Significance of β116 His (G18) at $\alpha 1\beta 1$ Contact Sites for $\alpha\beta$ Assembly and Autoxidation of Hemoglobin†

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ABSTRACT: The role of heterotetramer interaction sites in assembly and autoxidation of hemoglobin is not clear. The importance of β^{116His} (G-18) and γ^{116Ile} at one of the $\alpha 1\beta 1$ or $\alpha 1\gamma 1$ interaction sites for homodimer formation and assembly in vitro of β and γ chains, respectively, with α chains to form human Hb A and Hb F was assessed using recombinant $\beta^{116His\rightarrow Asp}$, $\beta^{116His\rightarrow Ile}$, and $\beta^{112Cys\rightarrow Thr,116His\rightarrow Ile}$ chains. Even though β chains (e.g., 116 His) are in monomer/tetramer equilibrium, β^{116Asp} chains showed only monomer formation. In contrast, β^{116Ile} and $\beta^{112Thr,116Ile}$ chains showed homodimer and homotetramer formation like γ-globin chains which contain 116 Ile. Assembly rates in vitro of β^{116Ile} or $\beta^{112Thr,116Ile}$ chains with α chains were 340-fold slower, while β^{116Asp} chains promoted assembly compared to normal β-globin chains. These results indicate that amino acid hydrophobicity at the G-18 position in non-α chains plays a key role in homotetramer, dimer, and monomer formation, which in turn plays a critical role in assembly with α chains to form Hb A and Hb F. These results also suggest that stable dimer formation of γ-globin chains must not occur in vivo, since this would inhibit association with α chains to form Hb F. The role of β^{116His} (G-18) in heterotetramer-induced stabilization of the bond with oxygen in hemoglobin was also assessed by evaluating autoxidation rates using recombinant Hb tetramers containing these variant globin chains. Autoxidation rates of $\alpha_2\beta_2{}^{116Asp}$ and $\alpha_2\beta_2{}^{116Ile}$ tetramers showed biphasic kinetics with the faster rate due to α chain oxidation and the slower to the β chain variants whose rates were 1.5-fold faster than that of normal β-globin chains. In addition, NMR spectra of the heme area of these two hemoglobin variant tetramers showed similar resonance peaks, which are different from those of Hb A. Oxygen-binding properties of $\alpha_2\beta_2{}^{116His\rightarrow Asp}$ and $\alpha_2\beta_2{}^{116His\rightarrow Ile}$, however, showed slight alteration compared to Hb A. These results suggest that the β116 amino acid (G18) plays a critical role in not only stabilizing $\alpha 1\beta 1$ interactions but also in inhibiting hemoglobin oxidation. However, stabilization of the bonds between oxygen and heme may not be dependent on stabilization of $\alpha 1\beta 1$ interactions. Tertiary structural changes may lead to changes in the heme region in β chains after assembly with α chains, which could influence stability of dioxygen binding of β chains.

The atomic-level structure of intersubunit contact sites in hemoglobin (Hb) has been clarified by X-ray crystallographic analysis (*1*). The packing of chains in Hb results in interlocking by contacts involving side chains between unlike α and β subunits. The $\alpha\beta$ contacts are of two types. The $\alpha 1\beta 1$ or $\alpha 2\beta 2$ contact sites involving B, G, and H helices and the GH helix are packed and remain unchanged when hemoglobin molecules switch from the deoxy to oxy configuration (*1*, *2*). In contrast, $\alpha 1\beta 2$ or $\alpha 2\beta 1$ undergoes a change in interactions with changes in the ligation state of heme. The allosteric properties of Hb are caused directly by structural changes of $\alpha 1\beta 2$ interfaces, and the stability of Hb is linked to $\alpha 1\beta 1$interfaces (*2*). In fact, the $\alpha 1\beta 1$ sites are more extensive than the $\alpha 1\beta 2$ sites, while there is little contact between α and α, or β and β (*1*, *2*).

Although assembly of the $\alpha\beta$ dimer is postulated to be the main rate-limiting step in heterotetramer assembly in vivo and is theorized to be governed by electrostatic attractions between α and β partner subunits (*3*, *4*), amino acids at $\alpha 1\beta 1$ sites have been documented to be the rate-limiting step in a variety of in vitro studies using recombinant globin chains (*5*−*7*). We also previously showed that γ chain amino acids at $\alpha 1\gamma 1$ interaction sites, especially the G14 and G18 positions, are critical for assembly in vitro of γ-globin homo (γ_2)- and hetero ($\alpha\gamma$)-dimers (*8*). Amino acids at the $\alpha 1\beta 1$ interface in Hb A differ from those at the $\alpha 1\gamma 1$ interface in Hb F at four positions (e.g., β51 Pro, β112 Cys, β116 His, and β125 Pro vs γ51Ala, γ112Thr, γ116Ile, and γ125Glu, respectively). These four substitutions probably account for the increased stability of Hb F and for its decreased

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dissociation into monomers compared to Hb A. Furthermore, our results showed that Ile at γ116 instead of His, which is present at β116 (G18), is responsible for stable γ chain dimer formation and that substitution with His for γ116 Ile results in increased assembly in vitro with α chains at a rate similar to that of β and α chain assembly (*8*). From these results we speculated that γ chains assemble in vivo with α chains prior to forming stable γ_2 dimers, possibly binding to α chains as partially folded nascent γ-globin chains prior to release from polyribosomes. However, reasons for differences in assembly between γ- and β-globin chains with α chains in vitro and in vivo are not yet understood completely.

Hemoglobin variants with amino acid substitutions at the $\alpha 1\beta 1$ interface usually show no or slight alteration in oxygen-binding properties, while stability is usually reduced which leads to varying degrees of hemolysis (*9*). However, the role of the $\alpha 1\beta 1$ subunit interface has been recently overlooked, and recent NMR studies suggested that there is communication between the $\alpha 1\beta 1$ and $\alpha 1\beta 2$ subunit interfaces during oxygenation of Hb molecules (*10*). In addition, even though it was reported that the $\alpha 1\beta 1$ contact of human hemoglobin plays a key role in stabilizing the bond with oxygen (*11*), the detailed role of the $\alpha 1\beta 1$ interface of assembled dimers and tetramers of hemoglobin in stability to autoxidation is not well understood. To understand further the importance of the G-18 position in $\alpha 1\beta 1$ or $\alpha 1\gamma 1$ and $\beta 1\beta 2$ or $\gamma 1\gamma 2$ interfaces in $\alpha_2\beta_2$ or $\alpha_2\gamma_2$ and β_4 or γ_4 tetramers, respectively, as well as in α-non-α chain assembly and autoxidation of hemoglobin, we engineered $\beta^{116His\rightarrow Asp}$, $\beta^{116His\rightarrow Ile}$, and $\beta^{112Cys\rightarrow Thr,116His\rightarrow Ile}$ chains. The ability of these β chain variants to form homodimers and tetramers as well as to assemble in vitro with α chains to form functional hetero-tetramers was evaluated. In addition, tetramer autoxidation rates and effects of amino acid change on the heme environment also were assessed by measurements of functional properties and dimer/tetramer equilibration and by NMR and CD spectroscopy.

MATERIALS AND METHODS

Expression of Soluble Recombinant Human β-Globin Chain Variants in E. coli. Two β116 globin chain variants and one β112/β116 variant (e.g., $\beta^{116His\rightarrow Asp}$, $\beta^{116His\rightarrow Ile}$, and $\beta^{112Cys\rightarrow Thr,116His\rightarrow Ile}$ chains) were constructed and expressed using the plasmid pHE2β (*12*, *13*). The basic strategy for generation of these variants by site-specific mutagenesis of the normal β chain involves recombination/PCR as described previously (*13*, *14*). Clones were subjected to DNA sequence analysis of the entire β-globin cDNA region using site-specific primers and fluorescently tagged terminators in a cycle sequencing reaction in which extension products were analyzed on an automated DNA sequencer. Plasmids were transfected into *Escherichia coli* (JM 109) (Promega Co., Madison, WI), bacteria were grown at 30 °C, the cultures were induced to express β-globin chains, and soluble β-globin chain variants were isolated and purified as described (*13*). Authentic human hemoglobin and α and β^A-globin chains were purified from erythrocyte lysates from normal controls according to previous methods (*15*). Removal of *p*-chloro mercuribenzoate from α and β chains was accomplished using 20 mM dithiothreitol (DTT), and globin chains were isolated after gel filtration on a Superose 12 column (Pharmacia Biotech Inc., Piscataway, NJ). Removal of catalase from β chains was performed also by cation-exchange chromatography on a Source 15S column, and purification of γ-globin chains was described previously (*8*).

Biochemical Characterization of Purified β-Globin Chains. Molecular mass and sample purity was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) as described (*16*). In addition, mutations in each purified β-globin chain variant were confirmed using mass spectral analysis. Electrospray ionization mass spectrometry (ES-MS) was performed on a VG BioQ triple quadrapole mass spectrometer (Micromass, Altrincham, U.K.) using the multiply charged ion peaks from the β-globin chain (M_r =15,867.2 Da) as reference for mass scale calibrations (*17*). Data analysis employed the MassLynx software package (Micromass, Altrincham, U.K.).

Purified β globin chains also were analyzed by cellulose acetate electrophoresis, and mobilities were compared with those of authentic human globin chains. Absorption spectra of purified β-globins in the CO form were recorded using a Hitachi U-2000 spectrophotometer (Hitachi Instruments, Inc. Danbury, CT). Circular dichroism (CD) spectra of hemoglobins were recorded using an Aviv-Model 62DS instrument employing a 0.1 cm-light path cuvette at ~20 μM hemoglobin, and temperature was controlled with a thermo-electric module. Globin concentration was determined spectrophotometrically using a millimolar extinction coefficient of 13.4 at 540 nm for carbonmonoxy hemoglobin (*18*). Oxygen-dissociation curves of hemoglobin tetramers were determined in 50 mM Bis-Tris buffer containing 0.1 M NaCl and 5 mM EDTA, pH 7.2, at 20 °C using a Hemox Analyzer (TCS Med. Co., Huntingdon Valley, PA) (*19*). NMR spectroscopy of single globin chains and heterotetramers containing normal and variant globin chains in 0.1 M Bis-Tris, pH 6.85 in 90% H_2O, 10% D_2O was performed using a DMX 400 NMR spectrophotometer (Bruker Instrument Inc., Billericia, MA) at 22 °C (*20*).

Subunit Dissociation and Association of β_4 Homotetramers and $\alpha_2\beta_2$ Heterotetramers. Dissociation of β-globin homotetramers and $\alpha_2\beta_2$ heterotetramers was studied by fast protein liquid chromatography (FPLC) on a Superose 12 gel-filtration column. Hemoglobin was mixed with vitamin B_{12}, which served as an internal marker for determination of total column volume (V_t). Different concentrations of β-globin chain solutions in a constant volume (0.3−200 μM in 25 μL) were injected onto a Superose 12 column, and gel filtration was accomplished using 0.1 M Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4, at room temperature (22 °C) (*5*).

Assembly in Vitro of β Chains with α Chains to Form $\alpha_2\beta_2$ Heterotetramers. $\alpha_2\beta_2$ heterotetramer formation was assessed by mixing purified β chain variants (~75 μM) with equimolar amounts of α globin chain in the CO form in 0.1 M Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4, at room temperature (22 °C), and tetramer formation was monitored by HPLC using a POROSHQ 4.6 mm/100 column (PerSeptive Biosystems Inc, Framingham, MA) (*5*, *13*). Tetramer formation of $\beta^{116His\rightarrow Ile}$ and $\beta^{112Cys\rightarrow Thr,116His\rightarrow Ile}$ chains with α chains was monitored by cellulose acetate electrophoresis on Titan III membranes at pH 8.6 with Super-Heme buffer (Helena Laboratories Beaumont, TX), since these heterotetramers could not be separated from single

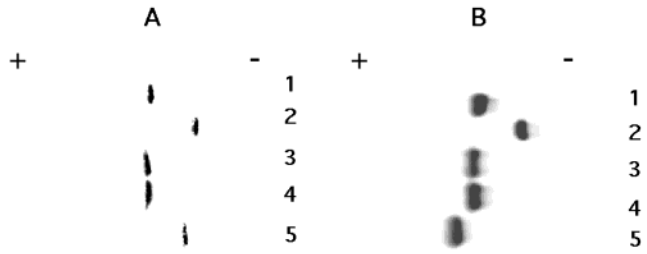

FIGURE 1: Cellulose acetate electrophoresis of purified β-globin chain variants and their corresponding $\alpha_2\beta_2$ tetramers. Purified β-globin variants (A) and their corresponding in vitro assembled $\alpha_2\beta_2$ tetramers (B) were analyzed by electrophoresis on cellulose acetate membranes. Panel A: lanes 1 and 2, β^A and β^S globins, respectively (purified from human red blood cells); lane 3, $\beta^{116His\rightarrow Ile}$ chains; lane 4, $\beta^{116His\rightarrow Ile,112Cys\rightarrow Thr}$ chains; lane 5, $\beta^{116His\rightarrow Asp}$ chains. Panel B: lane 1, Hb A; lane 2, Hb S; lane 3, Hb βC116I; lane 4, Hb β, H116I, and C112T; and lane 5, Hb βH116D.

chains by HPLC. Quantitation of percentage of formed heterotetramers of $\beta^{116His\rightarrow Ile}$ and $\beta^{112Cys\rightarrow Thr,116His\rightarrow Ile}$ chains with α chains was achieved by densitometric scanning after cellulose acetate electrophoresis (Helena, Beaumont, TX).

Autoxidation Measurements. Autoxidation rates of $\alpha_2\beta_2$ tetramers containing β116 variants [Hb βH116I ($\alpha_2\beta_2^{116I}$) and Hb β H116D ($\alpha_2\beta_2^{116D}$)] in the oxy form were measured in 0.1 M MES buffer (pH 6.5) at 35 °C in the presence of 0.1 M EDTA and were compared to rates for human oxy Hb A ($\alpha_2\beta_2$) as described previously (*11*, *21*).

RESULTS

Expression and Purification of Soluble β Globin Chain Variants. We previously reported expression and characterization of soluble human γ-globin chains and two γ chain variants (e.g., $\gamma^{112\ Thr\rightarrow Cys}$ and $\gamma^{116\ Ile\rightarrow His}$ chains) in bacteria in an effort to understand the basis for the slow assembly rate in vitro of γ with α chains (*8*). To define further the role of the amino acids at the G18 position of non-α chains in homo- and heterotetramer formation, three β-globin chain variants (e.g., $\beta^{116His\rightarrow Asp}$, $\beta^{116His\rightarrow Ile}$ and $\beta^{112Cys\rightarrow Thr,116His\rightarrow Ile}$ chains) were expressed and characterized. Automated DNA sequence analysis of the entire β-globin cDNA region in expression vectors using site-specific primers showed expected sequences for the two variant cDNAs. The three purified β116 variants migrate mainly as 16 kDa monomers with small traces of dimers (32 kDa) on SDS−PAGE (data not shown). Addition of 20 mM DTT to the chains in solution prior to SDS−PAGE converts the dimers to monomers, indicating that small amounts of β93 Cys oxidize and form disulfide-linked dimers. Electrophoretic mobility on cellulose acetate of purified $\beta^{116His\rightarrow Asp}$ chains was similar to $\beta^{6Glu\rightarrow Val}$ (β^S) and $\beta^{112Cys\rightarrow Asp}$ chains (Figure 1) and showed a single band (*5*), while the mobility of $\beta^{116His\rightarrow Ile}$ and $\beta^{112Cys\rightarrow Thr,116His\rightarrow Ile}$ chains was identical to that of normal β chains.

Mass spectral analysis of the three β chain variants using electrospray ionization mass spectrometry (ES-MS) resulted in values of 15,845.3, 15,843.1, and 15,841.3 for $\beta^{116His\rightarrow Asp}$, $\beta^{116His\rightarrow Ile}$ and $\beta^{112Cys\rightarrow Thr,116His\rightarrow Ile}$ chains, respectively, which are in agreement with expected masses of 15,845, 15,843, and, 15,841, respectively. Carbonmonoxy forms of all three variants showed typical absorption spectral characteristics of human hemoglobin chains with peaks at 568, 540, 419, 344, and 276 nm (*6*, *18*), indicating correct heme insertion into the variant β-globin chains.

Characterization of β-Globin Homotetramer Formation. Effects of the different β116 amino acids on homotetramer, dimer, and monomer formation were assessed by size-exclusion chromatography, and results were compared with those of normal β^A chains (*5*, *8*). Normal β^A-globin chains in solution exist as homotetramers (β_4) rather than monomers at relatively high chain concentrations (e.g., >100 μM). The position of the β chain elution peaks depends on concentration; the higher the concentration, the shorter the retention time since β chains are in self-equilibrium as tetramers, dimers, and monomers (Figure 2A,B). Typical examples of elution patterns of normal β chains and β116 β chain variants at high (~75 μM) (a′−d′) and low (~2.5 μM) (a−d) concentrations are shown in Figure 2A. The shape and symmetry of elution profile of these β chains did not show significant differences, but the positions of the elution peaks for high versus low applied concentrations as well as for different β chain variants were different when standardized to the elution time for the internal standard, vitamin B12 peak (dotted lines). At lower concentrations of normal β chains, the monomeric form predominates (Figure 2B), while we previously reported that the dimeric form predominates for γ chains at the same hemoglobin concentration at which monomers predominate for β^A chains (*8*). We also found previously that $\beta^{112Cys\rightarrow Asp}$ chains exist as monomers at the same concentration at which β^A chains exist as tetramers (*5*, *6*). Results of effects of concentration on elution position of $\beta^{116His\rightarrow Asp}$ chains (Figure 2B) were similar to those of α and $\beta^{112Cys\rightarrow Asp}$ chains (*6*, *8*), indicating that $\beta^{116His\rightarrow Asp}$ monomers predominate under these conditions. In contrast, like γ chains, results for $\beta^{116His\rightarrow Ile}$ chains show that dimers predominate upon dilution; even though dissociation to dimers from tetramers for this variant was not identical to that of γ chains. Homotetramer formation of $\beta^{116His\rightarrow Ile}$ chains occurred more readily than normal γ chains. In addition, results for $\beta^{112Cys\rightarrow Thr,116His\rightarrow Ile}$ and $\beta^{112Cys\rightarrow Thr}$ chains were similar to those for $\beta^{116His\rightarrow Ile}$ and normal β^A chains, respectively. Furthermore, the CD spectra of $\beta^{116His\rightarrow Ile}$ and $\beta^{112Cys\rightarrow Thr,116His\rightarrow Ile}$ chains in the UV region almost were superimposed on the spectrum of authentic human native β chains (Figure 3). However, ellipticity around 210 nm for $\beta^{116His\rightarrow Asp}$ chains was lower than that for normal β, $\beta^{116His\rightarrow Ile}$, and $\beta^{112Cys\rightarrow Thr,116His\rightarrow Ile}$ chains. This may be due to formation of monomers for $\beta^{116His\rightarrow Asp}$ chains versus tetramers for $\beta^{116His\rightarrow Ile}$ and $\beta^{112Cys\rightarrow Thr,116His\rightarrow Ile}$ chains.

Assembly in Vitro of β116 Variants and α-Globin Chains. We evaluated heterotetramer assembly rates in vitro of the three β116 variants with α chains in an effort to elucidate further the role of β116 His and γ116 Ile in assembly with α chains. The time course for tetrameric hemoglobin formation expressed as relative amounts of variant Hb tetramer formed as a function of time is shown as a first-order plot in Figure 4. At a hemoglobin concentration of ~75 μM, like normal β and $\beta^{112Cys\rightarrow Asp}$ chains (*5*), the rate of assembly of $\beta^{116His\rightarrow Asp}$ and $\beta^{116His\rightarrow Ile}$ chains with α chains was faster and slower, respectively, than that of normal β chains. This indicates that differences at the β116 (G18) position in β versus γ chains play a critical role in assembly in vitro of β and γ with α chains. The apparent first-order rate constant of $\beta^{116His\rightarrow Ile}$ chain heterotetramer formation is

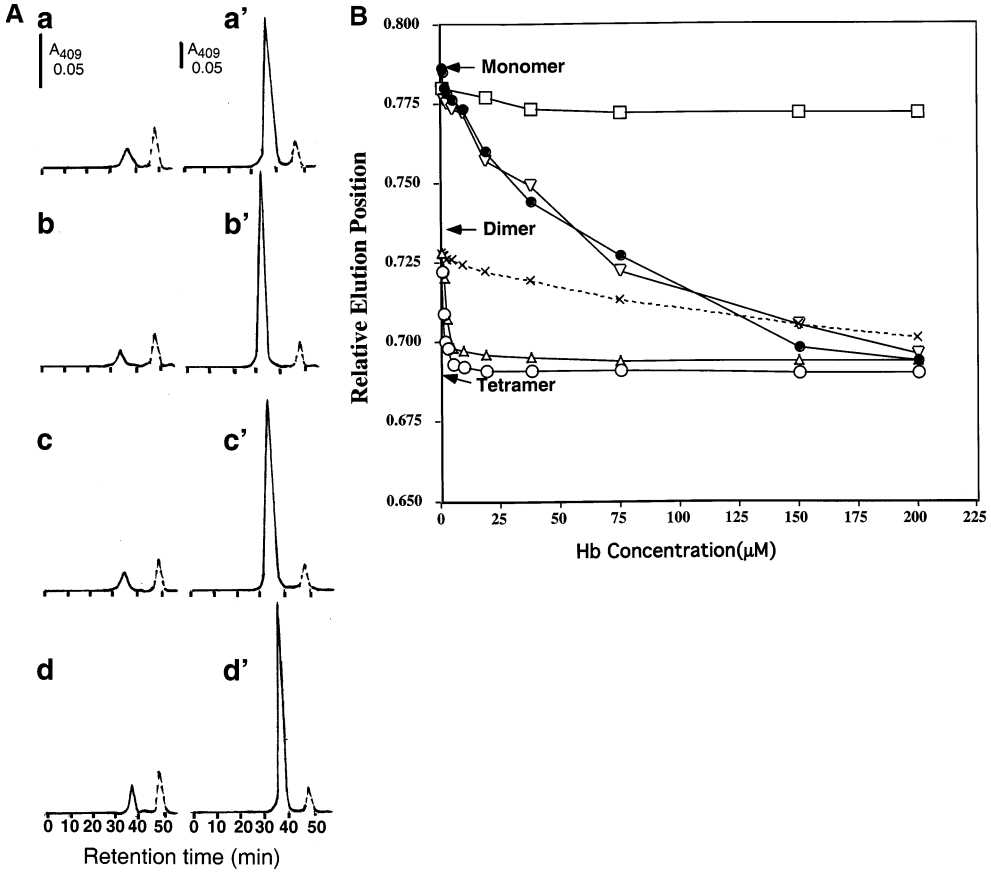

FIGURE 2: (A) Size-exclusion chromatography profile of normal β-globin chains and β116 chain variants. Purified β (a and a′), $\beta^{116His\rightarrow Ile}$ (b and b′), $\beta^{116His\rightarrow Ile,112Cys\rightarrow Thr}$ (c and c′), and $\beta^{116His\rightarrow Asp}$ (d and d′) chains were mixed with an internal marker [vitamin B12 for monitoring of total bed volume (V_t) in 25 μl in 0.1 M Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4] and applied to a Superose 12 column at room temperature. Chromatographic profiles of globin chains at higher (~75 μM) (a′−d′) and lower (~2.5 μM) (a−d) concentrations are shown. The dotted line indicates the elution peak of vitamin B 12, and the *y* axis represents optical density at 409 nm. (B) Relative elution on size-exclusion chromatography of purified β-globin chains as a function of concentration. Plots of relative elution position (*y* axis) as a function of concentration of globin chains (*x* axis) are shown for $\beta^{116His\rightarrow Ile}$ chains (○), $\beta^{116His\rightarrow Ile,112Cys\rightarrow Thr}$ chains (△), $\beta^{112Cys\rightarrow Thr}$ chains (▽), and $\beta^{116His\rightarrow Asp}$ chains (□) in comparison with normal human β chains (●) and recombinant human γ chains (×) in 0.1 M Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4. Relative elution position of each sample was determined by calculating V_e/V_t, where V_e and V_t represent elution volume of sample and total bed volume of column, respectively, obtained from the results in Figure 2A. Each value represents the mean from three independent experiments.

1.3×10^{-2} min^{-1}, which indicates a 3.4×10^2-fold slower rate of assembly with α chains than that of normal β chains. Heterotetramer assembly of $\beta^{112Cys\rightarrow Thr,116His\rightarrow Ile}$ chains with α chains was equivalent to that of $\beta^{116His\rightarrow Ile}$ chains. These results are consistent with our previous findings concluding that monomer concentration is more critical than electrostatic interaction for assembly of α and β chains, (*7*).

Effects of β116 Variants on Functional Properties and Heterotetramer Dissociation. Functional properties of β116 variant hemoglobin tetramers are shown in Table 1.

$\alpha_2\beta_2{}^{116His\rightarrow Asp}$ has similar cooperativity and oxygen affinity ($P_{50} = 4.3$, $n = 2.6$) compared to Hb A ($P_{50} = 4.0$, $n = 2.7$), while $\alpha_2\beta_2{}^{116His\rightarrow Ile}$ and $\alpha_2\beta_2{}^{112Cys\rightarrow Thr,116His\rightarrow Ile}$ show a slightly higher oxygen affinity, as evidenced by the lower P_{50} values of 3.2. The *n* values for all three variants were similar to Hb A (Table 1).

To evaluate further the effects of β116 amino acid substitution (e.g., His to Ile and Asp) on dimer−tetramer equilibrium, the different β116-variant $\alpha_2\beta_2$ heterotetramers were subjected to gel-filtration chromatography using a Superose 12 HR column at various hemoglobin concentrations (Figure 5). Decreasing concentrations of $\alpha_2\beta_2$ variant tetramers in the CO form in constant volume (25 μl) were loaded on the column. At decreasing Hb A concentrations, the relative elution position [defined as the ratio of elution volume (V_e) to total column volume (V_t) assessed using vitamin B12] approached 0.74, which corresponds to the value for αβ dimers. A plot of relative elution position as a function of hemoglobin concentration in Figure 5 showed similar curves for $\alpha_2\beta_2{}^{112Cys\rightarrow Thr}$, $^{116His\rightarrow Ile}$, $\alpha_2\beta_2{}^{116His\rightarrow Ile}$, and Hb A ($\alpha_2\beta_2$). In contrast, plots for $\alpha_2\beta_2{}^{116His\rightarrow Asp}$ were right-shifted from that of Hb A, and the relative elution position approached 0.765 at lower concentrations, which corresponds to the value for β chain monomers determined using $\beta^{116His\rightarrow Asp}$ and α chains.

Autoxidation of β116 Hemoglobin Tetramer Variants. It is known that the oxygenated form of Hb A is oxidized easily to the ferric met form (met Hb) with generation of superoxide anion (*22*, *23*). Autoxidation rates of Hb variants in 0.1 M MES buffer (pH 6.5) at 35 °C in the presence of 0.1 M EDTA was assessed by plots of $\ln\{[HbO_2]_t/[HbO_2]_0\}$ as a function of time, where $[HbO_2]$ is oxy hemoglobin in solution. The ratio of HbO_2 concentration after time "*t*" to that at time $t = 0$ can be monitored by changes in absorbance at 576 nm (Figure 6). The first-order plots are shown in Figure 6 for autoxidation of $\alpha_2\beta_2{}^{116His\rightarrow Asp}$ and $\alpha_2\beta_2{}^{116His\rightarrow Ile}$

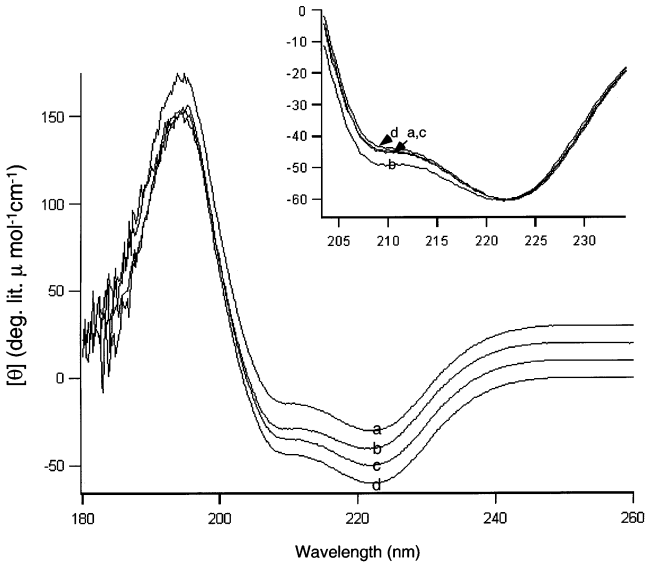

FIGURE 3: Circular dichroism spectra of purified β-globin chain variants. Circular dichroism (CD) spectra were determined for purified β-globin chain variants in the CO form in 10 mM phosphate buffer, pH 7.0 at 10 °C. Spectra are shown for $\beta^{116His\rightarrow Asp}$ chains (b), $\beta^{116His\rightarrow Ile,112Cys\rightarrow Thr}$ chains (c), and $\beta^{116His\rightarrow Ile}$ chains (d) with results compared to human β-globin chains (a). Inset represents Soret region of absorption spectra between 200 and 240 nm for each globin chain normalized to values at 222 nm after subtraction of the buffer spectrum.

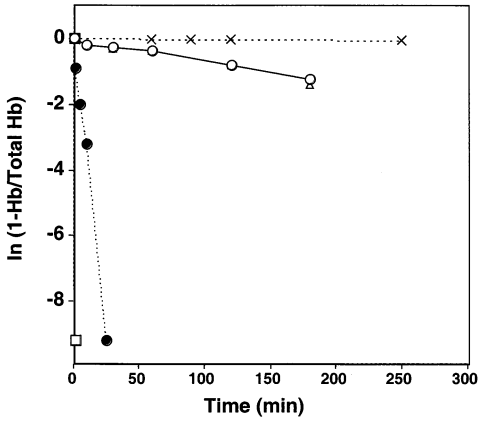

FIGURE 4: Rates of formation of hemoglobin heterotetramers. Time course of formation is shown for Hb F and Hb A after mixing of equimolar α chains with recombinant $\beta^{116His\rightarrow Ile}$ chains (○), $\beta^{116His\rightarrow Ile,112Cys\rightarrow Thr}$ chains (△), $\beta^{116His\rightarrow Asp}$ chains (□), normal human β chains (●), and recombinant human γ chains (×) in 0.1 M Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4 at room temperature (22 °C). Concentration of individual globin chains was ~75 μM, and the y axis indicates amount of heterotetramer formed relative to total β (or β^x) or γ chain, respectively. Values represent the mean of three independent experiments and S. E. M. is within ±2%. Inset shows expanded view of early time points (0−120 min).

variants compared to Hb A. Hb A shows a biphasic autoxidation curve as previously described, and it is characterized by first-order kinetics resulting from two different rate constant components (*21*, *24*). The slope for the fast (a_f) and slow (a_s) component rates for oxidation of Hb A were 0.14 and 0.02 h^{-1}, respectively, as shown by the dotted lines in Figure 6. These values for fast (a_f) and slow (a_s) rates for oxidation of $\alpha_2\beta_2{}^{116His\rightarrow Asp}$ and $\alpha_2\beta_2{}^{116His\rightarrow Ile}$ were 0.14 and 0.031 h^{-1}, respectively (Figure 6). Tsuruga and Shikama found that this biphasic autoxidation curve of Hb

Table 1: Oxygen-Binding Properties of β116-Variant Hemoglobin Tetramers[a]

hemoglobin	P_{50} 2,3BPG (−)	P_{50} 2,3BPG (+)	n_{max}
$\alpha_2\beta_2$	4.0 ± 0.1	12.5 ± 0.2	2.7 ± 0.1
$\alpha_2\beta_2{}^{116His\rightarrow Asp}$	4.3 ± 0.1	13.4 ± 0.2	2.6 ± 0.1
$\alpha_2\beta_2{}^{116His\rightarrow Ile}$	3.2 ± 0.1	10.8 ± 0.2	2.4 ± 0.2
$\alpha_2\beta_2{}^{112Cys\rightarrow Thr,116His\rightarrow Ile}$	3.2 ± 0.1	9.6 ± 0.1	2.6 ± 0.1

[a] Oxygen equilibrium curves of hemoglobins were determined using ~50 μM Hb concentration in 50 mM Bis-Tris/HCl buffer, pH 7.2, containing 100 mM NaCl and 5 mM EDTA at 20 °C. P_{50} represents the partial oxygen pressure required to give 50% oxygen saturation of hemoglobin. n_{max} values were calculated from the Hill plot of oxygen-equilibrium curves. Concentration of 2,3-BPG (Biphosphoglycerate) when present (+) is 2 mM. ± indicates SD of 3 or 4 independent measurements.

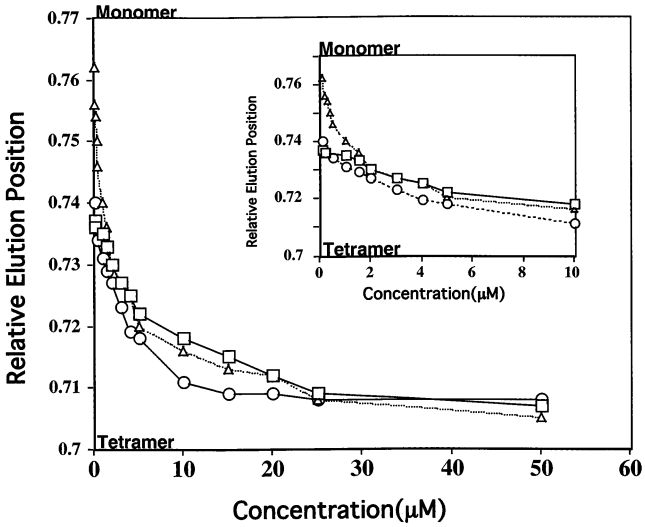

FIGURE 5: Effect of β116 amino acid on dissociation of $\alpha_2\beta_2$ tetramers. Varying concentrations ranging from 0.1 to 50 μM hemoglobin tetramer in 0.1 M Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4, at room temperature (22 °C) were loaded onto a Superose 12 column, and gel-filtration chromatography was performed at a flow rate of 0.5 ml/min in the same buffer at room temperature (23 °C). Relative elution position of each sample was determined by calculating V_e/V_t, where V_e and V_t represent elution volume of sample and total bed volume of column, respectively. Each value represents the mean from three independent experiments. Samples included Hb A from human red blood cells (○) and in vitro assembled β116 variant $\alpha_2\beta_2$ tetramers [Hb β H116D (△) and Hb β H116I (□)], with the inset showing expanded view of early time points.

A could be described by first-order kinetics containing two rate constants as follows (*24*):

$$[HbO_2]_t/[HbO_2]_0 = P(\exp - k_f t) + (1 - P)\exp(-k_s t) \quad (1)$$

In this equation, k_f and k_s represent first-order rate constants for the initial fast autoxidation and then second slow autoxidation, respectively, and P is the molar fraction of the rapidly reacting hemes. The value of P was allowed to vary from 0.4 to 0.6, and we used $P = 0.48$ as previously calculated (*11*, *21*). The k_s value is nearly equal to that of a_s, while k_f can be calculated from the experimental data using eq 1. They also found that the rate constant k_f is due to autoxidation of α chains and that the rate constant k_s is due to autoxidation of β chains in tetrameric Hb O_2 (*24*). Calculated k_f value was 0.26 h^{-1} for Hb A in our results,

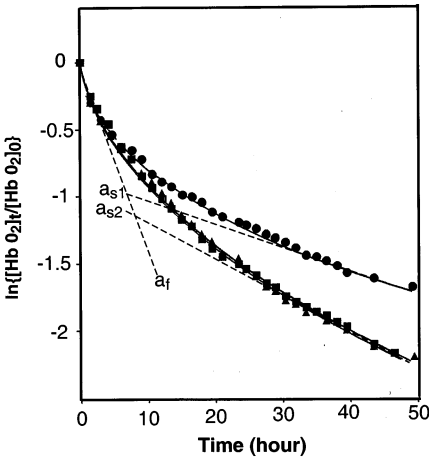

FIGURE 6: First-order plots for the autoxidation of β^{116Hb} variants compared to Hb A in the oxy form. The oxidation of in vitro assembled β116 variant $\alpha_2\beta_2$ tetramers [Hb βH116I ($\alpha_2\beta_2{}^{116I}$) (■) and Hb β H116D ($\alpha_2\beta_2{}^{116D}$) (▲)] were compared to human Hb A ($\alpha_2\beta_2$) (●) in the oxy form in 0.1 M MES buffer (pH 6.5) at 35 °C in the presence of 0.1 M EDTA. Results were assessed by plots of $\ln\{[HbO_2]t/[HbO_2]O\}$ as a function of time. The values a_f and a_s represent slopes of fast and slow oxidizing components of the biphasic curves.

which is 13 times larger than k_s or a_s. This value was higher compared to the value previously reported (7 times) (*24*). The k_f and k_s values for the $\alpha_2\beta_2{}^{116His\rightarrow Asp}$ and $\alpha_2\beta_2{}^{116His\rightarrow Ile}$ variants were the same. The a_f value for the β116 variants was similar to that of Hb A, while the k_s or a_s values for the β116 variants were about 1.5-fold higher than that of Hb A. These results indicate that autoxidation of β chains is affected by changes in amino acid at the G-18 position and that both $\beta^{116His\rightarrow Asp}$ and $\beta^{116His\rightarrow Ile}$ chains in the hemoglobin tetramers are autoxidized 1.5-fold faster than β chains in Hb A heterotetramers even though autoxidation of α chains remains the same in the hetero-teramers.

Proton NMR Spectra for β116 Chain Variants and Hemoglobin Heterotetramers. NMR spectra of recombinant $\beta^{116His\rightarrow Asp}$ and $\beta^{116His\rightarrow Ile}$ chains and in vitro assembled hemoglobin heterotetramers in the carbonmonoxy form were compared to those of native human α and β chains and Hb A in order to define effects of amino acid change on the heme environment and subunit interface interactions. The resonances between 15 and 24 ppm as well as from 12.2 to around −2 ppm originate from protons in heme groups and/or protons in amino acid residues located in the heme pocket of hemoglobin, respectively (*25*, *26*). As shown in Figure 7, a peak from the γ1-methyl proton of Val (E11) appeared around −1.74 and −1.92 ppm for the carbonmonoxy-α and -β subunits, respectively, which serves as a marker for heme environmental structure of globin chains (*25*, *26*). Resonances between 2 and 24 ppm for individual recombinant β116 chains and heterotetramers were similar to those of normal human β chains and human Hb A, respectively (*20*). In contrast, the resonance at 0 to −2 ppm for $\beta^{116His\rightarrow Asp}$ chains differs significantly from that of normal β chains, with the 1.92 ppm peak in β chains shifted to −1.76 ppm in $\beta^{116His\rightarrow Asp}$ chains (Figure 7). However, this peak for $\beta^{116His\rightarrow Ile}$ chains was the same as that for normal β chains. It is also noteworthy that the difference in resonance between $\beta^{116His\rightarrow Asp}$ and β^{A} chains is similar to that comparing α and β^{A} chains. Corresponding signals for $\alpha_2\beta_2{}^{116His\rightarrow Asp}$ were also different

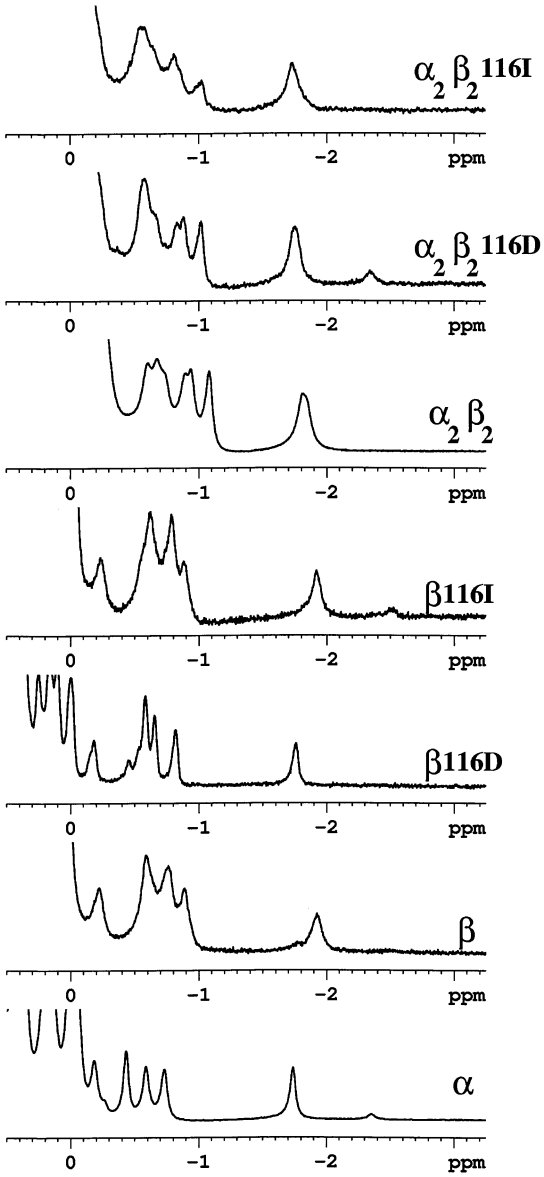

FIGURE 7: Exchangeable and heme proton NMR spectra. Hyperfine-shifted and exchangeable proton resonance between −3.2 and 0.5 ppm for recombinant $\beta^{116His\rightarrow Ile}$ chains (β 116I) and $\beta^{116His\rightarrow Asp}$ chains (β 116D) were compared to human α and β globin chains. Top three panels represent results from in vitro assembled β116 variant $\alpha_2\beta_2$ tetramers [Hb βH116I ($\alpha_2\beta_2{}^{116I}$) and Hb β H116D ($\alpha_2\beta_2{}^{116D}$)] compared to human Hb A ($\alpha_2\beta_2$) in the CO form in 0.1 M Bis-Tris buffer, pH 6.9, in 90% H_2O and 10% D_2O at 22 °C.

from Hb A (−1.75 vs −1.81), but signals for $\alpha_2\beta_2{}^{116His\rightarrow Ile}$ were similar to those of $\alpha_2\beta_2{}^{116His\rightarrow Asp}$ rather than those of Hb A.

DISCUSSION

Role of γ116 Ile and β116 His in γ and β Chains, Respectively, for Assembly with α Chains. Our current results for the β116 Ile variant showed much slower assembly with α chains than normal β chains in addition to stabilization of β_4 tetramers. These results are consistent with those in which the assembly in vitro of $\gamma^{116Ile\rightarrow His}$ with α chains was similar to that with β^{A} chains, and with the finding that this change lead to destabilization of γ_4 tetramers (*8*). These findings indicate that hydrophobicity of amino acid side chains at G18 in β and γ chains plays a critical role in equilibration of

monomer−dimer and teramers, which in turn plays a key role in assembly of γ and β chains with α chains. Our present results suggest that β116 Ile hydrophobic interactions with β chain sites (e.g., β115 Ala and β122 Phe) can stabilize $\beta1-\beta2$ interactions in β_4 homotetramers, which leads to formation of stable β_2 dimers and results in slow assembly with α chains in vitro. However, this hydrophobic interaction may not occur with β^{116His} when β chains assemble with α chains. A hydrogen bond involving adjacent amino acids on the G-helix of α chains may predominate (e.g., α110 Ala, α114 Pro and/or α117 Phe) at $\alpha1\beta1$ sites to promote stable $\alpha\beta$ hetero-dimer formation, as shown by X-ray diffraction results (*1*).

Our current findings also show that rate of assembly of β116 Ile chains with α chains was 1000-fold faster than that of γ chains, but 340-fold slower than that of β chains containing β^{116His}. Our previous findings showed that substitution of γ116 Ile with His increased assembly with α chains to a rate similar to that of β chain assembly (*8*). In addition, we showed that the relative elution position of normal γ chains on gel chromatography was not concentration-dependent like that of β chains; and, that stable dimer formation was favored at lower γ chain concentrations (*8*). We also showed that $\gamma^{116\ Ile\rightarrow His}$ chains promoted dissociation to monomers and dimers (*8*). Our current findings show that relative elution position of $\beta^{116His\rightarrow Ile}$ chains as a function of concentration was similar to that of γ chains and extrapolated to dimers on dilution; however, the dissociation pattern of homotetramers to dimers was not the same as that of γ chains. These results may in part explain the faster assembly in vitro of $\beta^{116His\rightarrow Ile}$ chains than normal γ chains with α chains to form heterotetramers. Our findings also demonstrate that interactions of α chains with γ chains in vitro are slightly different from those with β116 Ile chains. In addition, assembly of $\beta^{112Cys\rightarrow Thr,116His\rightarrow Ile}$ with α chains was similar to $\beta^{116His\rightarrow Ile}$ chains. These findings and our previous results showing similar assembly rates for $\gamma^{112\ Thr\rightarrow Cys}$ and γ chains with α chains (*8*) indicate that the difference between β^{112Cys} and γ112 Thr at the G14 position may not contribute to the different assembly rates in vitro of β and γ chains with α chains. Further elucidation of the molecular details causing stability of homo- and heterotetramers of β and γ chains is now required by X-ray analysis of γ_4 and Hb F in the liganded form.

The assembly of human Hb subunits (α and β chains) into stable Hb A heterotetramers ($\alpha_2\beta_2$) in vitro has been explored, and a three-step mechanism has been elucidated at least for in vitro assembly (*3*). The α chains are in monomer/dimer equilibrium and favor the monomeric form, whereas β-like globin chains are in monomer/tetramer equilibrium and favor the tetrameric form. It is generally assumed that dissociation of these oligomeric subunits into monomers must occur before these two different chains can combine to form $\alpha\beta$ dimers, which then associate to form tetrameric Hb ($\alpha_2\beta_2$). Our studies help explain the slow assembly of γ chains by the amino acids at $\alpha1\gamma1$ as well as $\gamma1\gamma2$ interaction sites and indicate that differences in amino acids at $\alpha1\beta1$ and $\alpha1\gamma$ interaction sites between Hb A and Hb F, respectively, as well as formation of stable γ_2 dimers controlled by amino aids at the $\gamma1\gamma2$ interaction sites lead to different assembly rates in vitro. However, this slower assembly of γ chains with α chains than $\beta^{112Cys\rightarrow Thr,116His\rightarrow Ile}$ or $\beta^{116His\rightarrow Ile}$ chains in vitro probably does not occur in vivo. Therefore, we proposed that γ-globin chains fold co-translationally and that partially folded nascent γ-globin chains associate with free α chains, which may exist transiently waiting to form $\alpha\gamma$ hero-dimers during or soon after translation before stable γ chain homo-dimers can form (*8*). In fact, we showed that rates of assembly of radiolabeled γ and β chains made in cell-free transcription/translation system with α chains were similar (*27*). These results further support the contention that α chains bind to nascent non-α chains and act as folding catalysts to promote functional tetrameric hemoglobin formation in vivo. Interaction sites at the G and H helices of nascent non-α chains may be responsible for this association with α chains prior to γ chain release from polyribosomes.

Role of β116 His in β Chain for Functional Properties of Tetramers. Our present results with Hb β116D also show similar functional properties as Hb A, even though α and β interactions of $\alpha_2\beta_2^{112Cys\rightarrow Asp}$ and $\alpha_2\beta_2^{116His\rightarrow Asp}$ tetramers are weaker because of instability of $\alpha1\beta1$ interaction sites. These results contrast with those for $\alpha_2\beta_2^{40\ Arg\rightarrow Asp}$ and $\alpha_2\beta_2^{101\ Glu\rightarrow Asp}$, in which substitutions located at the $\alpha1\beta2$ interface exhibited lower cooperativity and much increased oxygen affinity but did not affect the $\alpha1\beta1$ interfaces (*28*). These results reinforce the contention that allosteric transition between oxy and deoxy forms of tetrameric hemoglobin is mainly influenced by amino acid residues located at $\alpha1\beta2$ but not at $\alpha1\beta1$ interaction sites (*1*). Our present results also indicate that some interactions at the $\alpha1\beta1$ interface which involve β112 Cys and β116 His also contribute to stability of the $\alpha1\beta2$ interface.

Relationship between Amino Acids at the $\alpha1\beta1$ Interface and Antoxidation of Hemoglobin Tetramers. When α and β chains assemble, oxidation of hemoglobin is inhibited; however, the hemoglobin heterotetramers do exhibit a biphasic autoxidation curve characterized by two different rate constants; a fast one due to α chain and a slow one due to β chain oxidation (*21*, *24*). The stability to autoxidation of tetrameric ($\alpha_2\beta_2$) hemoglobin is similar to that of $\alpha\beta$ dimeric hemoglobin, since the rate of autoxidation does not depend on hemoglobin concentration (*24*). Yasuda et al. also recently reported that formation of $\alpha1\beta1$ contacts produce a conformational constraint on the heme environment in β chains where the distal (E7) His at position 63 is tilted slightly away from the bounded dioxygen, so as to prevent the proton-catalyzed displacement of O_2^- from the FeO_2 center by an entering water molecule (*11*). These findings are consistent with our present results on substitution of β116 His for Ile and Asp, which affect stabilization of $\alpha1\beta1$ interactions and lead to conformational constraint of the heme environment to promote oxidation.

The NMR ring-current-shifted resonances are sensitive to orientation and/or conformation of the heme group relative to the amino acid residues in the heme pocket (*25*). Our gel-chromatography results of $\beta^{116His\rightarrow Asp}$ chains like $\beta^{112Cys\rightarrow Asp}$ chains also showed monomer formation predominated (*5*). The α chains are known to favor formation of monomers rather than tetramers, and NMR resonance signals of the heme region in these chains differ from those of β chains (*14*, *26*, *29*). This difference was thought to be mainly attributed to local structural differences between α and β chains (*26*). However, our present results indicate that the

NMR signal difference between α and β chains is caused not only by the local structural difference between α and β chains, but may also reflect the tendency of α chains to form monomers. Furthermore, these results suggest that some conformational changes in the heme region affect monomer, dimer, and tetramer equilibration. These results also indicate that α and β assembly influences heme environmental structure and can cause changes in local structure not only at His E7 but also at Val E11 in the β chain. This then can cause a change in autoxidation rate of β chains after assembly with α chains, resulting in inhibition of autoxidation of hemoglobin.

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