these residues form a cluster, all members of which make direct contact with the side chain of βW37. In the present study, we have examined the effects of modifications of three members of the cluster, side chain deletions of αY140 and βW37, and enzymatic removal of αR141. All of the modifications of these three residues have global affects, increasing the ligand affinities of both the α and the β subunits of an otherwise unliganded hemoglobin tetramer. Therefore, their effects are communicated between or among subunits within the T quaternary structure. The heme group located closest to the hinge region is that of the α subunit. It seems quite reasonable that the above cluster of amino acids has a direct effect on the ligand affinity of the nearby α heme. The cluster forms part of a pocket in which the side chain of αY140 resides in the T quaternary state. According to Perutz (*2*), ligand binding to the α subunit initiates movement of the side chain of α140 out of its pocket. However, its interaction with βW37 and other members of the cluster resists this movement with the effect of lowering ligand affinity. Removing the side chain of αY140 or removing the constraint on that side chain by deleting the βW37 side chain is expected to increase the ligand affinity of the α subunit. How modifications of these residues affect the ligand affinity of the β subunit is less clear.

A recent report by Ackers et al. (*33*) offers new insight into the pathway by which the removal of Arg 141α is communicated to the β subunit. A hemoglobin tetramer with the desArg modification on only one of the two α subunits was examined. Such an asymmetric hemoglobin molecule has 16 distinct states of ligand binding, and these were obtained through the use of FeZn hybrids with fully liganded heme groups. By measuring the tetramer stabilities of all 16 states in the absence of IHP, the effects of the single modification on the energetics of ligand binding to both αβ dimers were determined. The results show that within the unliganded tetramer, the ligand affinities of both subunits in the structurally unmodified dimer are normal. However, both the α and the β subunits of the structurally modified dimer have increased ligand affinities. The conclusion is that the absence of Arg 141α in one αβ dimer alters the ligand affinity of the β subunit in the same dimer; therefore, communication of this chemical modification is not across the α1β2 interface to the β subunit of the neighboring dimer but rather within the single dimer unit. This same pattern, which Ackers has termed dimer autonomy, is observed with the βD99H substitution. However, no similar study of a mutation on the critical β subunit residues of the hinge region of the α1β2 interface has been carried out. Because the hinge region is some distance from the heme group of the involved β subunit, it is not clear which β subunit would be modified if only a single mutation of the β37 residue were present in a hemoglobin tetramer. Drs. Jeffrey Kavanaugh and Arthur Arnone have proposed that members of the Trpβ37 cluster might control the ligand affinity of the α subunit involved in the interface and through it the β subunit of the same αβ dimer (manuscript in preparation). If true, then a single βW37 mutation in a hemoglobin tetramer would be expected to alter the properties of the normal β subunit, not those of the mutant β subunit. It is important to keep an open mind about the pathways of communication responsible for the global effects resulting from modifications of key residues in the hinge region of the α1β2 interface. These pathways are of special interest as they may be involved in ligand-linked changes in the functional properties of the T quaternary structure of hemoglobin.

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