Mutations of the βN102 Residue of HbA Not Only Inhibit the Ligand-Linked T to R^e State Transition, but Also Profoundly Affect the Properties of the T State Itself[†]

Laura D. Kwiatkowski, Hilda L. Hui, Ellen Karasik, Judith E. Colby, and Robert W. Noble*

Department of Medicine, University at Buffalo, VA Western New York Healthcare System, Building 20, 3495 Bailey Avenue, Buffalo, New York 14215

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ABSTRACT: The properties of three HbA variants with different mutations at the β102 position, βN102Q, βN102T, and βN102A, have been examined. All three are inhibited in their ligand-linked transition from the low affinity T quaternary state to the high affinity R^e quaternary state. In the presence of inositol hexaphosphate, IHP, none of them exhibits cooperativity in the binding of oxygen. This is consistent with the destabilization of the R^e state as a result of the disruption of the hydrogen bond that normally forms between the β102 asparagine residue and the α94 aspartate residue in the R^e state. However, these three substitutions also alter the properties of the T state of the hemoglobin tetramer. In the presence of IHP, the first two substitutions result in large increases in the ligand affinities of the β-subunits within the T state structure. The βN102A variant, however, greatly reduces the pH dependencies of the affinities of the α and β subunits, $K_1(\alpha)$ and $K_1(\beta)$, respectively, for the binding of the first oxygen molecule in the absence of IHP. In the presence of IHP, the T state of this variant is strikingly similar to that of HbA under the same conditions. For both hemoglobins, $K_1(\alpha)$ and $K_1(\beta)$ exhibit only small Bohr effects. In the absence of IHP, the affinities of the α and β subunits of HbA for the first oxygen are increased, and both exhibit greatly increased Bohr effects. However, in contrast to the behavior of HbA, the ligand-binding properties of the T state tetramer of the βN102A variant are little affected by the addition or removal of IHP. It appears that along with its effect on the stability of the liganded R^e state, this mutation has an effect on the T state that mimics the effect of adding IHP to HbA. It inhibits the set of conformational changes, which are coupled to the K_1 Bohr effects and normally accompany the binding of the first ligand to the HbA tetramer in the absence of organic phosphates.

In recent years, more and more evidence has appeared that brings into question the precision with which the two-state model of Monod, Wyman, and Changeux (MWC model[1]), (*1*) and/or later adjustments to that model (*2*, *3*, *4*), describes the allosteric effects accompanying the stepwise binding of four oxygen molecules to hemoglobin. It has long been known that the binding of the first ligand to a heme group of an otherwise unliganded hemoglobin molecule is associated with a Bohr effect (*5*), but it was postulated that this resulted from a local transition in the tertiary structure of the subunit to which the ligand bound. As long as this tertiary transition was not communicated to the neighboring subunits, the symmetry of the hemoglobin tetramer, so fundamental to the MWC model, was maintained. However, by using a variety of techniques to determine the ligand-linked changes in tetramer stability resulting from every possible ligand-binding step in the saturation process, Ackers, Holt, and their co-workers (*6*−*9*) have shown convincingly that in the absence of organic phosphates, the binding of the first ligand to human HbA alters the ligand affinities of the remaining heme groups and does so in an asymmetric way. The ligand affinity of the unoccupied heme group, which is located on the same $\alpha\beta$ dimer to which the single ligand is bound, is increased. At the same time, the affinities of the unoccupied heme groups on the neighboring dimer are either changed very little or somewhat decreased. Using symmetrical FeZn hybrids of HbA, Karasik et al. (*10*) measured the affinities of binding of the first oxygen to the two types of subunits in unliganded HbA in the absence and presence of IHP. In the absence of IHP, they observed a distinct Bohr effect for both the α and β subunits, as expected. However, they found that in the presence of IHP, both of these Bohr effects were greatly attenuated. Furthermore, they noted that IHP reduced

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* Corresponding author. Tel: 716-862-6538. Fax: 716-862-6526. E-mail: rnoble@buffalo.edu.

[1] Abbreviations: IHP, inositol hexaphosphate; Hb, hemoglobin; HbA, human adult hemoglobin; T state, the quaternary structure normally assumed by hemoglobin when it is deoxygenated; R^e state, the ensemble of quaternary structures normally assumed by hemoglobin when it is full-liganded or oxygenated; MWC model, Monod, Wyman, and Changeux model or two state model; K_1, the intrinsic dissociation equilibrium constant for the reaction of the first ligand molecule with an otherwise unliganded hemoglobin molecule; $K_1(\alpha)$, the K_1 dissociation constant for an α subunit; $K_1(\beta)$, the K_1 for a β subunit; βN102T and other notations of single amino acid replacement are used to refer to a HbA molecule containing a mutant subunit with both the V1M substitution and the indicated mutation; DTT, dithiothreitol; DE52, diethylaminoethyl cellulose used for ion exchange chromatography; Cl$^-$ Tris, the buffer 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; Cl$^-$ bisTris, same as above but with the pH adjusted by titration with solid bisTris base; p50(O_2), partial pressure of oxygen required for half saturation; IPTG, isopropylβ-D-thiogalactopyranoside.

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the ligand affinities of both types of subunits in approximately the same pH-dependent manner. This requires that the binding of a single ligand at either type of subunit reduces the protein's affinity for IHP. Among other things, this necessitates a linkage between the binding of a ligand on the α subunit and the IHP affinity of the anion binding site, which lies between the two β subunits some distance from the α heme group. It is difficult to see how this could be accomplished by local changes in tertiary structure alone. The authors suggested that the conformational transition triggered by the binding of a single ligand might result not only in the K_1 Bohr effect and the ligand-linked reduction of the affinity of the IHP binding site, but also in the asymmetric distribution of ligand affinities of the remaining heme sites as identified by Ackers. A corollary to this hypothesis is that the presence of IHP, by stabilizing the structure with a high IHP affinity and inhibiting the structural transition responsible for the K_1 Bohr effects, is also likely to interfere with the changes in the ligand affinities of the remaining unoccupied heme sites that accompany the binding of the first ligand in the absence of this allosteric effector.

There is also accumulating structural evidence that hemoglobin cannot be adequately described by only two distinct conformational states. Mueser et al. (*11*) have crystallographically shown that ligand-saturated hemoglobin can exist not only in well-documented R and R2 structures but also in a range of different structural forms. For example, they crystallized the CO derivative of bovine Hb under three different solution conditions, obtained three crystals with different crystal lattices, and found three different structures for liganded bovine Hb. One was within reasonable range of the classic R2 structure, but the other two were neither R nor R2 but intermediate between these two structures. They concluded that in solution liganded hemoglobin must exist as an ensemble of structural states, which they referred to as R^e. The results of NMR studies on hemoglobin in solution by Lukin et al. (*12*) are consistent with this observation. However, unliganded HbA seems always to have the same structure regardless of the crystal lattice in which it is encased or the conditions under which the crystal is grown. Recently, however, Kavanaugh et al. (*13*) reported that the crystalline T states of normal and mutant hemoglobins differ from one another in ways that correlate with the ligand affinities of these T states. Furthermore, the binding of oxygen to these hemoglobins within their T state crystals results in further conformational changes, the magnitudes of which again correlate with ligand affinity. Thus, one can now speak of the ensemble of T conformational states, T^e, and this ensemble can be further divided into high-affinity T and low-affinity T forms. The existence of functionally distinct T conformational states has also been convincingly established by studies of the properties of T state hemoglobins immobilized in silica gels. Oxygen equilibrium measurements established variations in the ligand affinities of T state HbA, depending upon the conditions of its incorporation into the gel environment (*14*, *15*). Measurements of resonance Raman spectra and CO recombination kinetics of iron−zinc hybrids of HbA immobilized in gels demonstrated functional and structural plasticity in the T state (*16*). In the studies described above, the characterization of T state properties was facilitated by preventing the normal, ligand-linked transition to the high affinity R^e ensemble of quaternary states. This was accomplished by placing them in a constraining environment, such as a crystal lattice or a silica gel, or by limiting ligand binding to only two sites on the hemoglobin tetramer in the case of FeZn hybrids.

More recently, Samuni et al. (*17*) have examined the properties of the T states of the βN102A and βW37G variants of HbA, both in solution and entrapped in silica gels, to compare the structural and functional features of the very different T states of these two hemoglobins. This study was facilitated by the fact that under appropriate conditions, the liganded derivatives of these two variants remain largely in a T state conformation in solution even when saturated with the ligand. The evidence that this is true for the βW37G variant comes from studies of the properties of a series of different βW37 variants (*18*, *19*). For the βN102A variants, the authors acknowledged their reliance on previously reported studies from this laboratory as well as the results reported here. They used visible resonance Raman spectroscopy to assess proximal strain at the hemes and UV resonance Raman spectroscopy to evaluate the structural state of the α1β2 interface. From the latter, one can infer the quaternary state of the protein. Their results reinforce the conclusion reached in the current study that under appropriate conditions, the βN102A variant remains in the T quaternary state even when saturated with the ligand. They reported evidence for the existence of an array of functionally distinguishable T states of both hemoglobins, consistent with their earlier studies of HbA (*16*).

In the transition from the unliganded T quaternary structure of HbA to the ligand-saturated R^e conformational ensemble, there is considerable movement of the two αβ dimers with respect to one another. This movement results in significant alterations in the regions of contact between the dimers, the α1β2 and identical α2β1 interfaces, along with the α1α2 contacts. As a result, a number of specific, energetically favorable interactions that exist in the T state are disrupted and replaced by a smaller set of contacts specific to the R^e structures. One of the latter is a hydrogen bond that forms only in the R^e state between the asparagine residue at position 102 of the β subunit and the aspartate residue at position 94 of the α subunit, bridging the gap between the FG corners of the β subunit of one dimer and the α subunit of the opposite dimer. In the T state, these residues are too far apart to form a hydrogen bond. In the R^e state, the β102 asparagine also makes contact with the β37 tryptophan residue of the same subunit. Replacements of the asparagine residue at position β102 are found in a number of naturally occurring variants of human hemoglobin. These include hemoglobin Kansas, βN102T (*20*), hemoglobin Beth Israel, βN102S (*21*), and hemoglobin Saint Mandé, βN102Y (*22*), all of which exhibit reductions in both ligand affinity and cooperativity of oxygen binding, consistent with the destabilization of the R^e conformations as a result of the loss of normal βN102−αD94 H-bonding interactions. Of these naturally occurring variants, Hb Kansas has been studied in greatest detail. Bonaventura and Riggs (*20*) reported that ligand-saturated Hb Kansas was far more dissociated into dimers than HbA. Gibson et al. (*23*) examined the equilibria and kinetics of the reactions of Hb Kansas with oxygen and carbon monoxide. They concluded that in the absence of organic phosphates, in spite of reductions in both ligand affinity and cooperativity, ligand binding is associated with a transition

from the T to R conformation, as with HbA. However, the R state of Hb Kansas appeared to have a lower affinity than that of HbA. In addition, Hb Kansas exhibited increased heterogeneity between its α and β subunits, with the β subunit in the fully liganded hemoglobin having an unusually rapid rate of ligand dissociation. Riggs and Gibson (*24*) examined the reactions of ligands with the isolated α and β chains of Hb Kansas and found the mutant β chains to have an oxygen affinity roughly 6-fold lower than that of normal β chains. Atha et al. (*25*) examined the concentration dependence of the equilibrium of oxygen binding to Hb Kansas. This permitted the extrapolation to the oxygen-binding properties of the tetramer. The data indicated that at pH 7.5 in the absence of organic phosphates, oxygen binds to tetrameric Hb Kansas with a Hill coefficient less than unity below 25% saturation. The Hill coefficient then increases to a maximum of 1.5 at 80% saturation and then falls to unity as saturation is achieved. Although there is cooperativity in ligand binding, the free-energy change associated with the binding of the fourth oxygen molecule to Hb Kansas is significantly less negative than that for the binding of an oxygen molecule to the αβ Kansas dimer. In HbA, the binding of the fourth oxygen has a higher affinity than that of the αβ dimer (*9*). Although there appears to be significant cooperativity in the binding of oxygen to Hb Kansas in the absence of IHP, there is persuasive evidence that in the presence of IHP, even CO-saturated Hb Kansas remains in its low affinity, T quaternary state (*26*, *27*). As such, this variant has been used as a model of a liganded T state of human hemoglobin. In 1995, Yanase et al. (*28*) reported the preparation of the β102A variant by expression in yeast. This variant exhibits a lower oxygen affinity than any of the naturally occurring β102 variants. It also dissociates more readily into αβ dimers when liganded than HbA (*29*).

Currently, there is great interest and significant controversy concerning the sequence of structural and functional changes that accompany the binding of ligand to the T state of human HbA (*6*−*10*, *30*). A hemoglobin variant whose ligand-linked T to R^e state transition was blocked in solution but whose T state accurately reproduced the properties of the T state of HbA would be extremely useful for studies of the apparent complexities of T state properties. For this reason, our laboratory has carried out measurements of the properties of three different βN102 variants, βN102T (Hb Kansas), βN102Q, and βN102A. We have carried out detailed measurements of the equilibrium and kinetics of the reactions of these hemoglobins with oxygen and CO and characterized the differences in their functional properties, particularly those of their T states. We examined these variants in their standard state but also in their cross-linked state, to eliminate dissociation into αβ dimers, and as symmetrical FeZn hybrids so that the properties of their two types of subunits could be assessed independently. Some of these properties have been reported in articles directed at issues other than the very unusual behavior of this class of variants (*31*, *32*). As will be seen, of the three β102 variants examined, it is the T state of βN102A that most closely approximates the properties of the T state of HbA. However, its response to the binding of a single ligand to one of its subunits differs significantly from that of the T state of HbA, indicating that this mutation does a great deal more than destabilize the R^e quaternary state of human hemoglobin.

As already mentioned, in the liganded R^e state structure of human hemoglobin, the β102 asparagine side chain forms a hydrogen bond with the side chain of aspartate α94 (*28*, *29*). It is reasonable to expect that the loss of this interaction would destabilize the R^e quaternary structure of hemoglobin, perhaps delaying the transition in quaternary structure from T to R^e in the course of ligand binding, and increase the tendency of the liganded molecule to dissociate into dimers. However, if the loss of this interaction were the only source of the unusual properties of β102 mutants, then one would expect β102 mutants to behave similarly. This interaction can be eliminated in another independent way, by the deletion of the aspartate side chain of residue α94. Therefore, we have examined the properties of a hemoglobin variant with the α94 aspartate changed to a glycine residue in order to determine whether this variant is also inhibited in its ligand-linked transition to the R^e state.

MATERIALS AND METHODS

Native human HbA was prepared as described by Doyle el al. (*33*) using a Dintzis deionizing column to remove endogenous organic phosphates (*34*). HbA_0 was purified from HbA essentially by the procedure of Williams and Tsay (*35*). Isolated α and β chains were prepared by first reacting HbA_0 with PMB as described by Bucci and Fronticelli (*36*). The chains were then separated on a DE52 column as described by Geraci et al. (*37*). The sulfhydryl groups of the isolated chains were regenerated with dithiothreitol, DTT, and the chains with free sulfhydryls were further purified by chromatography on DE52. The α and β globins were prepared from α and β chains by a modification of the method of Rossi-Fanelli et al. (*38*) as described by Hui et al. (*39*). Cross-linked derivatives of HbA and hemoglobin variants with α subunit mutations were prepared by reaction of the hemoglobins with bis(3,5-dibromosalicyl)fumarate using essentially the method of Chatterjee et al. (*40*) and Snyder et al. (*41*) as described by Kwiatkowski et al. (*18*). The α chain dimers were prepared from cross-linked HbA as described by Kwiatkowski, et al. (*18*). Cross-linked derivatives of hemoglobin variants with mutations on the β subunits were prepared by combining cross-linked dimers of α subunits with either the variant β chains or the variant β globin plus hemin as previously described (*18*).

Variant β globin genes were generated by cassette mutagenesis or by PCR mutagenesis, using the QuikChange Site-Directed Mutagenesis Kit available from Stratagene (LaJolla, CA), on the T7 expression plasmid of Hernan et al. (*42*), as described by Noble et al. (*31*). The variant αD94G globin gene was also generated by PCR mutagenesis, using the QuikChange kit. The mutation was created using the T7 expression plasmid pET17b (Novagen, Madison, WI)-based expression vector, which coexpresses α and β globins as described by Noble et al. (*31*). T7 expression plasmids containing either a β globin gene or both an α and β globin gene were transformed into competent *E. coli* BL21(DE3) (Novagen, Madison, WI). As described by Hernan et al. (*42*), the transformed strains were grown in LB media containing ampicillin and induced with IPTG. Mutant β globins were isolated from the β globin expression system as previously described (*42*). Mutant α globins were obtained from the coexpression system by first isolating the expressed hemoglobin, removing the heme, and purifying the mutant α

globin in 8 M urea by chromatography on a CM52 column as previously described (*39*). All mutant globins had their N-terminal valine residues replaced by methionine.

The formation of normal and variant hemoglobins and symmetrical FeZn hybrids from isolated chains of HbA, mutant globin chains, and heme has been described (*42*, *31*). The variant hemoglobins were formed by combining the variant globins with hemin and normal partner subunits. Thus, the normal partner subunit of a variant never contained the V1M substitution. The αV1M substitution appears to have no significant effect on the functional properties of HbA. The *β*V1M substitution causes minor alterations in the properties of HbA. The effects of this mutation have been extensively examined by Doyle et al. (*33*). It somewhat lowers the ligand affinity of the Hb molecule but has no effect on ligand-binding kinetics (*31*). It appears that it slightly alters the equilibria between the conformational states of the hemoglobin tetramer.

Oxygen equilibria were measured tonometrically, as previously described by Noble et al. (*31*), at a hemoglobin concentration of 160 *μ*M in porphyrin equivalents. A 500 mL tonometer with a 2 mm attached cuvette was used. Spectrophotometric measurements were carried out with a Cary 14 spectrophotometer modified by OLIS (On Line Instrument Systems, Bogart, GA) for computer control and on-line data acquisition. All measurements were carried out at 20° in 100 mM Cl^- bisTris buffer at pH 7 and 100 mM Cl^- Tris buffer at pH 7.5 and 8. In order to maintain heme groups in their reduced ferrous state, the enzyme system of Hayashi et al. (*43*) was used.

The kinetics of CO combination with unliganded hemoglobins were measured by rapid mixing in an OLIS stopped-flow apparatus as described by Doyle et al. (*33*). The procedure was essentially that of Gibson (*44*) as described by Noble et al. (*31*). The reactions were followed at 420 and 435 nm using a 1.7 cm path length cell. A dithionite concentration of approximately 2 mM was used to maintain anaerobicity. The concentrations of CO and hemoglobins (in porphyrin equivalents) were generally 20 and 2 *μ*M, respectively. Although liganded hemoglobins dissociate significantly into *αβ* dimers at this concentration, no measurable dissociation is found with unliganded derivatives of HbA or the *β*N102 variants. Were a significant dimer population to exist in solutions of these hemoglobins when unliganded, then these dimers would combine with CO much more rapidly than the Hb tetramers, and a rapid kinetic phase would be observed. However, none were observed. Again, measurements were carried out at 20° in the same buffer used for the equilibrium measurements at pH 7. Kinetic transients were fitted to single exponentials or sums of two exponential functions using successive integration and Levenberg−Marquardt fitting routines supplied by OLIS and the Levenberg−Marquardt fitting routines supplied in Sigma Plot (SPSS Science).

The kinetics of CO recombination following flash photolysis were measured by a modification of the technique of Gibson (*45*) as described by Doyle et al. (*33*) using an apparatus consisting of an optical system and three photographic strobe units (Sunpak Auto 544) equipped with thyrister-quenching devices. Data were collected and processed by an OLIS Model 4000 data acquisition and control system. The strobes were adjusted to give a rectangular pulse approximately 0.5 mS long, and data collection was delayed until 1.2 mS after initiation of the pulse. To further isolate the signal from flash intensity, the flash was filtered through a solution of auramine, which has a very high extinction coefficient at the monitoring wavelengths, 420 and 435 nm. Partial photolysis was achieved by shielding the cuvette from most of the flash intensity so that the absorbance change produced by photolysis was between 5 and 10% of that associated with full photolysis.

Table 1: Equilibria of O_2 Binding to HbA and Three *β*N102 Variants and Their Cross-linked Derivatives[a]

	−IHP		+IHP	
variant	p50(O_2)	n_{Max}	p50(O_2)	n_{Max}
HbA	4.6	2.8	51	2.0
XL99α	12.7	1.8	77	1.8
*β*V1M[b]	6.7	2.8	59	1.8
*β*N102A	26	1.6	120	0.9
XL99α	107	0.95	142	1.0
*β*N102Q	14	1.1	49	0.83
XL99α	15	1.1	48	0.92
*β*N102T	30	1.06	126	0.71
XL99α	27	1.27	127	0.73

[a] Measurements were carried out at pH 7 at an Hb concentration of 160 *μ*M in heme equivalents in the absence and presence of 100 *μ*M IHP. The p50(O_2) (Torr) and the maximum Hill coefficient, n_{max}, are reported. [b] The data for the *β*V1M variant are also included, since all of the variant *β* subunits have this substitution at their N-termini.

RESULTS

Effects of the Mutations of the β102 Sequence Position of HbA on the Equilibria of Oxygen Binding. The comparison of the oxygen-binding properties of HbA and the three *β*N102 variants appears in Table 1. Because of the reported propensity of *β*N102 variants to dissociate into *αβ* dimers with their high ligand affinity, their cross-linked derivatives were also examined. This permitted the examination of the tetrameric Hb molecule without the complication of dissociation. Equilibrium measurements were carried out in the absence and presence of 1 mM IHP. Dissociation into dimers in the course of ligand binding results in a ligand-dependent increase in affinity leading to apparent cooperativity in ligand binding independent of the properties of the tetramer. For all three variants studied here, the replacement of *β*102 Asn with another amino acid results in a reduction in oxygen affinity. However, the magnitude of the reduction varies considerably. Comparing the uncross-linked *β*N102 variants in the absence of IHP and using the oxygen partial pressure required for half saturation, p50(O_2), of 6.7 Torr for the *β*V1M variant of HbA in the absence of IHP as our reference, we obtain a roughly 4-fold increase in p50(O_2) for *β*N102A, a 2-fold increase for *β*N102Q, and a 4.5-fold increase for *β*N102T. Oxygen binding to *β*N102A appears to be somewhat cooperative under these conditions, whereas both *β*N102Q and *β*N102T exhibit little cooperativity. In the absence of IHP, cross-linking reduces the affinity of oxygen binding to HbA by somewhat less than 3-fold and reduces the affinity of *β*N102A 4-fold, while having little effect on the oxygen affinities of the *β*N102Q and *β*N102T variants. In the absence of IHP, cross-linking also reduces the cooperativity of oxygen binding to HbA and the *β*102A variant, while slightly increasing the cooperativity of *β*N102T

Table 2: Kinetics of CO Combination with HbA and Three βN102 Variants[a]

variant	−IHP	+IHP
HbA	0.18 A[b]	0.09 A
XL99α	0.16 A	0.09 A
βV1M	0.17 A	0.09 A
XL99α	0.15 A	0.08 A
βN102A	0.067	0.07 (50%)[c]
		0.03 (50%)[c]
XL99α	0.09 (50%)[c]	0.09 (50%)[c]
	0.035 (50%)[c]	0.032 (50%)[c]
βN102Q	0.34 (50%)[c]	0.24 (50%)[c]
	0.16 (50%)[c]	0.09 (50%)[c]
XL99α	0.48 (50%)[c]	0.38 (50%)[c]
	0.09 (50%)[c]	0.08 (50%)[c]
βN102T	0.10 (97%)	0.11 (50%)[c]
		0.05 (50%)[c]
XL99α	0.24 (50%)[c]	0.18 (50%)[c]
	0.06 (50%)[c]	0.05 (50%)[c]

[a] Measurements were carried out at pH 7 at an Hb concentration of 2 μM in heme equivalents in the absence and presence of 100 μM IHP. Second-order rate constants are reported in units of μM^{-1} s^{-1}. [b] Denotes accelerating kinetics. [c] Denotes a fit to the data in which the absorbance changes resulting from each of the two kinetic components were forced to be equal to one another.

and having no apparent effect on βN102Q. In the presence of IHP, the effects of cross-linking are uniformly modest. The addition of IHP greatly reduces oxygen affinities for all of the uncross-linked hemoglobins, and in its presence, none of the βN102 variants exhibits cooperativity in oxygen binding. With the addition of IHP, the p50(O_2) of cross-linked βN102A is increased by 33%, whereas the p50(O_2) values of cross-linked βN102Q and cross-linked βN102T are increased to roughly the same values exhibited by their uncross-linked counterparts in the presence of IHP. Again, no cooperativity in oxygen binding is observed for the cross-linked variants in the presence of IHP. In evaluating these data, it is important to remember that cross-linking is not without functional consequences. It reduces the oxygen affinity of HbA but appears to have little effect on the properties of its T state as indicated by its lack of effect on CO combination kinetics (*18*). This reduction in affinity may be due in part to a preferential stabilization of the T state with respect to the R^e state of the protein, but it cannot be said to be fully understood.

Effects of β102 Mutations on the Kinetics of CO Combination. The kinetics of combination of CO with deoxygenated HbA and the three βN102 variants following rapid mixing were measured at pH 7 in the absence and presence of IHP. The kinetics of CO combination were also measured for the cross-linked derivatives of these four hemoglobins. The rate constants determined from these measurements are reported in Table 2. The kinetics of CO combination with HbA exhibit acceleration. In contrast, none of the βN102 variants exhibits accelerating CO combination kinetics. In the majority of cases, the reactions decelerate, and the kinetics can be well fitted to the sum of two exponential functions of equal amplitude. The two exceptions are the kinetics of βN102A and βN102T in the absence of IHP, which are well fitted to single-exponential functions. With the βN102 variants, a small, fast kinetic phase is often observed that is not eliminated either by cross-linking the hemoglobin or by the addition of IHP, which precludes a population of $\alpha\beta$ dimers. It was concluded that these fast phases were the result of small amounts, less than 3%, of rapidly reacting impurities in these preparations. They have had no significant effect on any of the conclusions drawn from the measurements on these samples. The addition of IHP reduces the overall rate constant for CO combination with HbA by roughly a factor of 2. IHP also reduces the rates of CO combination with the β102 variants but to a variable extent. In the presence of IHP, all of the βN102 variants bind CO with biphasic kinetics. Cross-linking HbA has little effect on the kinetics of CO combination. However, in the absence of IHP, it converts the kinetics of CO combination with βN102A to a biphasic reaction and does the same for the slow kinetic phase of CO combination with βN102T. Adding IHP to the cross-linked derivatives of the βN102 variants has little or no effect on the rates of their reactions with CO.

Effects of β102 Mutations on the Kinetics of CO Recombination Following Flash Photolysis. In measuring CO recombination, one begins with a fully liganded Hb molecule, with its particular dimer−tetramer equilibrium. This is distinctly different from the measurement of CO combination with unliganded Hb, which in general has much less tendency to dissociate into dimers. Although the rate of the transition from the R^e quaternary structure of Hb to the low-affinity T quaternary structure is much faster than the rate of CO recombination, the rate of assembly of dimers into Hb tetramers is slow compared to the rate of CO recombination. Therefore, in CO recombination, one typically observes the sum of the rapid reaction of CO with the population of dimers and the much slower reaction of CO with unliganded T state tetramers in a proportion that reflects the dimer−tetramer equilibrium of the fully liganded Hb molecule.

Under the conditions of our CO recombination experiments, normal human HbA is roughly 60% dimer and 40% tetramer at pH 7 in the absence of IHP. The equilibrium constant for the assembly of the liganded tetramer of Hb Kansas (βN102T) from two $\alpha\beta$ dimers has been determined by Atha et al. (*25*) to be 9.1×10^3, which indicates the presence of 2% tetramers at the Hb concentration used in the CO recombination measurements. Under the same conditions, the kinetics of the recombination of CO with the βN102A and βN102Q variants at 2 μM heme show no sign of a slow tetramer process, indicating that the proportion of tetramer present cannot be more that 2 or 3% in either case. However, in the presence of IHP, similar experiments indicate that the βN102Q variant is 40% tetrameric.

As already mentioned, cross-linking is carried out to eliminate the dissociation of the hemoglobin tetramer into $\alpha\beta$ dimers. To ensure that cross-linking is complete, the kinetics of CO combination following rapid mixing are compared to that of CO recombination following flash photolysis. In Figure 1, the kinetics of CO combination and recombination with cross-linked βN102T variant in the presence and absence of IHP are shown. Under both solution conditions, the kinetics observed by flow and flash are virtually identical. No rapid kinetic phases, as would result from the presence of $\alpha\beta$ dimers, are observed following photolysis. Similar results were achieved with the cross-linked derivatives of the βN102A and βN102Q variants. No $\alpha\beta$ dimers were detected by flash photolysis in any of the preparations of cross-linked hemoglobins.

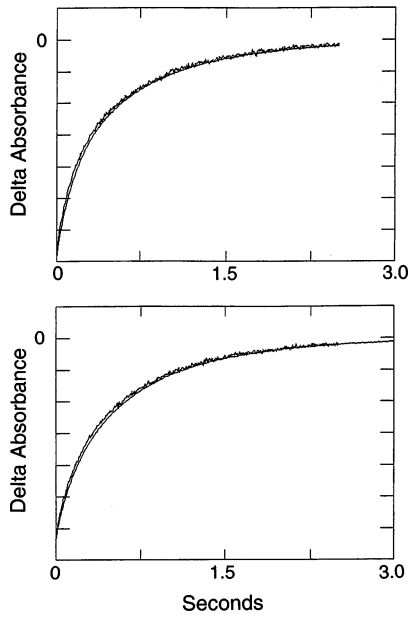

FIGURE 1: Comparison of the kinetics of the reaction of CO with the unliganded cross-linked βN102T variant following rapid mixing in a stopped flow apparatus and the kinetics of CO recombination with this cross-linked hemoglobin following complete removal of the bound ligand by flash photolysis. In all experiments, the CO concentration was 20 μM, and the Hb concentration was 2 μM in heme equivalents. The upper graph compares the time courses of CO combination and recombination in the absence of IHP, whereas the lower graph contains data obtained in the presence of 1 mM IHP. In all other aspects, the solutions used in all of the studies were identical.

Differences in the rates of CO combination to unliganded and triliganded hemoglobin are one indication of cooperativity in ligand binding. Measurements of CO recombination following full and partial (5−10%) flash photolysis yield these data. These experiments have been carried out with cross-linked hemoglobins to eliminate the rapid kinetic phases that would result from the presence of $\alpha\beta$ dimers. In Figure 2, the time courses of CO recombination following full and partial photolysis in the presence and absence of IHP are shown for cross-linked HbA and the cross-linked derivatives of the three βN102 variants. With HbA (Figure 2A), the rate of CO recombination with a predominantly triliganded hemoglobin population is much greater than the rate for fully unliganded hemoglobin both in the absence and presence of IHP. For the βN102 variants, the picture is very different. In the presence of IHP, no significant differences between fully and partially photodissociated hemoglobins are observed with any of the βN102 variants. In the absence of IHP, fully and partially unliganded βN102A (Figure 2B) behave identically, whereas a small difference of questionable significance is observed with βN102T (Figure 2D). Under these same conditions, CO is seen to combine significantly more rapidly with triliganded βN102Q (Figure 2C) than with the fully unliganded variant. However, the rate of the rapid kinetic phase of this reaction is less than what would be expected if it were the result of the presence of triliganded R^e state molecules.

Effects of Mutations at the αD94 and βW37 Positions on the Kinetics of CO Recombination with Hemoglobin. In Figure 3, the results from similar experiments carried out on the cross-linked derivatives of the αD94G, βW37Y, and βW37G variants are shown. As already mentioned, the αD94 mutation eliminates the R^e state interaction between this aspartate side chain and the β102 asparagine residue. The βW37 variants were examined because this residue also makes contact with the βN102 residue in liganded HbA. As with the βN102 variants, the cross-linked αD94G variant exhibits little or no tendency to switch to its R^e state in response to ligand binding. In the absence of IHP, this hemoglobin reacts significantly more rapidly when triliganded than when fully unliganded. However, neither reaction has a rate constant consistent with the presence of a population of R^e state hemoglobin. In the presence of IHP, the time courses of the recombination reactions following full and partial flash photolysis are superimposable. In contrast, the βW37Y variant exhibits larger differences in the rate of CO recombination following full and partial photolysis, whether or not IHP is present. βW37G again exhibits relatively small differences in the rates of CO recombination to fully and partially liganded tetrameric hemoglobin, but the difference persists in the presence of IHP. However, as will be discussed, the interpretation of the origins of the effects of this mutation on functional properties is complex.

Effects of β102 Mutations on the Equilibria and Kinetics of the Reactions of the First Ligand with Unliganded Hb. Symmetrical FeZn hybrids permit the independent examination of the reactions of the α and β subunits of the unliganded T state of a hemoglobin with ligands. In their unliganded state, these hybrids have two identical, potential ligand-binding sites. As a result, the kinetics of CO combination can be precisely fitted to a two-step sequential process. The initial rate constant is a measure of the kinetics of the reaction of the first ligand to an otherwise unliganded hemoglobin molecule at the subunit type that possesses the heme groups in the hybrid under examination. Likewise, the equilibria of ligand binding to such hybrids can be precisely fitted to a two-step Adair equation. The rates of combination of the first CO molecule with an α subunit or a β subunit of otherwise unliganded Hb tetramers, $l'_1(\alpha)$ and $l'_1(\beta)$, have been reported by Hui et al. (*32*) for HbA and the three βN102 variants along with the equilibrium dissociation constants, $K_1(\alpha)$ and $K_1(\beta)$, for the reaction of an oxygen molecule with the same subunits of these otherwise unliganded hemoglobins. These were reported as a part of an examination of the relationship between oxygen affinity and the rate of CO combination for the subunits of a number of unliganded variants of HbA in the presence of IHP.

The data from which the equilibrium constants, $K_1(\alpha)$ and $K_1(\beta)$, for HbA and the βN102 variants were determined are shown in Figure 4. Here, the fractional saturation with oxygen at 20° and pH 7 in the presence of IHP is plotted as a function of the partial pressure of oxygen for the $[\alpha(Fe)]_2$-$[\beta(Zn)]_2$, upper panel, and $[\alpha(Zn)]_2[\beta(Fe)]_2$, lower panel, hybrids. The curved lines are the fits of each data set to a second-order Adair equation. The resulting values of K_1 are shown in Table 3. These clearly demonstrate one of the largest differences among these three variants. If one examines the values of $K_1(\alpha)$ for the β102 variants, one observes little variation from the value of this constant for HbA, the largest deviation being roughly 25%. However, although the value of $K_1(\beta)$ for the βN102A variant is nearly equal to that for HbA, that for the βN102Q variant indicates an oxygen affinity almost 10-fold greater than that of $K_1(\beta)$

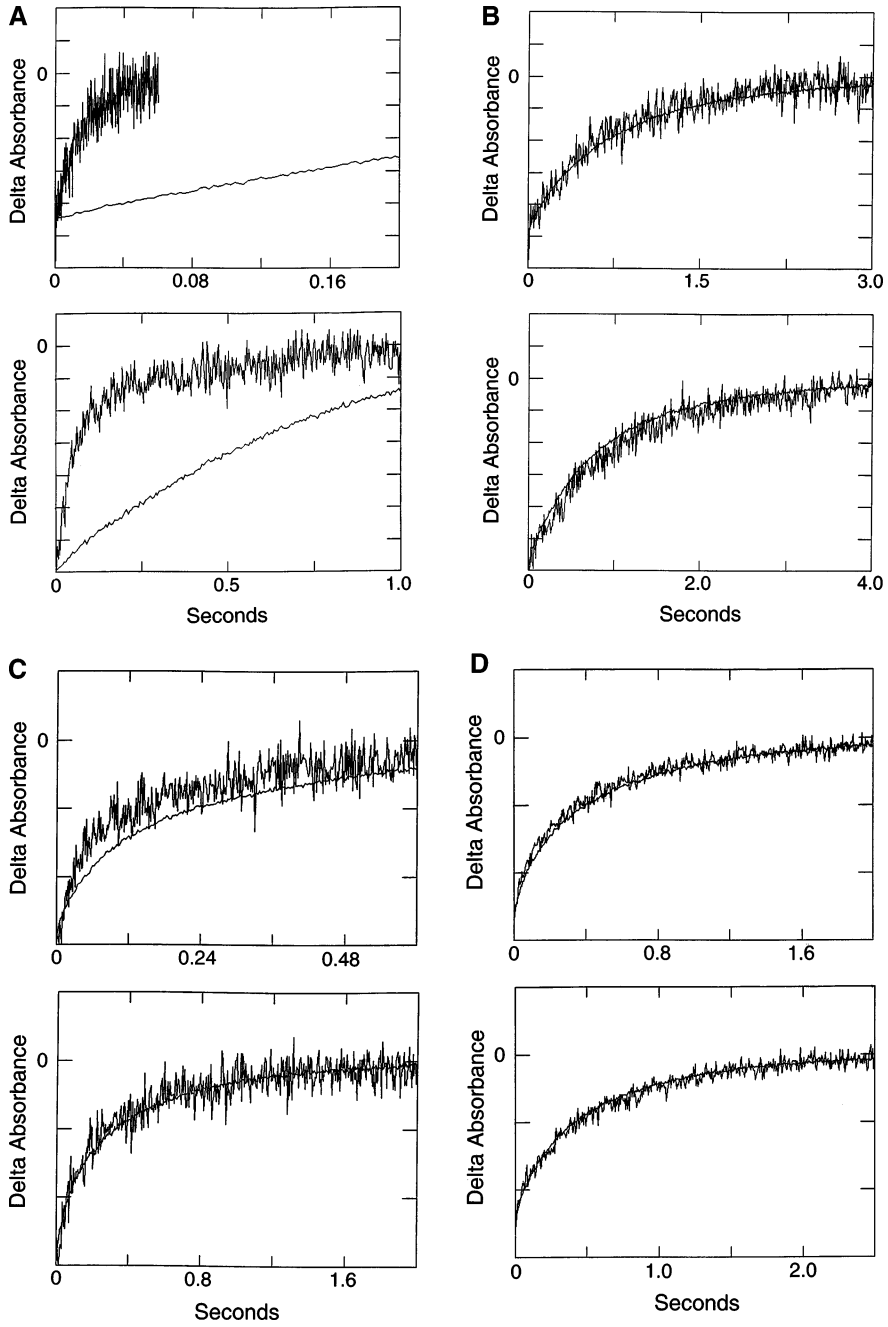

FIGURE 2: Comparison of the kinetics of CO recombination following full and partial photodissociation for the cross-linked derivatives of (A) HbA, (B) βN102A, (C) βN102Q, and (D) βN102T. Experimental conditions were as described in Figure 1. In every case, the data obtained by partial flash photolysis can be identified by their higher relative noise level. In each panel, the upper graph contains data obtained in the absence of IHP, whereas the lower graph contains data obtained in the presence of 1 mM IHP.

for HbA and that for the βN102T an oxygen affinity more than 4-fold greater than that for HbA. Aside from any effects these two mutations may have on the ligand-linked structural transitions and overall oxygen affinity of human hemoglobin, they have a decided effect on the properties of the β subunit within the deoxygenated T quaternary structure.

The values of $l'_1(\alpha)$ and $l'_1(\beta)$ were measured both in the absence and presence of IHP and appear in Table 4. Examining the data for HbA, one sees that adding IHP has less effect on the kinetic properties of the α subunit than on the rate constant for the β subunit, which is reduced by more than half. As with the data for the equilibria of oxygen binding, these kinetic results demonstrate once again the relative invariance of the properties of the α subunits of the β102 variants and the rather large variations in the properties of the β subunits among these variants. Again, the βN102Q variant has the greatest effect on β subunit properties, a 6- to 10-fold increase in rate constant, depending upon the absence or presence of IHP. In the case of the βN102T variant, the β subunit rate constant is increased by 2- to 3-fold. The effect of IHP on $l'_1(\beta)$ varies considerably. For none of the β102 variants is it as great as that for normal HbA. For βN102T, the decrease is 38%, and for βN102Q, it is 32%. For βN102A, it is only 25%, which is barely significant. In fact, the kinetics of the T state of this variant in the absence of IHP resembles the kinetics of HbA in the presence of this allosteric effector far more than that of HbA in its absence.

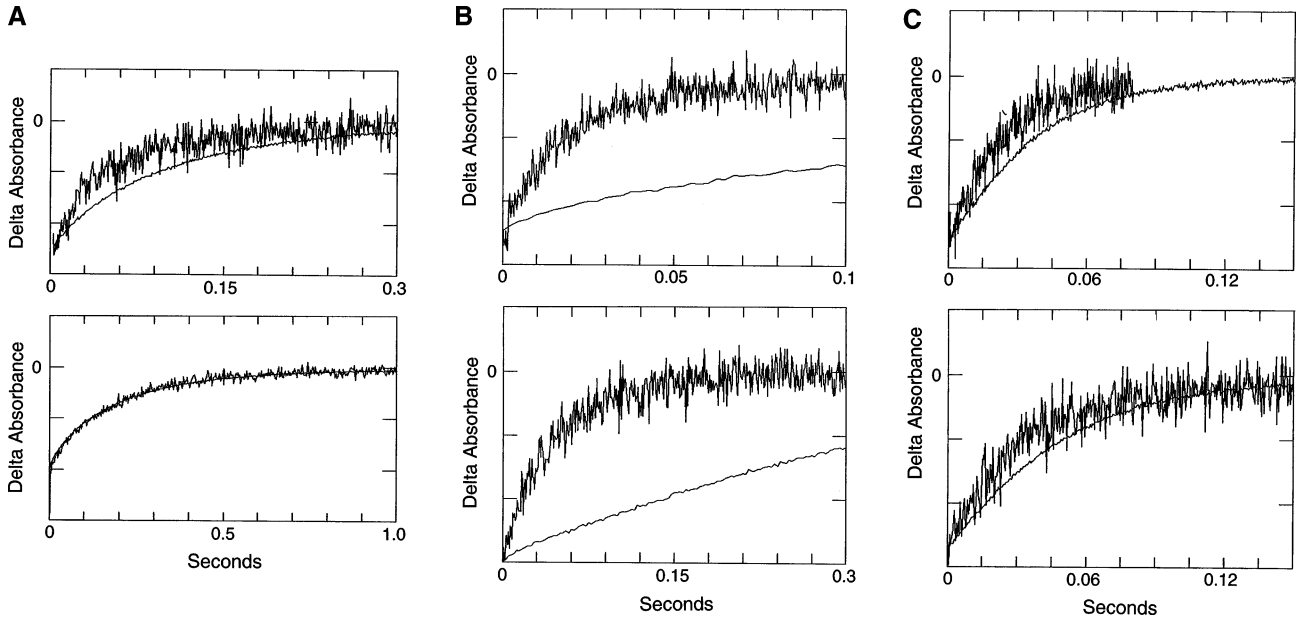

FIGURE 3: Comparison of the kinetics of CO recombination following full and partial photodissociation of the cross-linked derivatives of (A) αD94G, (B) βW37Y, and (C) βW37G. Experimental conditions were as described in Figure 1. Again, the upper and lower graphs in each panel represent data obtained in the absence and presence, respectively, of 1 mM IHP.

From the data in Tables 1, 3, and 4, it is quite clear that of the three βN102 variants examined here, the T state of βN102A most closely resembles that of HbA. However, in the absence of IHP, the βN102A variant most resembles the T state of HbA when the latter is in the presence of IHP. As previously mentioned, Karasik et al. (*10*) demonstrated that the addition of IHP to HbA drastically reduced the Bohr effects of $K_1(\alpha)$ and $K_1(\beta)$. These facts raise the possibility that the βN102A mutation might itself reduce these K_1 Bohr effects in the absence of IHP. To examine this possibility, the pH dependencies of $K_1(\alpha)$ and $K_1(\beta)$ for βN102A were measured in the absence and presence of IHP in the pH range from 7 to 8. The results are shown in Figure 5, along with the data for HbA in the same pH range from Karasik et al. (*10*). The upper panel shows the pH dependencies of $K_1(\alpha)$ and the lower panel those for $K_1(\beta)$. It is evident that the data for HbA in the absence of IHP is totally unlike the data for HbA in the presence of IHP or for the βN102A variant in the presence or absence of IHP. The latter three data sets in both panels of the Figure exhibit far smaller pH dependencies than the data for HbA in the absence of IHP. The removal of the side chain of the βN102 residue clearly results in the attenuation of the K_1 Bohr effects of both the α and β subunits in the absence of IHP to similar extents.

DISCUSSION

Differences among the Three βN102 Variants. The three β102 variants examined in this study exhibit very different properties. Whereas the T state of the βN102A variant in the presence of IHP exhibits properties very similar to those of HbA, those of the other two variants do not. The largest deviations from HbA behavior are seen in the properties of the β subunits. As can be seen in Tables 3 and 4, in the presence of IHP, the affinities of the β subunits of the βN102Q and βN102T variants for the first oxygen molecule and the rates of combination of the first CO molecule to the β subunits are all significantly greater than those for the β subunits of HbA and the βN102A variant.

βN102Q Variant. It is interesting that the βN102Q replacement results in such a large change in β subunit properties. It suggests that a stereochemical problem is introduced by the elongation of this side chain by a single carbon atom. As can be seen in Table 3, the two types of subunits of this variant have very different properties. Such subunit heterogeneity in the absence of cooperativity would be expected to result in a Hill coefficient less than unity, and this is found to be the case in the presence of IHP (Table 1). In the absence IHP, one finds a Hill coefficient slightly above unity. The intrinsic heterogeneity of the subunits increases the significance of this otherwise modest Hill coefficient. The extent to which ligand-linked dissociation into dimers contributes to this cooperativity was explored by cross-linking the two dimers. As seen in Table 1, the effect is minimal. Although flash photolysis of the uncross-linked derivative of this hemoglobin indicates substantial dissociation into dimers, it must be remembered that CO recombination experiments are carried out at a hemoglobin concentration of 2 μM in porphyrin equivalents, 80-fold lower than that used in equilibrium measurements. Because of the higher overall ligand affinity of the tetramer of this variant in the absence of IHP, as indicated by the properties of the cross-linked protein, the ligand-linked change in its dimer−tetramer equilibrium must be far less than that for the βN102A variant. The addition of IHP reduces the oxygen affinities of both the cross-linked and uncross-linked forms of this variant to the same extent and also reduces their Hill coefficients, although not exactly to the same extent. The absence of significant cooperativity in the binding of oxygen to this variant in the presence of IHP is supported by the values of $K_1(\alpha)$ and $K_1(\beta)$ measured under these same conditions. The predicted $p50(O_2)$ for an equal mixture of sites with these two affinities as computed by the relationship

$$\log p50 = \frac{(\log p50\alpha + \log p50\beta)}{2}$$

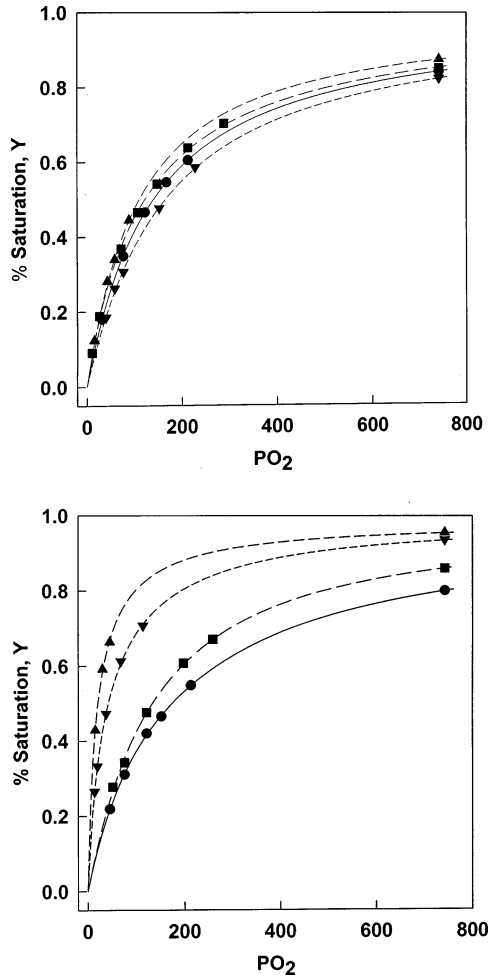

FIGURE 4: Measurements of equilibria of oxygen binding, at pH 7 in the presence of IHP, to FeZn hybrids of HbA (●) and variants with substitutions at the βN102 position, βN102A, (■); βN102Q, (▲); and βN102T, (▼). Fractional saturation is plotted as a function of the partial pressure of oxygen, pO_2 (Torr). Data for the $[\alpha(Fe)]_2$-$[\beta(Zn)]_2$ hybrids are shown in the upper panel, whereas data for the $[\alpha(Zn)]_2[\beta(Fe)]_2$ hybrids appear in the lower panel. The lines are the results of fitting each of the data sets to a second-order Adair equation, from which the values of K_1 were obtained.

Table 3: Dissociation Equilibrium Constant for the Reaction of the First Oxygen Molecule with an Otherwise Unliganded Hb Tetramer at Either an α Subunit, $K_1(\alpha)$, or a β Subunit, $K_1(\beta)$[a]

variant	IHP	$K_1(\alpha)$	$K_1(\beta)$
HbA[b]	−	75	70
	+	138	156
βN102A	+	112	154
βN102Q	+	113	16
βN102T	+	175	36

[a] Data obtained in the presence of IHP are from Hui et al. (*32*). Measurements were carried out on the two symmetrical FeZn hybrids of HbA and each of the three βN102 variants. The units of the equilibrium constants are in Torr. Experiments were carried out at pH 7 at an Hb concentration of 160 μM in porphyrin equivalents. When present, the concentration of IHP was 100 μM. [b] Data for HbA in the absence of IHP were plotted in Figure 1 of Karasik et al. (*10*)

is 43 Torr, which compares well with the measured $p50(O_2)$ of 49 Torr.

βN102T Variant (Hb Kansas). As with the βN102Q variant, the subunit heterogeneity observed in the T state of the βN102T variant suggests that the Hill coefficient observed in the absence of IHP indicates significant cooperativity. Surprisingly, cross-linking this variant increases the cooperativity of oxygen binding while having little effect on the value of $p50(O_2)$. This mutation has the effect of increasing the oxygen affinity of the β subunit, in the otherwise unliganded tetramer in the presence of IHP, by 4-fold while decreasing that of the α subunit by a modest 27%. If one assumes that there is no cooperativity in oxygen

Table 4: Second-Order Rate Constants for the Combination of the First CO Molecule with an Otherwise Unliganded Tetramer of HbA and Three βN102 Variants[a]

variant	IHP	$l'_1(\alpha)$	$l'_1(\beta)$
HbA	−	0.13	0.11
	+	0.10	0.046
βN102A	−	0.12	0.053
	+	0.11	0.04
βN102Q	−	0.12	0.65
	+	0.11	0.44
βN102T	−	0.10	0.24
	+	0.09	0.15

[a] This data first appeared in Table 2 of Noble et al. (*31*). Measurements were carried out on the two symmetrical FeZn hybrids of each of the hemoglobins at pH 7 at an Hb concentration of 2 μM in porphyrin equivalents in the absence and presence of 100 μM IHP. Second-order rate constants are reported for the initial combination of ligand with an α subunit, $l'_1(\alpha)$, or a β subunit, $l'_1(\beta)$, in units of μM^{-1} S^{-1}.

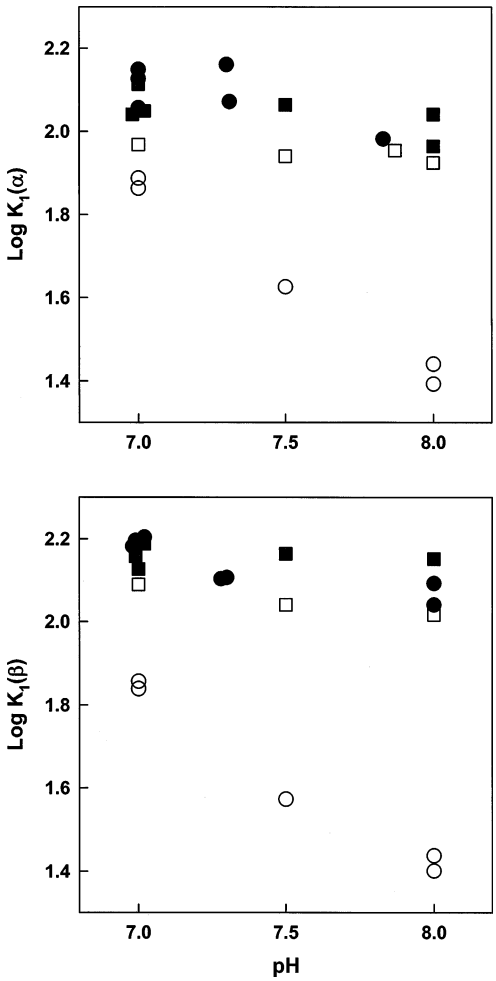

FIGURE 5: Effects of IHP and the βN102A substitution on the pH dependence of $K_1(\alpha)$ and $K_1(\beta)$. The symbols indicate the following: HbA in the absence of IHP, (○); HbA in the presence of IHP, (●); βN102A in the absence of IHP, (□); and βN102A in the presence of IHP, (■). Data for HbA are from Karasik et al. (*10*).

binding to this hemoglobin under these conditions, then these values of $K_1(\alpha)$ and $K_1(\beta)$ predict an overall p50(O_2) for the Hb tetramer of 79 Torr, well below the observed value of 126 Torr. They also predict a Hill coefficient of 0.86, well above the observed value of 0.71. It is tempting to suggest the possibility of negative cooperativity in this case, but that should await further experimentation.

Riggs and Gibson (*24*) found the isolated β chains of Hb Kansas (βN102T) to have an abnormally low affinity for oxygen, some 6-fold lower than that of isolated β_A chains. This suggests that the β Kansas subunit within the $\alpha\beta$ dimer of Hb Kansas has a lower affinity than that of the β subunit of HbA, again within the dimer. In addition, the data in Table 3 indicate that in the low-affinity T state tetramer, the oxygen affinity of the β subunit of the βN102T variant is more than 4-fold greater than that of the normal β subunit. This means that the incorporation of a Hb Kansas β chain into the unliganded Hb Kansas tetramer results in a much smaller decrease in oxygen affinity than the incorporation of a normal β chain into its unliganded tetramer. Combining the data on the FeZn hybrids in Table 3 with the data of Riggs and Gibson (*24*), one computes that this reduction in the affinity for the β subunit of Hb Kansas is some 24-fold less than that experienced by the normal β chain. These results indicate that the β subunit of Hb Kansas is decidedly abnormal both in its own functional properties and in its functional response to the combination with α chains to form the quaternary T state structure of unliganded hemoglobin. It also suggests that the effect of the binding of an oxygen molecule to a β subunit of unliganded Hb Kansas on dissociation of the Hb tetramer into $\alpha\beta$ dimers will be 24-fold less than that for HbA. CO recombination with the βN102Q variant in the absence of IHP occurs with the rapid rate indicative of $\alpha\beta$ dimers. This recombination reaction displays no unusually slow component that one might expect if the βN102Q subunit also had an abnormally low ligand affinity in the $\alpha\beta$ dimer.

It appears that neither the βN102Q nor the βN102T variant represent an adequate model for the T state of HbA. The β subunits of these two variants are distinctly abnormal. It is quite possible that the crystal structures will offer explanations for the effects of these two substitutions on the properties of the β subunits within the T quaternary state. These structures have not been determined yet, but work is under way in another laboratory.

βN102A Variant. The βN102A variant presents a very different picture. First, it appears that the majority of the cooperativity observed for the βN102A variant in the absence of IHP results from a ligand-linked dissociation to high affinity $\alpha\beta$ dimers. This conclusion is based upon the effects that cross-linking has on the functional properties of this variant, that is, eliminating cooperativity and reducing its oxygen affinity by 4-fold, resulting in a p50(O_2) of 107 Torr. There is no evidence to suggest that the deoxygenated derivative of this variant dissociates significantly into $\alpha\beta$ dimers. However, given the very low ligand affinity of the tetramer, there must be a very strong linkage between ligand binding and dimer formation. The oxygen affinity of the cross-linked tetramer in the absence of IHP (p50(O_2) = 107 Torr) is only slightly greater than that observed when IHP is added, the p50(O_2) equaling 142 Torr when the hemoglobin is cross-linked and 120 Torr when not cross-linked. The lack of cooperativity in the presence of IHP is evidenced not only by the Hill coefficient but also by the values of $K_1(\alpha)$ and $K_1(\beta)$ in Table 3. If one assumes that there is no cooperativity in the presence of IHP, then the measured p50(O_2) should be predicted by the values of $K_1(\alpha)$ and $K_1(\beta)$ measured under these same conditions. The predicted value of p50(O_2) for this variant in the presence of IHP when not cross-linked is 131 Torr, which compares well with the measured value of 120 Torr. It also suggests that within the error of these measurements, when cross-linked in the absence of IHP, this variant is very nearly in its minimum affinity T state and binds oxygen without significant cooperativity. The possibility of residual cooperativity in oxygen binding to the uncross-linked βN102A tetramer cannot be addressed by the present data. As already pointed out, the reduction of the oxygen affinity of HbA that accompanies cross-linking may be due in part to a preferential stabilization of the T state with respect to the R^e state of the protein. If so, this would be expected to contribute to a reduction in cooperativity. However, it is unlikely that this stabilization would be sufficient to produce the effects observed with the βN102A variant in the absence of what is effectively a large-scale assembly of dimers into tetramers. However, the kinetics of CO combination with deoxygenated βN102A in the absence of IHP exhibits no sign of subunit heterogeneity, even though the kinetics in the presence of IHP is heterogeneous, and the measurements of $l'_1(\alpha)$ and $l'_1(\beta)$ confirm subunit differences. The masking of subunit heterogeneity is a common kinetic reflection of cooperativity. Whether it could result from ligand-linked dissociation into dimers depends on how the rates of the dimer dissociation reaction depend on fractional saturation, which is not known for this variant.

The crystal structure of the unliganded T state of the βN102A variant has been reported (*13*), and it appears to be very similar to that of HbA, both in its quaternary and tertiary structure. This is not surprising because the β102 aspartate side chain makes no contacts in the T state. It is also consistent with the fact that crystals of the unliganded derivatives of HbA and the βN102A variant have been shown to have very similar oxygen affinities (*31*).

Possible Origins of the Different Effects of Cross-Linking on the βN102 Variants. The relatively small effects that cross-linking has on the oxygen equilibria of the βN102Q and βN102T variants are difficult to understand. When saturated with the ligand both exhibit greater dissociation into dimers than HbA. However, both of these variants have β subunits with oxygen affinities that, in the T state tetramer, are greater than normal and that appear to be much less affected by the transition from T state tetramer to dimer than in normal HbA. In addition, both variants exhibit cooperative ligand binding in the absence of IHP when cross-linked. This means that the sequence of ligand-linked changes in tetramer stability in these hemoglobins must be significantly different from that found in either HbA or the βN102A variant. Because of their higher affinities, oxygen will tend to bind first to the β subunits of the βN102Q and βN102T variants and will result in relatively small decreases in tetramer stability. Only in the latter stages of oxygen binding will binding to low affinity α subunits become prominent with its large effect on tetramer stability. The result is that dimers will appear in the late stages of the saturation process and primarily affect the late Adair constants. With both HbA and

the βN102A variant, all subunits at the initiation of oxygen binding have low T state affinities, and the early binding steps have large effects on dimer stability. In the case of HbA, ligand affinity increases greatly with increasing saturation; therefore, the later stages of ligand binding have relatively little effect on tetramer stability. In fact, from the data of Ackers, Holt, and their co-workers (*6*−*9*), it appears that the final step of oxygen binding to HbA has a higher affinity than that of the dimer, resulting in dimer stabilization. The result of this difference in the pattern of ligand-linked tetramer destabilization is that HbA dissociation into dimers affects binding early in the saturation process and affects the entire saturation curve, whereas for the βN102Q and βN102T variants, the effects should appear primarily in the latter portion of the saturation process. The βN102A variant retains its low T state affinity over all or almost all of the saturation process and exhibits no cooperativity in the absence of IHP when cross-linked. From this, we can conclude that the change in tetramer stability resulting from the binding of four oxygen molecules is greatest for this variant, and as with HbA, major destabilization starts at the beginning of the saturation process. It is not surprising that this variant exhibits the greatest response to cross-linking. The fact that this variant shows no significant cooperativity in the absence of IHP when dissociation into dimers is prevented indicates that its R^e state is destabilized with respect to the T state to a much greater extent than is the case for the other two variants. This does not fully explain the apparent lack of significant effects of cross-linking the βN102Q and T variants, but it does explain why the effects are smaller than those for HbA and βN102A.

Role of the H Bond between the Side Chains of βN102 and αD94 in Cooperative Oxygen Binding. The loss of cooperativity in oxygen binding caused by the βN102A substitution results in large measure from the loss of the hydrogen bond between the asparagine side chain of this residue and the aspartate side chain of αD94. The fact that the αD94G substitution also greatly reduces or eliminates cooperativity is strong evidence that the interaction that occurs between the side chains of these two amino acids in the R^e state of HbA makes a critical contribution to the energetics of this quaternary structure. Its absence sufficiently destabilizes the R^e state with respect to the liganded T state that cooperativity, which results primarily from the transition between these two structures, is virtually eliminated. However, it is important to note that the effects of these two mutations are not identical. Unliganded αD94G reacts with CO twice as rapidly as HbA, indicating that this mutation also results in a significant increase in the ligand affinity of the T quaternary structure. Although such an increase in T state affinity will reduce the energetics of the linkage between ligand binding and the transition from the T to the R^e quaternary structure, the magnitude of the effect appears much too small to explain the loss of cooperativity exhibited by this variant.

An interaction also occurs between βW37 and βN102, which could contribute to R^e state stability. However, the effect of deletion of the βW37 side chain on the ligand affinity of the T quaternary state of hemoglobin is a drastic increase in ligand affinity, which acts to greatly decrease the destabilization of the T state that normally results from ligand binding. The result is that one expects a destabilization of the R^e state with respect to the liganded T state as a result of this mutation even without any real change in R^e state stability. Replacement of the β37 tryptophan side chain with tyrosine does not eliminate cooperativity even in the presence of IHP. However, elimination of the Trp side chain by replacement with glycine greatly reduces the difference in the rates of CO rebinding following full and partial photolysis. This Gly substitution increases the ligand affinity of the T state in the presence of IHP by approximately 40-fold. Therefore, it is impossible to assign the apparent change in cooperativity to a change in interaction with βN102.

βN102A Substitution Has Much the Same Effect on the T State of HbA as the Addition of IHP. From Tables 3 and 4, it can be seen that the substitution by alanine of the asparagine side chain of residue β102 does much more than destabilize the R^e quaternary state of HbA. It also has very much the same effect on the properties of the T state of HbA as the addition of IHP. Although the rate of binding the first CO molecule to an α subunit of HbA is reduced marginally by IHP addition, the rate for the binding of the first CO to a β subunit is reduced by roughly 60% (Table 4). In the absence of IHP, the βN102A substitution has these same effects on the hemoglobin molecule. The addition of IHP to HbA at pH 7 roughly halves the affinities of the α and β subunits for the first oxygen molecule, doubling $K_1(\alpha)$ and $K_1(\beta)$ (Table 3). As shown in Figure 5, at pH 7 in the absence of IHP, the βN102A substitution increases $K_1(\beta)$ by 70%, while increasing $K_1(\alpha)$ by 25%. These effects increase with increasing pH, and at pH 8, the addition of IHP to HbA increases $K_1(\alpha)$ and $K_1(\beta)$ approximately 3.3- and 4.4-fold, respectively. In the absence of IHP, the βN102A substitution increases $K_1(\alpha)$ and $K_1(\beta)$ by 3.2- and 4.1-fold, respectively. In addition, as shown in Figure 5, IHP addition and the βN102A substitution have in common the large reduction they cause in the pH dependences of $K_1(\alpha)$ and $K_1(\beta)$.

It Appears that in the Absence of IHP, the Binding of a Single Ligand Molecule to One Heme Group of an Otherwise Unliganded Hemoglobin Tetramer Produces a Complex Structural Transition. Among the changes resulting from this transition are the following: (1) an increase in the ligand affinity of the liganded heme group, the energy from which drives the transition, (2) an alteration of the p*K* values of one or more ionizable groups responsible for the K_1 Bohr effects, (3) an alteration in structure that recognizes the presence of the β102 asparagine side chain, and (4) a reduction in the affinity of the major anion binding site for IHP of sufficient magnitude to permit IHP to inhibit the transition. Although the complex set of asymmetric changes in the ligand affinities of the remaining, unliganded heme groups, as reported by Ackers, Holt and their co-workers (*9*) have not been shown to be inhibited by either IHP addition or the βN102A mutation, it is reasonable to presume that they are a result of the same structural transition. Using the findings of Holt and Ackers as a framework, one can postulate that the binding of a single ligand to the HbA tetramer in the absence of IHP produces a conformational transition, in the dimer to which the ligand is bound, from that of a low-affinity T state dimer to a high-affinity T state dimer. Whereas the increase in the ligand affinity of the liganded subunit drives this conformational transition, it results in an increase in the ligand affinity of the unoccupied heme in the αβ dimer to which the ligand is bound. One

can speculate that this conformational transition might involve the bending of the $\alpha\beta$ dimer as pointed out by Kavanaugh et al. (*13*). The similarity of the pH dependencies of $K_1(\alpha)$ and $K_1(\beta)$ and of the effects of IHP addition and the βN102A substitution on these dissociation constants supports the proposition that the conformational response of the dimer is very similar, whether the ligand binds to an α or a β subunit. Within this framework, it is not unexpected that the addition of IHP has only a limited effect on the properties of the unliganded βN102A variant. This structural and functional transition, normally observed when a single ligand binds to HbA, is greatly inhibited by this mutation, leaving very little for the addition of IHP to accomplish. In other words, the fact that the effects of adding IHP and the βN102A substitution are not additive is consistent with both doing more or less the same thing.

It needs to be mentioned that the term high-affinity T state appears in several places in the literature, and its meaning is often not the same. It is used to describe the T state of mutant hemoglobins whose mutations render the ligand affinity of the T state higher than normal. It has been used by Kavanaugh et al. (*13*) to describe the structure assumed by the crystalline T state when a deoxygenated Hb is first crystallized and subsequently saturated with the ligand. It has also been used to describe the properties of symmetrical FeZn hybrids of HbA when they are diliganded (*16*). In the present case, we are referring only to the monoliganded T state. From the work of Holt and Ackers, we have reason to believe that this T state is functionally and, therefore, structurally asymmetric. This is unlikely to be the case for the other high-affinity T state structures that have been examined.

It is striking that the transition from the low-affinity T state to the higher affinity, monoliganded T state involves a number of the same structures that are involved in the T to R^e state transition. IHP binds much more energetically to the T state than to the R^e state, but it also binds better to the low-affinity T than to the high-affinity T structure (*10*). The substitution of the β102 asparagine residue by an alanine inhibits both the ligand-linked transition from T low to T high and the ligand-linked transition from T to R^e, similar to the results from the addition of IHP. Both the T low to T high and the T to R^e transitions are associated with Bohr effects, but we do not know whether or not these involve the same ionizable groups.

SUMMARY

This study was first undertaken in order to better characterize the previously recognized differences among three variants of HbA with substitutions at the βN102 residue (*32*). The resulting data confirmed that all three variants are inhibited in the ligand-linked transition from the low-affinity T quaternary state to the high-affinity R^e state. This is particularly evident in the presence of IHP, as indicated by their reduced ligand affinities and the absence of cooperativity in ligand binding. The latter is demonstrated not only by the absence of cooperativity in the equilibria of oxygen binding but also by the measurements of CO recombination following full and partial photolysis. The identity of the rates at which CO recombines with unliganded and with triliganded tetramers indicates that ligand affinity does not increase as fractional saturation increases. A similar finding with the αD94G variant supports the proposition that it is the disruption of the H bond between the side chains of the α94 aspartate and β102 asparagine residues that is responsible for the destabilization of the R^e state in the βN102 variants. In examining the properties of the T states of these three variants in the presence of IHP, it was found that replacing the β102 asparagine with either threonine or glutamine increases the ligand affinity of the β subunits within the unliganded T state, $K_1(\beta)$, while having relatively little effect on the α subunits, $K_1(\alpha)$. However, replacement of the asparagine with alanine, effectively deleting the side chain, results in little change in the ligand affinities of either α or β subunits, suggesting that this variant might be a useful model of the T state of HbA. However, the properties of this variant in the absence of IHP showed this not to be the case. In the presence of IHP, the affinities of the α and β subunits of unliganded HbA for the first ligand molecule exhibit only very small Bohr effects, which are greatly increased when IHP is absent. In contrast, the βN102A variant exhibits little K_1 Bohr effect in the presence or absence of IHP, indicating that the presence of the β102 asparagine side chain is a requirement for the ligand-linked conformational transition that results in the K_1 Bohr effect in the absence of organic phosphates.

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