Articles

Preparation and Kinetic Characterization of a Series of βW37 Variants of Human Hemoglobin A: Evidence for High-Affinity T Quaternary Structures†

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ABSTRACT: Four variants of human β globin in which the Trp at position 37 has been replaced with a Tyr, Ala, Gly, or Glu have been expressed in *Escherichia coli*. These globins have been combined with normal human α chains and heme to form tetrameric hemoglobin molecules. A technique for the preparation of α chain dimers, which are cross-linked between their α99 lysine residues, has been developed, and these α dimers were combined with two of the β globins, βW37G and βW37E, to form the corresponding cross-linked variants. The kinetics of CO binding to the deoxygenated derivatives following rapid mixing and of CO rebinding following flash photolysis have been examined as functions of pH in the presence and absence of the organic phosphate inositol hexaphosphate, IHP. The kinetic measurements indicate that replacement of the tryptophan with other residues destabilizes the hemoglobin tetramer, resulting in considerable dissociation of even the deoxygenated hemoglobins into αβ dimers at micromolar protein concentrations. Substitutions at β37 also alter the properties of the deoxygenated hemoglobin tetramer. The alteration of the functional properties of the T states of these variants as well as the tendency of the deoxygenated derivatives to dissociate into αβ dimers increases in the order HbA < βW37Y < βW37A < βW37G < βW37E. Stabilizing the βW37G or βW37E tetramers by addition of IHP or by cross-linking does not restore the normal functional properties of the T state. Measurements of the geminate rebinding of CO establish a kinetic difference between the normal R state tetramer and the αβ dimer consistent with quaternary enhancement, the greater affinity of oxygen for the R state tetramer than for the αβ dimer. Kinetics of geminate rebinding also suggest that quaternary enhancement may be altered by substitutions at the β37 position.

The naturally occurring mutant forms of human hemoglobin have been of tremendous importance in the elucidation of the relationship between structure and function in this complex protein. Recently developed techniques for the expression of globin sequences in microorganisms, combined with site-directed mutagenesis, have made possible the production of hemoglobin variants with virtually any amino acid substitution at any chosen sequence position (Nagai & Thøgersen, 1987; Doyle et al., 1992).

The human hemoglobin, HbA,[1] tetramer is composed of two equal halves, the αβ dimers. The ligand-saturated tetramer dissociates freely into αβ dimers, establishing an equilibrium which includes a concentration-dependent, but generally significant, percentage of the dimers (Mills et al., 1976). These dimers have high ligand affinity and exhibit no cooperativity in their reaction with oxygen or other ligands (Mills & Ackers, 1979). The critical nature of the interface between the two dimers in the hemoglobin tetramer becomes evident when one considers that it is the formation of this interface in the joining of two deoxygenated dimers that transforms the high-affinity deoxy dimers into the low-affinity T state of the intact, tetrameric hemoglobin molecule and permits the cooperative transitions that result in the formation of the high-affinity R state upon ligation.

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[1] Abbreviations: IHP, inositol hexaphosphate; HbA, human adult hemoglobin; HbA_0, purified major component of human adult hemoglobin; Hb, hemoglobin; rHbA, recombined hemoglobin composed of native α chains and β chains with a Val to Met substitution at position 1; bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; PMB, *p*-(hydroxymercuri)benzoate; HbXL99α, human hemoglobin A which has been cross-linked between the two α99 lysine residues with a fumarate linkage; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; DTT, dithiothreitol.

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The tryptophan residue at position β37 is found in the interface between the two $\alpha\beta$ dimers and is an invariant residue among the vertebrate hemoglobins. It appears to serve an important role in maintaining the stability of the tetramer. Three naturally occurring mutations of human hemoglobin at this sequence position have been reported: Hb Rothschild (βW37R) (Gacon et al., 1977), Hb Hirose (βW37S) (Yamaoka, 1971; Sharma et al., 1980), and Hb Howick (βW37G) (Owen et al., 1993). Although Hb Rothschild is reported to exhibit a reduced oxygen affinity, the oxygen affinities of the other two mutants are greater than that of HbA. In addition to these naturally occurring mutants, both the βW37F and βW37T variants have been produced by expression in microorganisms (Ishimori et al., 1992; Vallone et al., 1996). Because of the apparent variety of effects caused by modifications at this site, it was decided to prepare a number of different β37 variants and to carry out a concerted examination of their properties in order to examine the relationships between the structure of this amino acid residue and the functional properties of the hemoglobin molecule.

In this paper we report the preparation of four β chain variants, βW37Y, βW37A, βW37G, and βW37E, and the development of a procedure for the preparation of cross-linked hemoglobins which is applicable to variants of the β chain. The βW37Y variant is as conservative a substitution as can be made with a naturally occurring amino acid. βW37A deletes most of the side chain but retains the tertiary nature of the α carbon while βW37G deletes the side chain entirely. The βW37E variant is a radical substitution of a large hydrophobic residue by one with a negative charge. We report the results of measurements of the kinetics of CO combination with the deoxygenated hemoglobins following rapid mixing and CO recombination following complete photodissociation on the millisecond time scale. Rates and yields of geminate rebinding of carbon monoxide were measured by photolysis on the nanosecond time scale. Three accompanying papers report the following: the high-resolution structures of the deoxygenated derivatives of all four of these β37 variants (Kavanaugh et al., 1998); measurements of equilibria of oxygen binding and tetramer dissociation into $\alpha\beta$ dimers under a variety of experimental conditions (Kiger et al., 1998); and the resonance Raman spectroscopy of the heme regions of this set of hemoglobin variants (Peterson & Friedman, 1998).

EXPERIMENTAL PROCEDURES

Native human HbA was obtained from hemolysates of freshly drawn blood as described by Doyle et al. (1992). This material is kinetically indistinguishable from purified HbA_o (Steinmeier & Parkhurst, 1975) and was used for kinetic comparison of mutant and normal human hemoglobin. Isolated α and β chains of human hemoglobin were prepared by a modification of the method of Bucci and Fronticelli (1965) as described by Hernan et al. (1992). All β globins from *Escherichia coli* contain the additional βV1M substitution. It has been been shown by Doyle et al. (1992) that HbA and the βV1M variant are indistinguishable in the kinetics of their reactions with CO.

Cross-Linked HbA. The cross-linked derivative HbXL99α was prepared by the reaction of deoxy-HbA with bis(3,5-dibromosalicyl) fumarate as described by Chatterjee et al. (1986) and Snyder et al. (1987). HbXL99α was reduced anaerobically with dithionite and stripped of all ions as previously described (Riggs, 1981). HbXL99α was purified by anion-exchange chromatography using a DE-52 column equilibrated with 0.015 M HCl plus Tris, pH 8.0. Elution of HbXL99α from this column was accomplished isocratically with 0.035 M NaCl in the same buffer. If necessary, further purification of HbXL99α was accomplished by HPLC using a TSK gel DEAE-5PW anion-exchange column as described elsewhere (Fowler et al., 1992). These are the conditions used in all HPLC purifications of cross-linked α chains and cross-linked hemoglobins referred to in this study.

Preparation of Cross-Linked α Dimers. Subunits of HbXL99α were prepared by a modification of the method of Bucci and Fronticelli (1965) for the preparation of α and β chains of native human hemoglobin. Cross-linked α dimers were prepared from the reaction of HbXL99α with *p*-(hydroxymercuri)benzoate (PMB, Sigma). A concentration of HbXL99α between 0.2 and 0.5 mM in heme equivalents, a chloride concentration of 1.0 M, a pH of 5.4, a 15−20 molar ratio of PMB to HbXL99α tetramer, and at least a 16 h incubation at 4 °C under CO were found to be important requirements for optimizing the splitting reaction. The PMB-bound α dimers were separated from unsplit HbXL99α and PMB-bound β chains by DEAE-cellulose chromatography using a linear gradient from 0.015 M HCl plus Tris, pH 8.0, to 0.015 M HCl plus bis-Tris, pH 7.0. The first fraction off this column contained the cross-linked α-PMB dimers which were further purified by preparative HPLC (Fowler et al., 1992). The free sulfhydryl groups of cross-linked α-PMB dimers were regenerated essentially as described by Parkhurst and Parkhurst (1992) for the regeneration of normal α chains. The cross-linked α dimers were shown to be completely regenerated and homogeneous on non-SDS−PAGE and analytical HPLC analysis and fully dimeric on SDS−PAGE.

The Mutant Hemoglobins. Each mutant β globin gene was constructed with the oligonucleotide in vitro mutagenesis system from Amersham and expressed in *E. coli* BL21(DE3), as described by Hernan et al. (1992). Each β37 derivative contained two mutations. The β1 Val was replaced by Met, and the β37 Trp was replaced by Tyr, Ala, Gly, or Glu. The plasmid pETbDesVal was used as the starting material for all of the β37 modifications. The *Xba*I−*Hin*dIII fragment from pETbDesVal, containing the upstream T7 regulatory sequences and the synthetic β globin gene, was transferred to M13mp19 to give M13XHbDesVal. The β37 modifications were made with the following oligonucleotides: βW37Y was constructed with 5′-TGGTCTACCCTTACACCCAGAGGTT; βW37A was constructed with 5′-TGGTCTACCCTGCGACCCAGAGGTT; βW37G was constructed with 5′-TGGTCTACCCTGGAACCCAGAGGTT; and βW37E was constructed with 5′-TGGTCTACCCTGAAACCCAGAGGTT. Each of the modifications of the various β37 mutants were determined by M13 DNA sequencing. Expression of the mutant β globins was accomplished after the *Xba*I−*Hin*dIII fragment from each of the M13 clones carrying the β37 modification was purified, ligated into pET8c digested with *Xba*I and *Hin*dIII, and transformed into *E. coli* BL21(DE3). The transformed strains of *E. coli* BL21(DE3) were grown in large batches, and the mutant β globin was purified from the bacterial paste as described by Hernan et al. (1992).

The β globins were incorporated into tetrameric hemoglobins by mixing with heme (Fluka Biochemika Corp.) and either normal α chains or cross-linked α dimers, as previously described (Hernan et al., 1992). Prior to measurement of the kinetics of ligand binding the CO derivatives were converted to the oxygenated forms as previously described (Riggs, 1981).

Reconstitution of HbXL99α from Cross-Linked α Dimers and β Chains. Cross-linked α dimers were reacted with an excess of native β chains in the presence of CO and DTT as described by Kilmartin et al. (1975) for reassembly of HbA from α and β chains. Excess β chains were removed with a DE-52 column as also described in this reference. Purity of the sample was demonstrated by HPLC and by both SDS− and non-SDS−PAGE.

Inositol hexaphosphate, IHP, was obtained as the sodium salt from Sigma. The pH of the stock solution was adjusted by titration with the acid form of Amberlite IR 120 resin. This permitted acidification without the introduction of additional anions such as chloride.

Mass Spectrometry. Electrospray mass spectrometry was performed on the mutant Hb samples at the University of Illinois Mass Spectrometry Facility. The samples were diluted to a concentration of 10 pmol/μL into a 50:50 v/v acetonitrile: H_2O solution containing 0.1% formic acid for these measurements.

Kinetics of CO Binding. Three types of measurements of CO binding kinetics were carried out, all of which are sensitive to different parameters and have different interpretations. CO binding directly to the deoxygenated hemoglobin molecule was carried out by rapid mixing of a solution of deoxyhemoglobin with a solution of CO. With human HbA one typically observes a relatively slow combination reaction, the rate of which increases as the reaction proceeds. This autocatalytic behavior is one kinetic reflection of cooperative ligand binding to the hemoglobin tetramer. The next two types of measurements take advantage of the fact that the CO ligand can be photodissociated from hemoglobin with a high quantum yield. The first of these two techniques uses a light pulse of sufficient intensity and duration (100 μs or more) to achieve complete photodissociation of the ligand from the hemes and protein matrix. Before photolysis the initial state of the hemoglobin molecule is ligand saturated, and the protein will typically exist as a mixture of tetramers and αβ dimers. At the CO concentrations used in these studies, the second-order rate of CO rebinding is slow enough that the tetrameric molecules convert from the R quaternary state to the deoxygenated T state before CO recombines significantly. However, CO recombination occurs too rapidly for dimers to reassociate to form hemoglobin tetramers. Therefore, in the case of HbA the recombination of CO following complete photolysis is a kinetically heterogeneous process characterized by two kinetic phases, a rapid phase corresponding to CO binding to αβ dimers and a slow phase of CO binding to T state hemoglobin tetramers. Finally, if a short (nanosecond) pulse of light is used for photolysis, the CO molecule is dissociated from the heme group but still resides within the ligand binding pocket of the protein. From this position the ligand can either rebind to the heme via a first-order, geminate process or escape into the external solution from which rebinding is a much slower, second-order reaction.

Kinetics of CO Recombination Following Complete Photodissociation. Measurements were carried out as previously described (Doyle et al., 1992). The reactions are monitored by absorbance change at 420 or 435 nm. Concentrations of CO and hemoglobin (in heme equivalents) were 16−20 and 1.5−2.0 μM, respectively. Dithionite was present at a concentration of approximately 2 mM in order to maintain anaerobic conditions and to keep the heme groups in the ferrous state.

Kinetics of CO Combination with Deoxygenated Hemoglobin. These kinetics were measured with an OLIS (On line Instrument Systems Inc., Bogart, GA) U.S.A. stopped-flow apparatus which is similar to that described by Gibson and Milnes (1964). The procedures were essentially those of Gibson (1959) as described in Doyle et al. (1992). Reactions were followed at 420 and 435 nm using a 1.7 cm path-length cell. Reactant concentrations after mixing were the same as in the complete photodissociation experiments.

Kinetic data from stopped-flow and flash photolysis experiments were obtained under four different experimental conditions. Measurements at 20 °C were carried out at pH 6 and 7 in 100 mM HCl plus bis-Tris buffer and at pH 8 in 100 mM HCl plus Tris buffer. Measurements were also carried out at 21.5 °C and pH 7.4 in a buffer containing 100 mM Tris-HCl, 100 mM NaCl, and 1 mM EDTA as used by Kiger et al. (1997) for measurements of equilibria of oxygen binding. An on-line computer with OLIS instrumentation and software was used for acquisition and manipulation of all kinetic data. Data were fitted to single- or double-exponential functions using successive integration and Levenberg−Marquardt fitting routines as supplied by OLIS software and Levenberg−Marquardt fitting routines as furnished in Sigma Plot. None of the kinetic transients required a sum of more than two exponential functions to be fitted within the errors of the measurement. This does not mean that the transients result from the sum of no more than two kinetic processes but merely that the data are insufficient to define more than three independent variables, i.e., two rate constants and the relative contributions of the two kinetic processes to the total reaction. The larger the difference in the rates of the two processes which fit a transient, the greater the precision with which the three variables can be defined. When a transient is fitted by two processes whose rates differ by a factor of 2.5 or less, the assignment of three variables is associated with considerable uncertainty. The problem is that a sum of two such closely related kinetic functions can be fitted to a single exponential function with only modest residuals. These residuals are well outside experimental error but are insufficient to define with precision both a second reaction rate and the relative contributions of the two processes to the overall reaction. Furthermore, the estimated rate constants are strongly coupled to the estimated values of relative contribution, with the rates decreasing as the percent contribution of the rapid phase increases, making comparison of results difficult. For these reasons, whenever a standard fitting of a data set to a sum of two exponential functions resulted in two rate constants differing by a factor of 2.5 or less, we have fitted these data by arbitrarily setting the amplitudes of the two processes equal and permitting only the two rate constants to vary to obtain the optimal fit. In all cases this procedure

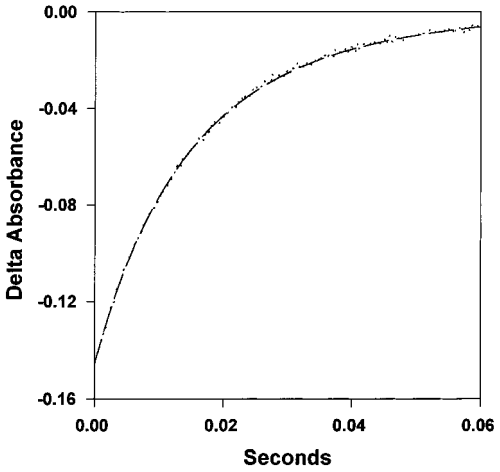

FIGURE 1: Time course of the absorbance change associated with the rebinding of CO to the βW37Y variant following complete photodissociation. The dotted line is the curve obtained by fitting the experimental data to the sum of two exponential functions of equal magnitude. Experimental conditions: 0.1 M HCl and bis-Tris, pH 6, 20 °C.

yields fits which are well within experimental error, as shown in Figure 1. Fits in which a 50:50 contribution from the two kinetic components is forced are noted as such. In some cases there is reason to suspect that kinetic heterogeneity of this magnitude results from the difference in the kinetics of CO binding to the α and to the β subunits within the $\alpha\beta$ dimer, in which case two kinetic processes of equal magnitude would be appropriate. However, it is important to understand that this ratio is not established by the data.

Single exponential functions were used to fit both kinetically homogeneous reactions and reactions exhibiting autocatalysis. In the latter case the fitted transient fails to precisely reproduce the data. Autocatalytic CO combination kinetics following rapid mixing are a normal feature of HbA and many variants of this protein and are a kinetic reflection of cooperative ligand binding. The data presented here are inadequate to determine the minimally four sequential processes and multiple intermediate states which define this reaction. The detailed analysis of kinetic cooperativity has been undertaken by Perrella and co-workers (Perrella et al., 1992). It is not the subject of the present study. Fitting to a single exponential function permits the comparison of the overall rates of these processes, and autocatalysis is used as an indicator of ligand binding associated with a ligand-linked transition in functional properties.

Rigorous error analysis for the fitted rate constants is difficult. Because of the large numbers of data points obtained in each kinetic experiment, the estimated errors of the fits are very small indeed. However, reproducibility between and among experiments indicates a more reasonable estimate of error to be $\pm$10% for both the rate constants and the fractional contributions of the kinetic components of the reactions.

Nanosecond Geminate Recombination of CO. Observed recombination rates and geminate yields were measured by monitoring the 420 nm absorption transient following flash photolysis. The protein samples were exchanged into 100 mM bis-Tris-HCl buffer, pH 7.0 (from Sigma), using a Sephadex G-25 column. Samples were saturated with CO, reduced by the addition of 10 mM dithionite, and sealed in a 0.2, 2, or 10 mm path-length cuvette just prior to the experiments. The cuvette path length varied depending on the protein concentration to maintain an appropriate optical density. Ten nanosecond (fwhm) pulses of the 532 nm harmonic of a Nd:YAG laser (Spectra Physics GCR 150) were used to photodissociate the CO. The rebinding was monitored using a xenon flash lamp (FX-200) aligned 90° relative to the laser pulse and focused through the sample. For the 0.2 mm path-length cuvette a brass block was machined so that it would fit in a typical 10 $\times$ 10 mm cuvette holder. This block held the flat surface of the cuvette at a 45° angle relative to both the laser and the flash lamp. After being passed through the sample, 420 nm light was isolated through an Oriel 1/4 meter monochromator and monitored with a photomultiplier tube (PMT). The PMT signal was fed directly into an oscilloscope (LeCroy 9350, 500 MHz). Traces from 30 to 100 laser shots were averaged to achieve suitable signal-to-noise ratios. The averaged data were fitted using Igor (WaveMetrics) to a single exponential.

Table 1: Theoretical Molecular Weights of Mutant Hemoglobins Compared to Values Measured by Electrospray Mass Spectrometry

variant	calculated	measured
βW37Y	15 878.4	15 876 $\pm$ 2, 15 890 $\pm$ 3[a]
βW37E	15 844.3	15 842 $\pm$ 2
βW37G	15 772.3	15 770 $\pm$ 3
βW37A	15 786.3	15 784 $\pm$ 2

[a] One preparation of this variant gave this value, which implies an extra carbon atom or its equivalent. No differences in functional properties were observed.

RESULTS

Electrospray Mass Spectrometry. The masses of the subunits of each of the mutant Hb samples were determined by electrospray mass spectrometry. In each sample the native α chains had a measured mass of 15126 $\pm$ 2 Da. This corresponds well to the calculated value of 15127.7 Da. Table 1 lists each of the measured and theoretical calculated values of each of the mutant β chains. In each case the measured values agree with the calculated values. These data provide additional confirmation of the mutations and demonstrate that no posttranslational modification of the mutated sequences has occurred.

Kinetics of CO Binding to Cross-Linked α Dimers. The kinetics of CO binding to cross-linked α dimers were compared to those of normal human α chains and are presented in Table 2. The rates of CO binding to these derivatives at pH 7.0, 20 °C, were measured by both flash photolysis and rapid mixing in a stopped-flow apparatus with similar results. The overall rate of CO binding to α chains is faster than that for cross-linked α chains, and the presence of IHP does not affect the rates of either of these high-affinity derivatives. The reduction in the value of the overall rate constant for CO binding observed upon cross-linking α chains parallels the reduction in R state affinity (Vandegriff, 1991) and overall oxygen affinity (Snyder et al., 1987) observed upon cross-linking HbA to form HbXL99α.

Kinetics of CO Binding to Reconstituted HbXL99α. Control experiments were carried out to demonstrate the ability of cross-linked α dimers to assemble with normal human β chains or β globin to form fully functional cross-

Table 2: Summary of Kinetic Properties of α Dimers and Reconstituted HbXL99α

derivative	second-order rate constants (10^6 M^{-1} s^{-1}) without IHP	+0.1 mM IHP
Deoxy-Hb + CO by Rapid Mixing		
α chains	4.5	ND
α dimers	3.2	ND
HbXL99α (std)	0.16 A[a]	0.09 A
r_c-HBXL99α[b]	0.16 A	0.11 A
r_g-HBXL99α[c]	0.18 A	0.10 A
CO Recombination Following Flash Photolysis		
α chains	4.0	4.0[d]
α dimers	3.1	3.1[d]
HbXL99α (std)	0.17 A	0.09 A
r_c-HbXL99α	0.17 A	0.09 A
r_g-HBXL99α	0.18 A	0.10 A

[a] A indicates an autoaccelerating reaction. [b] HbXL99α reconstituted from cross-linked α dimers and normal human β chains. [c] HbXL99α reconstituted from cross-linked α dimers, hemin, and human β globin derived from *E. coli.* [d] [IHP] = 1 mM.

linked hemoglobin. Unsplit, purified HbXL99α was the reference standard. In Table 2 are presented the rate constants for the reactions of CO with standard HbXL99α, r_c-HbXL99α (HbXL99α reassembled from α dimers and normal human β chains), and r_g-HbXL99α (HbXL99α reassembled from α dimers, hemin, and human β globin derived from *E. coli*) in the absence and presence of 0.1 mM IHP. The three cross-linked hemoglobins exhibit similar CO binding kinetics which are autocatalytic with overall rate constants which vary from 0.16×10^6 to 0.18×10^6 M^{-1} s^{-1} in the absence of IHP and from 0.09×10^6 to 0.11×10^6 M^{-1} s^{-1} in the presence of 0.1 mM IHP. Most importantly, no sign of a rapid second kinetic phase is observed following flash photolysis as would be expected for hemoglobins which are locked in their tetrameric form and are unable to dissociate into $\alpha\beta$ dimers.

Kinetics of CO Combination with Deoxygenated Hemoglobin Following Rapid Mixing. Kinetic data for the reaction of CO with native human HbA, the four β37 variants, and the cross-linked derivatives of βW37G and βW37E are reported in Table 3. For HbA the overall rate of CO combination is sensitive to both pH and the addition of IHP.

Modification of the β37 residue alters the kinetics of CO combination, with the magnitude of the alteration increasing in the order βW37Y < βW37A < βW37G < βW37E. The binding of CO to the deoxygenated βW37Y variant is more rapid than to normal human HbA at pH 6 and 7 but remains autocatalytic and sensitive to the addition of 0.1 mM IHP. However, at pH 7.4 and 8 the reaction begins with a very rapid process which accounts for 25% and 32%, respectively, of the total absorbance change. The addition of IHP eliminates this rapid reaction, yielding a single, slow, autoaccelerating reaction which is again somewhat more rapid than that observed with HbA.

The behavior of the βW37A variant is more perturbed. In the absence of IHP, one observes two kinetic processes at all pH values, the more rapid reaction with a rate constant of roughly 5×10^6 M^{-1} s^{-1} and the slower reaction with rate constants which vary from 0.72×10^6 M^{-1} s^{-1} at pH 6 to 1.6×10^6 M^{-1} s^{-1} at pH 8. Addition of IHP at pH 6 and 7 results in autocatalytic reactions which are slower than either of the two processes observed in the absence of IHP. At pH 7.4 and 8 the kinetics are more complex. While the addition of IHP significantly slows the overall process, it does not eliminate the heterogeneity of the reaction.

The βW37G and βW37E variants are similar to βW37A. In the absence of IHP the kinetics of CO combination are consistently heterogeneous and rapid, and upon addition of IHP at pH 6 and 7 the biphasic reaction disappears and is replaced by a single, slower process. However, the slower rates obtained in the absence of IHP and the rates observed in the presence of IHP increase for the most part in the sequence βW37A < βW37G < βW37E. In addition, in the presence of IHP, CO combination with βW37G and βW37E becomes kinetically homogeneous at pH 6 and 7 but does not appear to be autocatalytic.

The reactions of cross-linked βW37G and βW37E with CO at pH 7 in the absence of IHP are slightly autocatalytic with no sign of a second kinetic phase. Addition of 0.1 mM IHP slows both reactions to exactly the same rate observed for the un-cross-linked hemoglobins in the presence of IHP at pH 7.

Dependence of CO Combination Kinetics on IHP Concentration. IHP exhibits very different affinities for the different structural states of hemoglobin. To assess which structural states of hemoglobin might be involved in the observed effects of IHP on the properties of the βW37 variants, the dependence of the kinetics of CO combination with deoxygenated βW37A, βW37G, and βW37E on IHP concentration was examined at pH 7. The time courses for the binding of CO to the βW37A and βW37G variants of human HbA in the presence of different concentrations of IHP are shown in panels A and B of Figure 2. The concentration of hemoglobin in these studies was 2 μM in heme equivalents or 0.5 μM in Hb tetramers. Therefore, a total IHP concentration of 0.5 μM is the stoichiometric amount required to saturate all IHP binding sites. The full effect of IHP on this reaction is obtained for both of these variants at a total IHP concentration of 1 μM, i.e., a free IHP concentration of 0.5 μM. A similar set of data for the βW37E variant is shown in Figure 2C. This variant exhibits significantly less sensitivity to IHP with the maximal IHP effect occurring at a total concentration of 4 μM (3.5 μM free IHP).

Second-Order Kinetics of CO Recombination Following Complete Photodissociation. The results of the measurement of CO recombination at pH 7 in the presence and absence of IHP are presented in Table 4. The data for native HbA exhibit two kinetic processes and can be fitted by a sum of two exponential functions (see Experimental Procedures). The slower kinetic process is the reaction of CO with the deoxygenated T state tetramer. Its rate constant is similar to that determined by stopped-flow measurements and exhibits the same sensitivity to pH and IHP (Doyle et al., 1992). The rapid rate results from the recombination of CO with $\alpha\beta$ dimers (Edelstein, 1970). At the hemoglobin concentration used for these measurements, the rapid process represents 61% of the total absorbance change, but this is also dependent on both the presence and absence of IHP and on the pH (data not shown). Cross-linking HbA eliminates the dissociation of the ligand-saturated molecule into $\alpha\beta$ dimers.

Table 3: Kinetics of CO Combination Following Rapid Mixing

		second-order rate constants (10^6 M^{-1} s^{-1}) [contribution to total absorbance change (%)]								
pH	IHP	HbA	βW37Y		βW37A		βW37G		βW37E	
			Un-Cross-Linked Hemoglobin Derivatives							
6	−	0.15 A[a]	0.21 A		5.6 [17]	0.72 [83]	3.0 [65]	0.79 [35]	3.6 [70]	1.2 [30]
6	+	0.09 A	0.17 A		0.5 A		0.9		0.9	
7	−	0.15 A	0.24 A		5.4 [26]	1.1 [74]	4.6 [32]	1.5 [68]	5.0[b]	2.1[b]
7	+	0.09 A	0.18 A		0.75 A		1.0		1.3	
7.4	−	0.2 A	7.8 [25]	0.3 [75]	4.5 [40]	1.3 [60]	4.3[b]	1.9[b]	5.7[b]	2.4[b]
7.4	+	0.12 A	0.2 A		1.6[b]	0.9[b]	1.6 [95]		3.5[b]	1.72[b]
8	−	0.25	5.1 [32]	0.31 [68]	4.7 [66]	1.6 [34]	5.1[b]	2.0[b]	6.0[b]	2.5[b]
8	+	0.13 A	0.18 A		1.7[b]	0.74[b]	2.6[b]	1.2[b]	5.3[b]	2.2[b]
			Cross-Linked Hemoglobin Derivatives							
7	−	0.16 A					1.3 A		1.5 A	
7	+	0.09 A					1.0 A		1.3 A	

[a] A = autocatalytic reaction. [b] Percent contribution to total absorbance change was fixed at 50% for both kinetic phases.

In the absence of IHP the kinetics of the recombination of CO with the βW37 variants are remarkably uniform. Recombination data for pH values other than 7 are not presented because in the absence of IHP no significant pH dependence was observed in the kinetics for any of the variants. In all cases the data require a sum of two exponential functions to achieve a precise fit. Since the two rapid rate constants obtained by the standard fitting procedure differ by less than a factor of 2.5, the ratios of their contributions to the overall reactions have been fixed at unity (see Experimental Procedures). The addition of IHP slows the recombination process to different extents depending upon the variant being examined and the pH (data not shown). As with HbA, cross-linking the βW37G and βW37E variants eliminates the heterogeneity of the CO recombination reactions. In the absence of IHP slightly autocatalytic reactions are observed, and with both variants the rate constant is smaller than either of the rate constants obtained with the un-cross-linked derivative. In each case addition of IHP decreases the rate constant to the same value observed for CO combination with the un-cross-linked variant following rapid mixing in the presence of IHP. Cross-linking βW37G and βW37E permits the examination of the kinetics of CO rebinding to mostly triliganded tetramers of the variants without contribution from αβ dimers. This is done by reducing the intensity of the photolyzing flash to give between 5% and 10% photolysis. At pH 7 in the absence of IHP the rate constants for this recombination process were found to be 2.4×10^6 M^{-1} s^{-1} for the cross-linked βW37G variant and 2.2×10^6 M^{-1} s^{-1} for cross-linked βW37E. The fact that CO binds more rapidly to partially liganded cross-linked tetramers than to fully unliganded cross-linked tetramers is another demonstration of kinetic cooperativity in the binding of CO to these two hemoglobin variants. It should be noted, however, that similar measurements on HbA result in rate constants following full and partial flash photolysis which differ by some 30-fold or more rather than the 2-fold or less seen here.

Concentration Dependence of Geminate Recombination of CO with β37 Mutants. These measurements were taken to probe the effects of the mutations on the heme environment, specifically the heme environment of the R state. The protein is saturated with CO before photodissociation, and the geminate phase is complete before the R to T transition (Hofrichter et al., 1983). The observed geminate rates and yields were obtained from single exponential fits of the geminate transients. Representative transients are shown in Figure 3. The rates were analyzed by using a three-state model as previously described (Alpert, 1979; Bandyopadhyay, 1992; Campbell, 1985). The geminate rebinding rate and escape rate were calculated from the observed rate and geminate yield on the basis of the model:

$$\mathrm{Hb{-}CO} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} \mathrm{Hb\ CO} \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} \mathrm{Hb + CO}$$

where $K_{obs} = k_{21} + k_{23}$ and yield $= k_{21}/(k_{21} + k_{23})$.

Table 5 summarizes the results. The geminate yield of each one of these variants is lower than that of a reference hemoglobin, rHbA (Doyle et al., 1992). The perturbations are more significant for the substitutions of Ala, Glu, and Gly than for Tyr. The results in Table 5 were obtained at a heme concentration of 250 μM. At this concentration about 90% of the subunits of the reference hemoglobin are in tetramers.

To determine if the changes in geminate recombination kinetics observed in the mutants were related to the formation of dimers, we investigated the concentration dependence of geminate recombination in the reference hemoglobin. A change in concentration near the midpoint of the dimer/tetramer equilibrium will effect a change in the fraction of subunits in dimers. There was a clear, direct correlation between geminate yield and fraction of subunits in the dimer (Figure 4). This correlation can be attributed to a difference in the heme environments between dimers and tetramers. Furthermore, the geminate yield of pure dimers can be determined by extrapolation from the plot in Figure 4 to be 17.8%. The βW37A, βW37G, and βW37E also have geminate yields of about 17%, suggesting that the heme environments of these mutants are similar to that of hemoglobin dimers.

Measurements of geminate recombination yields as a function of heme concentration for each of the mutants are presented in Figure 5. The geminate yields of the βW37A, βW37G, and βW37E mutants remain at the dimer value over the entire concentration range. The geminate yield of the βW37Y mutant drops to the same value at concentrations below 30 μM [heme]. At this concentration about 70% of rHbA would be in the tetrameric form.

It is noteworthy that the observed rates of all the mutants were equal to the observed rate of rHbA. These rates

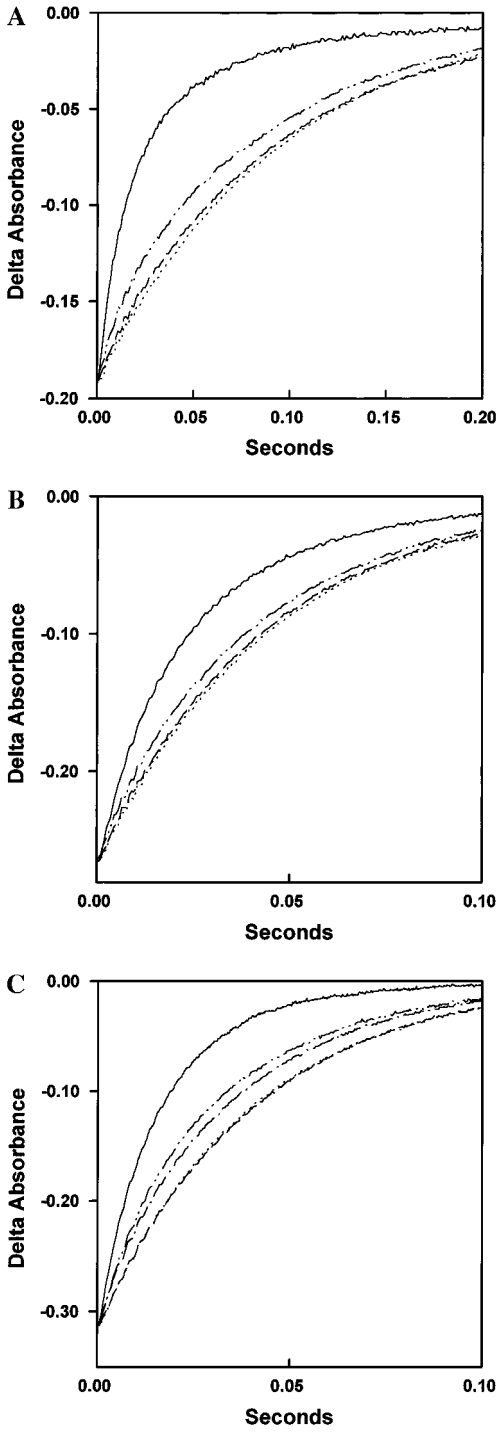

FIGURE 2: Effects of different concentrations of IHP on the time courses of the binding of CO to the βW37A, βW37G, and βW37E variants of human HbA. The Hb concentration in all cases was 2 μM in heme equivalents. (A) βW37A: (—) no IHP, (—••—) 0.5 μM IHP, (---) 1.0 μM IHP, and (•••) 100 μM IHP. (B) βW37G: (—) no IHP, (—••—) 0.5 μM IHP, (---) 1.0 μM IHP, and (•••) 100 μM IHP. (C) βW37E: (—) no IHP, (—••—) 0.5 μM IHP, (—•—) 1.0 μM IHP, (---) 4.0 μM IHP, and (•••) 100 μM IHP. (The last two kinetic transients superimpose so precisely that it is difficult to distinguish them.)

remained constant at the values listed in Table 5 over the entire concentration range for both the reference hemoglobin and the mutants. However, fitting to the three-state model demonstrates variation of the geminate rebinding rate, k_{21}, and the escape rate, k_{23}. Furthermore, the rates, k_{21} and k_{23}, change in a compensatory manner, a decrease in k_{21} being accompanied by an increase in k_{23}.

DISCUSSION

The magnitudes of the perturbations in functional properties resulting from the replacement of the βW37 residue increase in the order βW37Y, βW37A, βW37G, and βW37E. The smallest effect results from the relatively conservative replacement of Trp by Tyr. Replacing the Trp side chain with a methyl group is next while complete removal of the side chain causes an even larger effect. Finally, the radical substitution of the bulky, hydrophobic Trp by a negatively charged Glu results in the greatest disruption.

Kinetics of Second-Order Binding of CO. The kinetic results reported here can be accommodated for the most part within a model which assigns two distinct effects to alterations in the β37 residue of human HbA. One of these is the destabilization of the hemoglobin tetramer, which increases the dissociation of both the deoxygenated and liganded molecule into $\alpha\beta$ dimers. Such destabilization has already been reported for Hb Hirose (βW37S) by Sasaki et al. (1978) and for Hb Howick (βW37G) by Brittain (1994). The other effect is the alteration of the kinetics of the reaction of CO with the deoxygenated T states of these hemoglobins.

The kinetics of CO recombination with the βW37 variants in the absence of IHP are insensitive to pH, and the rate constants for the two components of these kinetic transients are similar to those reported for the α and β subunits within the $\alpha\beta$ dimer of HbA by Edelstein et al. (1970). These results indicate that in the absence of IHP the ligand-saturated derivatives of all of these variants are completely dissociated into $\alpha\beta$ dimers. This conclusion is reinforced by the values of the free energies of dimer assembly of these variants at pH 7.4 measured by Kiger et al. (1998), which indicate the presence of very small amounts of Hb tetramer at the Hb concentrations used in these kinetic experiments. The average values of the rate constants for the fast and for the slow reactions of the $\alpha\beta$ dimers of the four variants at pH 7 (Table 4) are 6.2×10^6 and 2.5×10^6 M^{-1} s^{-1}, respectively. The average of these two rate constants is 4.3×10^6 M^{-1} s^{-1}. The similarity of the kinetic properties of the $\alpha\beta$ dimers of HbA and the dimers of the βW37 variants is not unexpected given the fact that the β37 residue lies at the surface of the dimer.

We conclude that the kinetics of CO binding to the deoxygenated tetramers are seen whenever the reaction is autocatalytic or kinetically homogeneous. Such kinetics are uniformly observed for the βW37 variants following rapid mixing at pH 6 and 7 in the presence of IHP as well as in the reaction of CO with the cross-linked derivatives of βW37G and βW37E. Although the rate constants for the binding of CO with these tetramers are very large when compared to the rate constants for CO binding to deoxygenated HbA, they are uniformly smaller than either of the two rate constants associated with $\alpha\beta$ dimers. The cross-linked derivatives are constrained in their tetrameric state as indicated by the identity of the kinetics of CO combination following rapid mixing and CO recombination following flash photolysis. In the presence of IHP at pH 7 the rate constants for the combination of CO with βW37G and βW37E are the same for the un-cross-linked and cross-linked

Table 4: Kinetics of CO Recombination Following Flash Photolysis

pH	IHP	second-order rate constants (10^6 M^{-1} s^{-1}) [contribution to total absorbance change (%)]									
		HbA		βW37Y		βW37A		βW37G		βW37E	
		Un-Cross-Linked Hemoglobin Derivatives									
7	−	5.1 [61]	0.15 [39]	7.4[b]	2.9[b]	6.0[b]	2.3[b]	5.8[b]	2.4[b]	5.6[b]	2.4[b]
7	+	4.6 [31]	0.08 [69]	4.0 [80]	0.16 [20]	4.5 [41]	1.1 [59]	4.4 [20]	1.1 [80]	5.3 [47]	1.7 [53]
		Cross-Linked Hemoglobin Derivatives									
7	−	0.17 A[a]						1.3 A		1.5 A	
7	+	0.09 A						1.0 A		1.3 A	

[a] A = autocatalytic reaction. [b] The data were fitted by fixing the contributions of the two kinetic phases at *50%*.

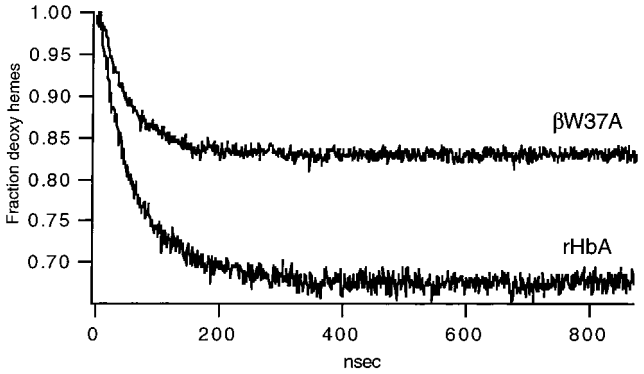

FIGURE 3: Experimental traces of the time courses of the geminate recombination of CO with the reference hemoglobin (rHbA) and βW37A at concentrations of 250 μM and 5 μM (heme), respectively, in 0.1 M bis-Tris, pH 7.0, 0.1 M Cl^-, and 0.01 M dithionite.

Table 5: Parameters for Geminate Rebinding of CO to Four β37 Variants and for a Reference Hb, $\alpha_2(r\beta)_2$, Composed of Normal α Chains and Recombinant β Chains

variant	K_{obs} (10^7 s^{-1})	yield (%)	K_{21} (10^6 s^{-1})	K_{23} (10^7 s^{-1})
$\alpha_2(r\beta)_2$	1.6 ± 0.1	31.1 ± 0.5	4.9 ± 0.1	1.1 ± 0.1
$\alpha r\beta$ (dimer)[a]	1.6	17.8	2.8	1.3
βW37Y	1.5 ± 0.1	22.6 ± 0.8	3.4 ± 0.1	1.2 ± 0.1
βW37A	1.7 ± 0.1	17.0 ± 0.7	2.9 ± 0.2	1.4 ± 0.1
βW37G	1.7 ± 0.1	17.1 ± 0.5	2.9 ± 0.2	1.4 ± 0.1
βW37E	1.6 ± 0.1	17.0 ± 0.3	2.7 ± 0.2	1.3 ± 0.1

[a] The geminate rebinding parameters for the $\alpha r\beta$ dimer were determined by extrapolating the data plotted in Figure 4 to pure dimer.

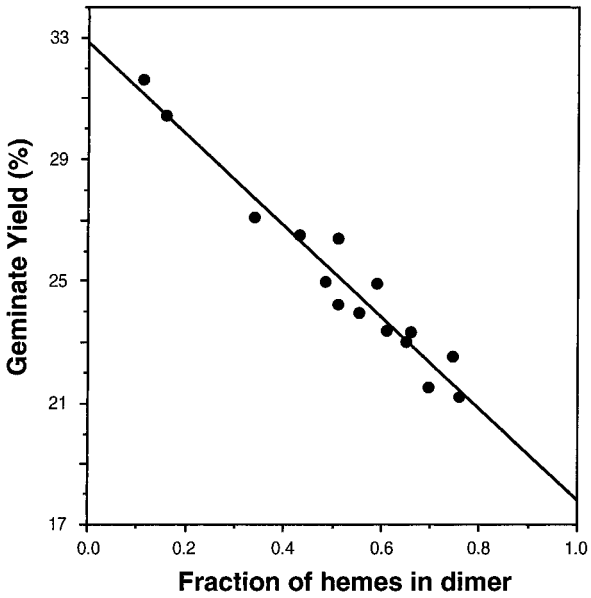

FIGURE 4: Geminate yield of the reference hemoglobin, rHbA, composed of normal human α chains and recombinant β chains with a Val → Met substitution of the N-terminal residue, as a function of dimer concentration. K_{eg} = [tetramer]/[dimer]^2 = (1 − Fd)/(Fd^2·[heme]), where Fd, fraction of dimer, equals fraction of hemes in $\alpha\beta$ dimers. $K_{eg} = 2.01 \times 10^5$ s^{-1} from Doyle et al. (1992). Heme concentration was varied from 1 to 250 μM at 25 °C in 0.1 M bis-Tris, pH 7.0, 0.1 M Cl^-, and 0.01 M dithionite. The line is a least-squares fit to the data.

derivatives of these variants. This demonstrates that IHP addition results in complete assembly of the un-cross-linked variants into tetramers and that cross-linking does not significantly alter the functional properties of the tetramers.

Under many conditions the kinetics of CO binding to the βW37 variants are composed of the binding of CO to the deoxygenated tetramer and to varying amounts of $\alpha\beta$ dimer. Therefore, the time course of the reaction results from three, not two, kinetic processes with different rate constants: the binding of CO to the α subunits and β subunits of the $\alpha\beta$ dimer and to the hemoglobin tetramer. When the tetramer reacts much more slowly than either subunit of the $\alpha\beta$ dimer, then fitting to the sum of two exponentials yields a rapid rate constant which is roughly the average of the properties of the subunits of the $\alpha\beta$ dimer and the slow rate constant of the tetramer. An example is the recombination of CO with the βW37Y variant at pH 7 in the presence of IHP (Table 4). A value of 4×10^6 M^{-1} s^{-1} is a reasonable average of the rate constants for the α and β chains within the $\alpha\beta$ dimer, and 0.16×10^6 M^{-1} s^{-1} represents the rate constant for the tetramer. It has already been shown (Doyle et al., 1992) that under such conditions the kinetic estimate of the free energy of dimer assembly into tetramer closely approximates the values obtained by equilibrium measurements. As the rate constant of the tetramer approaches that of the slower of the two subunits of the $\alpha\beta$ dimer, the kinetic separation of dimers and tetramers becomes less precise. As a result the slower of the two fitted rate constants may contain contributions from both the tetramers and the $\alpha\beta$ dimers, making the determination of the ratio of dimer and tetramer concentrations difficult. An example of this situation is the recombination of CO with the βW37E variant at pH 7 in the presence of IHP (Table 4). The smaller of the two rate constants is 1.7×10^6 M^{-1} s^{-1}, relatively close to the smaller of the rate constants for the $\alpha\beta$ dimer, 2.5×10^6 M^{-1} s^{-1}. The general slowing of the kinetics of CO recombination with βW37E upon IHP addition is consistent with a partial assembly of the $\alpha\beta$ dimers into hemoglobin tetramers, but the extent of assembly cannot be determined with reasonable precision.

The rapid mixing experiments indicate that in the absence of IHP the destabilization of the deoxygenated Hb tetramer increases in the sequence βW37Y < βW37A < βW37G <

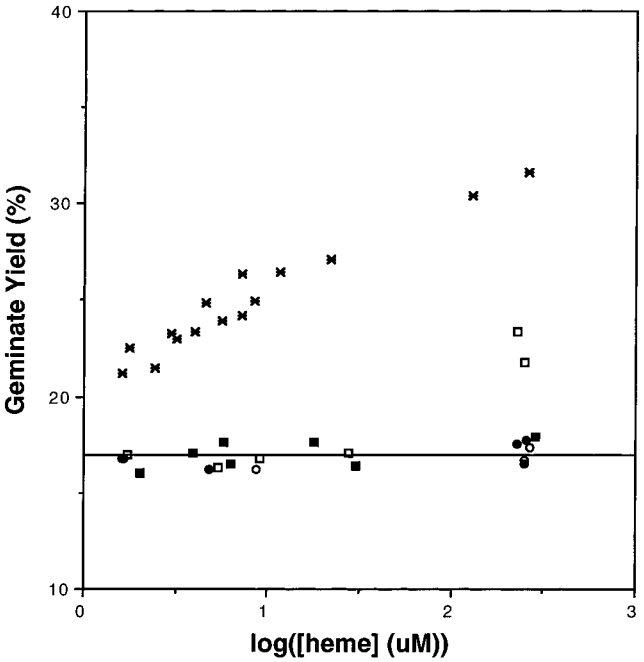

FIGURE 5: Geminate yield of reference hemoglobin (rHbA) and mutant hemoglobins as a function of log μM hemoglobin concentration. Hemoglobin concentrations were varied from 1 to 250 μM at 25 °C in 0.1 M bis-Tris, pH 7.0, 0.1 M Cl^-, and 0.01 M dithionite. The data for the reference hemoglobin, rHbA (×), is the same as that in Figure 4. The data for the four β37 variants are indicated as follows: βW37E (○), βW37G (●), βW37Y (□), and βW37A (■). The line represents the average geminate yield over the entire concentration range of the βW37E, βW37G, and βW37A mutants, a geminate yield of 17.0 ± 0.6%.

βW37E. Destabilization also appears to generally increase with increasing pH. In the absence of IHP, only the βW37Y variant at pH 6 and 7 appears to be fully tetrameric as indicated by autocatalytic CO binding kinetics. All of the other variants exhibit biphasic CO combination kinetics at all pH values as does the βW37Y variant at pH 7.4 and 8. For all of these biphasic reactions at least one of the kinetic processes has a rate constant consistent with CO binding to $\alpha\beta$ dimers. For the βW37E variant the kinetics at all pH values above 6 are consistent with complete dissociation into $\alpha\beta$ dimers. The dimer−tetramer assembly free energy of the deoxygenated βW37E variant at pH 7.4 has been determined from the concentration dependence of average molecular size as measured directly by large-zone analytical gel chromatography (Kiger et al., 1998). The result confirms that this variant is essentially completely dissociated into dimers at the hemoglobin concentration used for the kinetic measurements.

Addition of IHP to the deoxygenated derivatives of the βW37A, βW37G, and βW37E variants at pH 6 and 7 results in complete assembly into tetramers. This is also true for the βW37Y variant at pH 7.4 and 8. This shift in the dimer−tetramer equilibrium to essentially entirely tetramers is a result of the strong, preferential binding of IHP to the deoxygenated tetramer. The data in Figure 2 demonstrate that this assembly is complete at an IHP concentration of 1 μM for βW37A and βW37G and 4 μM for βW37E. The only known structure of hemoglobin to which IHP binds with sufficient energy to achieve saturation at these concentrations is the T quaternary state. The binding of IHP to the deoxygenated T state structure of human HbA can be discerned by the very large effect of this binding on the kinetics of CO combination with the T state tetramer (see Table 3). We find that at pH 7 at a HbA concentration of 2 μM in heme this transition in the kinetic properties of the deoxygenated T state of HbA results from virtually stoichiometric binding of IHP and is certainly complete at a total IHP concentration of 1 μM (data not shown). In contrast, the binding of IHP to the R state tetramer can be observed by examining the concentration dependence of the effect of IHP of the dimer−tetramer equilibrium as estimated by CO recombination following flash photolysis. This IHP-induced increase in the relative concentration of the HbA tetramer is maximal at an IHP concentration of 100 μM and is then reversed at 1 mM IHP as a result of IHP binding to the $\alpha\beta$ dimer (Doyle et al., 1992). The observation that in pH 7 inorganic phosphate buffer the deoxygenated derivatives of all of the βW37 variants crystallize in the T quaternary state (Kavanaugh et al., 1998) supports the conclusion that the deoxygenated tetramers of these variants exist in this quaternary structure in solution.

The data support a large variation in the oxygen affinities of the T states of these hemoglobin variants. The rate constants for CO combination with deoxygenated hemoglobins at pH 6 and 7 in the presence of IHP vary from 0.17×10^6 to 1.3×10^6 M^{-1} s^{-1}. The rate of CO combination is highly coupled to the oxygen affinity of the deoxygenated T state. It has already been observed that the 10-fold increase in the oxygen affinity of T state crystals of Hb Rothschild (βW37R) (Rivetti et al., 1993) in the absence of chloride ion is associated with a 4-fold increase in the rate of CO binding to the deoxygenated T state in solution in the presence of IHP (Kelly et al., 1994). The removal of the C-terminal arginine residues of the α chains of HbA results in a 14-fold increase in the O_2 affinity of the crystalline T state (Kavanaugh et al., 1995) and is associated with a roughly 4-fold increase in the rate of CO binding in solution in the presence of IHP (Bonaventura et al., 1974). These numbers suggest that the change in the activation free energy of the CO combination reaction equals roughly 55% of the change in the free energy of oxygen binding to the crystalline T state. If this is so, then the increase in the rate of CO binding to the βW37A and βW37G variants is consistent with an increase in the oxygen affinity of the T state of nearly 2 orders of magnitude, and the ligand affinities of these T states resemble that of the normal R state tetramer more closely than that of the deoxygenated T state of HbA. Certainly the kinetic properties exhibit this relationship. The functional differences among the deoxygenated T states of the β37 variants are paralleled by the magnitudes of small but significant changes in the crystallographic structures of the deoxygenated T states of these variants as reported in an accompanying paper (Kavanaugh et al., 1998) and by the magnitudes of the shifts in the stretching frequencies of the bond between the iron atoms and the proximal histidine residues of the deoxygenated derivatives of these mutants as measured by resonance Raman spectroscopy (Peterson & Friedman, 1998).

Geminate Recombination. The clear concentration dependence of geminate yield establishes beyond doubt that the heme environments of the ligand-saturated, R state tetramer of human HbA are different from those of the $\alpha\beta$ dimers (Figures 4 and 5). There has been a tendency to

equate all "high-affinity" forms of hemoglobin, i.e., the isolated subunits, $\alpha\beta$ dimers, and the R state tetramer. However, some time ago Ackers and co-workers [see Ackers and Johnson (1990)] presented data which indicated that the binding of the fourth ligand to the hemoglobin tetramer was associated with a greater affinity than ligand binding to the $\alpha\beta$ dimer. They called this phenomenon quaternary enhancement. Our results suggest that a kinetic equivalent of the quaternary enhancement effect can be described by a change in the geminate yield, most likely via a change in the geminate rebinding rate, k_{21}.

The kinetic equivalent of quaternary enhancement is readily apparent in the behavior of the reference hemoglobin, rHbA. The degree of quaternary enhancement is reflected in the increase in geminate yield from a dimer value of 17% to a tetramer value of 33%. The increase in geminate yield upon tetramer formation means that a smaller fraction of Fe−CO bond cleavage events result in CO escape into solution. This results in a decreased overall rate of dissociation of the ligand from the heme into the bulk solution and, consequently, an increased ligand affinity in hemoglobin tetramers. In contrast, this is not observed for the βW37A, βW37G, and βW37E mutants. This apparent loss of the quaternary enhancement effect can be explained by either of two scenarios: (1) the complete dissociation of the mutant tetramers into dimers or (2) the elimination of interactions in the tetramer required for quaternary enhancement.

The simplest explanation for the reduction in geminate yield observed in each of the mutants is the destabilization of the fully liganded tetramer into dimers (scenario 1). At all concentrations, βW37A, βW37G, and βW37E mutants displayed geminate recombination rates and yields equal to those of rHbA dimers, and the βW37Y mutant falls to the same value at low concentrations (Figure 5). The similarity in the values strongly suggests that the kinetic parameters observed are the result of dimer formation in each of these mutants. In addition, the data are consistent with previous geminate recombination studies on the naturally occurring Hb Rothschild (βW37R) (Campbell et al., 1985). They observed a reduced geminate yield in Hb Rothschild and attributed it to the dissociation of the mutant hemoglobin into dimers. However, this explanation may be an oversimplification of the data. For this explanation to adequately describe the data, the destabilization of the tetramers of βW37A, βW37G, and βW37E must be complete.

There is no direct evidence for the complete dissociation of βW37A, βW37G, and βW37E into dimers throughout the concentration range examined in the geminate recombination experiments. According to the estimates of the dimer/tetramer equilibrium for the fully liganded mutant hemoglobins, in the accompanying paper (Kiger et al., 1998), approximately 30−40% of the subunits are in the tetrameric form for each mutant at the maximum concentration examined (250 μM [heme]). Thus, the possibility exists that for certain mutants both the dimeric and tetrameric states have equal geminate yields.

An equivalence of geminate yields between dimers and tetramers can be described in terms of a lack of quaternary enhancement (scenario 2). The quaternary enhancement effect is defined as the free energy difference between the binding of the last ligand to a tetramer and the binding of a ligand to a dimer. A negative value implies a greater affinity to the tetramer. This effect is a factor in the cooperative ligand binding observed in HbA. Geminate rebinding to the hemoglobin tetramer should always reflect the property of the quaternary R state and be sensitive to quaternary enhancement since the geminate process occurs far more rapidly than the R to T transition (Hofrichter, 1983). Kiger et al. (1998) have shown that the Ala, Gly, and Glu mutants are not cooperative and do not display quaternary enhancement based on a thermodynamic study of the stepwise cooperative free energies. Thus both the kinetic and thermodynamic data are consistent with an absence of quaternary enhancement in these three mutants.

The concentration dependence of the geminate yield for βW37Y does suggest the existence of a quaternary enhancement effect in this variant. Indeed this variant cooperatively binds oxygen and displays a quaternary enhancement as demonstrated by the stepwise ligand binding free energies (Kiger et al., 1998). Furthermore, one can utilize the dimer to tetramer equilibrium estimated from the oxygen equilibrium data to extrapolate a value for the geminate yield of βW37Y tetramers. This value is 33.8%, which is very similar to that of native tetramers, indicating that although the βW37Y mutant tetramer is significantly destabilized, in terms of tetramer dissociation, the quaternary enhancement effect within the mutant tetramer is of approximately the same magnitude as HbA tetramers. This is consistent with the thermodynamic study.

In summary, an understanding of the structural origins of the difference between the ligand affinities of the deoxygenated T state and the R state of hemoglobin remains crucial if we are to truly understand the mechanisms of cooperative ligand binding to this protein. The results reported here demonstrate that deoxygenated T states can have functional properties which approach those of the hemoglobin R state, indicating that simply comparing R and T state structures is unlikely to yield the understanding we seek. T states with differing functional properties offer a valuable model system with which to explore the plasticity which T states can display and the structural elements of the T state which control or modulate function. The observation of a quaternary enhancement effect within the process of the geminate rebinding of CO suggests that this experimental tool may be valuable in developing our understanding of the nature of this component of cooperativity in hemoglobin.

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