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Consequence of β16 and β112 Replacements on the Kinetics of Hemoglobin Assembly

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The rates of α/β monomer combination of four β^A variants (β112C → S, β112C → D, β112C → T, and β112C → V) in the presence and absence of β16G → D (β^J) were measured in an attempt to assess the consequences of amino acid substitution at both a surface (β16) and an $\alpha_1\beta_1$ interface (β112) residue on oxyhemoglobin assembly. Rates of α/β monomer combination determined spectrally in 0.1 M Tris–HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4, at 21.5°C differed by over 40-fold (22 ± 2.0 to $0.49 \pm 0.1 \times 10^5$ M^{-1} s^{-1}), and were in the order: HbA β112S = HbJ β16D, β112S > HbA β112D = HbJ β16D, β112D > HbA > Hb J > HbA β112T = HbJ β16D, β112T > HbJ β16D, β112V > HbA β112V. This extensive kinetic investigation of single/double amino acid-substituted recombinant hemoglobin molecules, in conjunction with molecular modeling studies, has allowed examination of an array of unique α/β subunit interactions and assembly processes.

Key Words: **human recombinant hemoglobin; subunit assembly; association kinetics; spectroscopy; molecular modeling.**

Hemoglobin's physiologic properties depend on the orderly assembly of its subunits in erythrocytes and their precursors (1). This assembly of human hemoglobin subunits (α and β chains) into stable tetramers ($\alpha_2\beta_2$) *in vitro* has been explored (2–6) and a three-step mechanism has been elucidated (7–9). The α chains are in monomer/dimer equilibrium, whereas β chains are in monomer/tetramer equilibrium. 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. Two $\alpha\beta$ dimers then associate to form tetrameric hemoglobin ($\alpha_2\beta_2$). The assembly of the $\alpha\beta$ dimer is postulated to be the main rate-limiting step *in vivo* and is governed by electrostatic attractions between α and β partner subunits (3, 5). Indeed, our previous studies using recombinant hemoglobin variants showed that $\alpha_2\beta_2$ tetramer formation is promoted by the incorporation of additional negative charged residues into the β chain (10).

Recent kinetic assembly studies using carboxypeptidase modified hemoglobins [Des (His-146, Tyr-145) β and Des-Arg-141) α] suggested, however, that specific domains for α- and β-chain interaction are also critical for the assembly process (11). In addition, our mutagenesis studies showed that β112Cys (G14), located at one of the $\alpha_1\beta_1$ interaction sites on the G helix, was found to be essential to the formation of a stable $\alpha\beta$ dimer (12, 13). Thus, the relationship between electrostatic interaction of α and β chains (coulombic interactions), and specific residues at the $\alpha_1\beta_1$ subunit interface, specifically the β112Cys position, is worth exploring. In this report, we systematically evaluated the effects of differences in surface charge of the β chain and differences in β112 amino acid residues on the assembly of human hemoglobin by direct measurement of association rates of β 16, 112 variants with α chains.

MATERIALS AND METHODS

Expression and characterization of soluble recombinant human β-heme chain variants in E. coli. Four β112Cys (C) chain variants [β112Ser (S), β112Val (V), β112Thr (T) and β112 Asp (D)] in the presence and absence of β16Gly (G) → Asp (D) were constructed, and expressed using the pHE2β plasmid vector which contains cDNAs coding for each β-chain variant alone and methionine aminopeptidase as described previously (10, 12, 14). The basic strategy for generation of these variants by site-specific mutagenesis of the normal β chain involved recombination/polymerase chain reaction as described previously (10, 15). Clones were subjected to DNA sequence analysis of the entire β-globin cDNA region using site-specific

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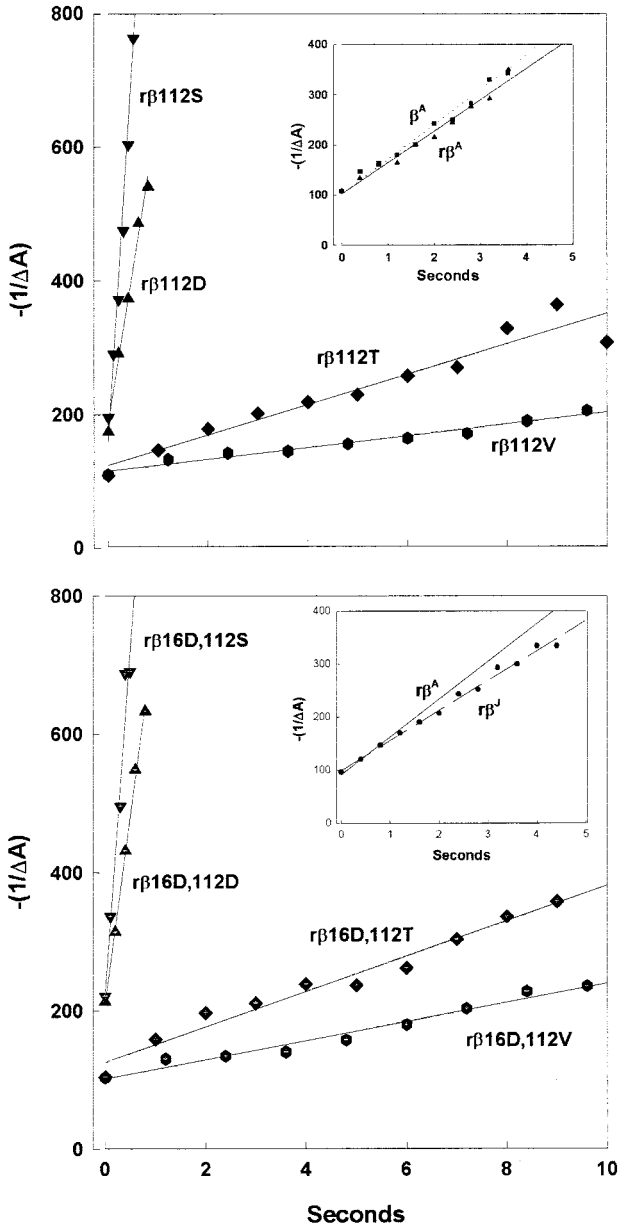

FIG. 1. Second order rate plots of subunit assembly of recombinant oxyhemoglobins. Kinetics of assembly of single substitution oxyhemoglobins (top). Rate plots of combination of α chains with β112C $\rightarrow$ S, β112C $\rightarrow$ D, β112C $\rightarrow$ T, and β112C $\rightarrow$ V chains. (Top, inset) Second order rate plots of assembly of native and recombinant β^A chains. Kinetics of assembly of double substitution oxyhemoglobins (bottom). Kinetic plots of association of α chains with β16G $\rightarrow$ D, 112C $\rightarrow$ S; β16G $\rightarrow$ D, 112C $\rightarrow$ D; β16G $\rightarrow$ D, 112C $\rightarrow$ T; β16G $\rightarrow$ D, 112C $\rightarrow$ V. (Bottom inset) Second-order plot of rβ^A (from top inset) and rβ^J with α chains. All studies were carried out as matched experiments with the single and double substituted recombinant β chains being reacted with the identical α chain controls. Experimental conditions were 0.1 M Tris–HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4, at 21.5°C. The change of absorbance was monitored at 415 nm and concentrations of α and β chains were 10 and 5 μM in heme before mixing, respectively.

primers and fluorescently-tagged terminators in a cycle sequencing reaction in which extension products were analyzed on an automated DNA sequencer (16). Plasmids were transfected into *E. coli* (JM 109) (Promega Co., Madison, WI), bacteria were grown at 30°C, and soluble β-globin chain variants were isolated as described (14). Human hemoglobin α-heme chains were purified from normal human red blood cells as described (17). Molecular mass and sample purity were assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Mutations of each purified β-globin chain variant were confirmed by electrospray ionization mass spectrometry (ES-MS) performed on a VG BioQ triple quadrapole mass spectrometer (Micromass, Altrincham, UK) using the α-globin chain (M_r 15,126.4 Da) as reference for mass scale calibrations (18).

Spectroscopic kinetic measurements of assembly. Spectral changes have been known to accompany the formation of oxyhemoglobin from oxygenated α and β subunits (19) and have been employed to monitor the rates of α and β chain assembly (6, 11, 20). Purified α and β chains equilibrated with standard experimental buffer (0.1 M Tris–HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4, at 21.5°C) were rapidly mixed in a 20-mm pathlength cell in a Kinetics Instrument Stopped Flow device temperature regulated at 21.5°C and on-line to an OLIS 3820 system. Protein concentration (on a heme basis) of α and β chains in the oxygenated form was determined on a Cary 2200 spectrophotometer ($\epsilon_{576\,nm}$ = 15.37 mM^{-1} cm^{-1}). Kinetic data were analyzed by the integrated second order rate equation: $1/\Delta A_T = (C_T/\Delta Ao)k_2t + 1/\Delta A_o$, where k_2 is a second order rate constant, t is time (second) $C_T = fC_o$, C_o = chain concentration (heme basis) and f = % monomer after mixing. The % monomer (f) present of the β chain variants prior to mixing with α chains in the standard experimental buffer was determined by $\Delta\epsilon$ (415 nm) values of 2.5 μM^{-1} cm^{-1} and 1.0 μM^{-1} cm^{-1} for β112T, β112V, and β112S, β112D, respectively (6, 11).

Molecular modeling of region of $\alpha_1\beta_1$ interface. The structures of the α (A) and β (B) chains of the oxyHbA dimer were obtained from the Protein Databank at the Brookhaven National Laboratories (entry 1HHO) and those of the recombinant β chains were determined using the automated protein modeling procedure, ProMod, at Swiss-Model following submission of its amino acid sequence (21). The superimpositions of the recombinant β chains over normal β chains of entry 1HHO were performed using MOLW (Molecular Images, Inc.) by matching the α-carbons (C_α) of residues 1–146 (22, 23), followed by visualization of Van der Waals surfaces of pertinent $\alpha_1\beta_1$ interface residues (24, 25).

TABLE I

Rates of Assembly of Recombinant β Chains with α Chains

Single substitution	$k'(10^{-5})$ M^{-1} s^{-1}	$k'(r\beta^x)/k'(r\beta^A)$
β^A(16G 112C)	3.7 ± 0.4	—
rβ^A(16G 112C)	3.4 ± 0.5	—
rβ^J(16D 112C)	2.8 ± 0.1	0.82 ± 0.1
rβ^A(16G 112D)	8.9 ± 0.1	2.6 ± 0.4
rβ^A(16G 112S)	22 ± 2.0	6.5 ± 1.0
rβ^A(16G 112T)	1.4 ± 0.4	0.41 ± 0.1
rβ^A(16G 112V)	0.49 ± 0.1	0.14 ± 0.04
Double substitution	$k'(10^{-5})$ M^{-1} s^{-1}	$k'(r\beta^{xx})/k'(r\beta^A)$
rβ^J(16D 112C)	2.8 ± 0.1	—
rβ^J(16D 112D)	9.3 ± 0.1	2.7 ± 0.4
rβ^J(16D 112S)	21 ± 4.0	6.2 ± 2.0
rβ^J(16D 112T)	1.3 ± 0.1	0.38 ± 0.06
rβ^J(16D 112V)	0.70 ± 0.1	0.21 ± 0.04

Note. Rate constants and standard deviations were determined as described under Materials and Methods.

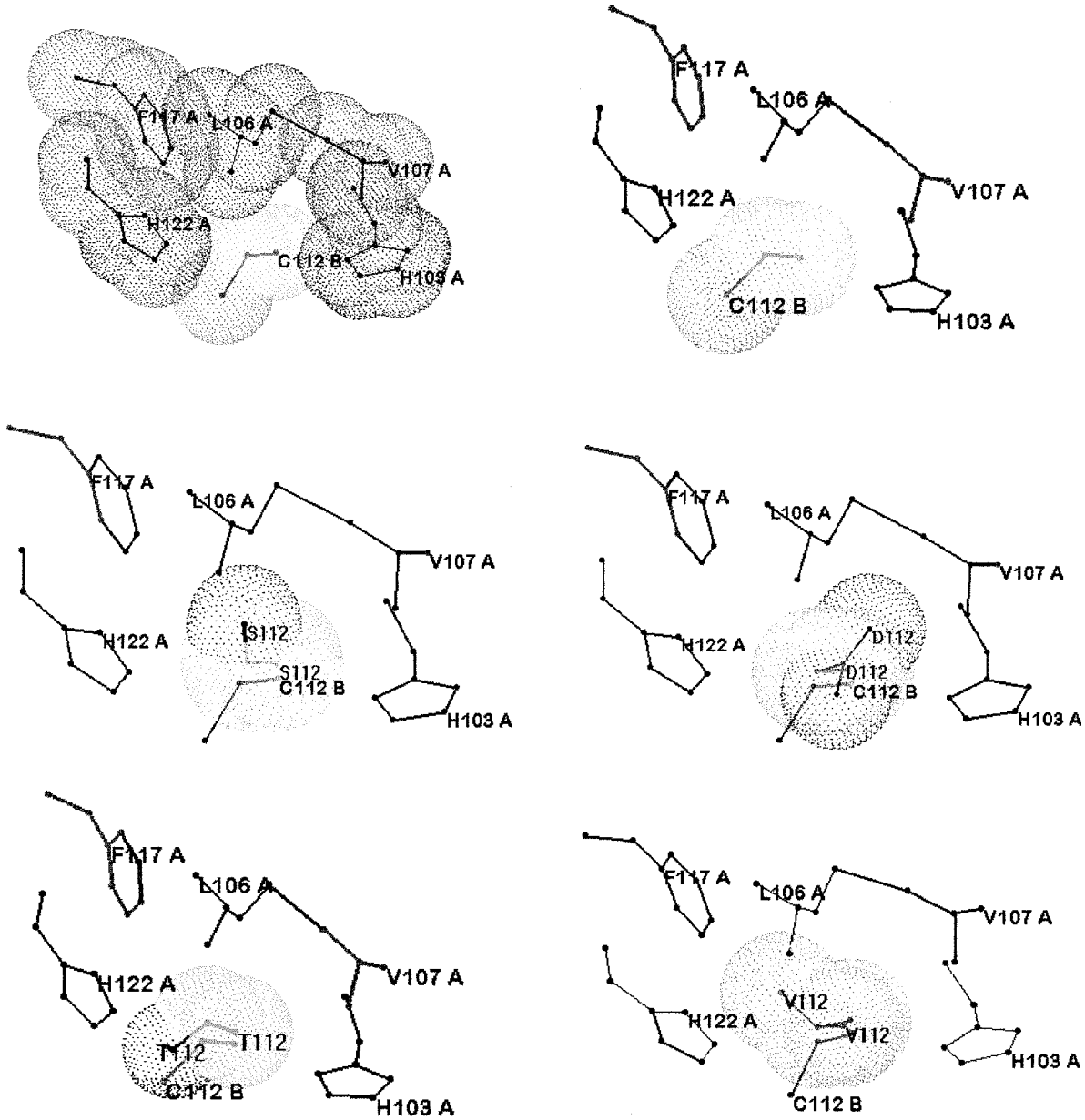

FIG. 2. Molecular modeling of $\alpha_1\beta_1$ interface region of recombinant hemoglobins. An area of the $\alpha_1\beta_1$ subunit interface of oxygenated HbA, which includes the β112C (designated C112B) residue, as well as, its α (A) chain contacts (α103H; α106L; α107V; α117F and α122H) is displayed revealing the van der Waal surfaces of all six residues (top left). Note the overall compactness of this contact region. This same structural segment is also depicted for the $\alpha\beta$112C (top right) as well as each recombinant hemoglobin (middle left, $\alpha\beta$112S; middle right, $\alpha\beta$112D; bottom left, $\alpha\beta$112T; bottom right, $\alpha\beta$112V). All β112 residues interact with their α partner chain as demonstrated in competition experiments (13) and by kinetic experiments reported here; however, β112S and β112D do not form $\alpha\beta$ structures comparable to native $\alpha\beta$ dimers. All molecular modeling was conducted as described under Materials and Methods.

RESULTS AND DISCUSSION

Kinetics of Assembly of β112 Variants with α Chain

The rates of assembly of our engineered β112 variants with α chains were measured by exploiting the Soret spectral changes that accompany formation of oxyhemoglobin (6, 11). These monomer combination reactions were carried out in a non-phosphate system (0.1 M Tris–HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4, at 21.5°C) at protein concentrations of 5 μM and 10 μM in heme for [α] and [β] chains, respectively. The time courses of these reactions could be described by an integrated second order rate expression and yielded linear rate plots (Fig. 1). Regression analysis readily permitted determination of both rate constant values and standard deviations (Table I). The rate of assembly

of native β^A and recombinant β^A ($r\beta^A$) subunits with their α partners were essentially identical and exhibited values of 3.7 ± 0.4 and $3.4 \pm 0.5 \times 10^5$ M^{-1} s^{-1}, respectively. In contrast, the rate of formation of rHbJ (β16Gly $\rightarrow$ Asp) was found to exhibit a small but significantly slower rate of assembly ($2.8 \pm 0.1 \times 10^5$ M^{-1} s^{-1}) than either its HbA or rHbA counterpart. This was intriguing since rHbJ, a more anodic (negatively charged) hemoglobin than HbA, would be expected to exhibit a faster rate of monomer assembly with its cathodic α chain partner (3, 6).

The rates of monomer assembly of β112Ser and β112Asp variants were 6.5- and 2.6-fold faster than that of $r\beta^A$ chain, while the β112Thr and β112Val mutants were 2.4- and 6.8-fold slower than that of its $r\beta^A$ counterpart. With the exception of β112Asp variant, these rβ chains exhibited rates that were consistent with competition experiments previously reported (13). Furthermore, the presence of an additional charge ($r\beta^J$) did not significantly alter the rates of these variants. Indeed, within the experimental error only $r\beta^J$112Cys $\rightarrow$ Val assembled faster (1.4-fold) than its single substituted counterpart than $r\beta^A$ 112Cys $\rightarrow$ Val.

Second order α/β monomer association reactions have been observed under the present experimental conditions with reported rates of $2–4 \times 10^5$ and $1–11 \times 10^5$ M^{-1} s^{-1} for β^A chains with altered charges (6) and/or carboxyl-terminal modifications (11), respectively. These observed rates correlated well with the focused kinetic study presented here and were well within the rate range for macromolecular assembly [between 10^4 and 10^6 M^{-1} s^{-1} at 25°C (26, 27)]. The two reportedly unstable rβ chains, those with the serine and aspartate 112 substitutions, combined with their α-chain partner in a more rapid manner than their parent β^A and β^J counterparts. Both substitutions interestingly enough also showed a decrease in color yield for the reaction; a finding also which may reflect an intrinsic instability of the assembled molecule. In contrast, $r\beta^A$, $r\beta^J$ and their β112Cys $\rightarrow$ Thr, β112Cys $\rightarrow$ Val double recombinant species demonstrated a slow full color yielding monomer reaction and the apparent formation of a stable tetrameric hemoglobin product. Taken together this would imply that a faster combination rate may mean a less structurally consequential coupling, while a slow rate may indeed imply a rapid recognition and slow structural accommodation (rate limiting step) which would be expected to be exhibited during efficient macromolecular assembly. Overall, these finding were also observed for the double substituted hemoglobin variants. The β16-residue change from glycine to aspartate was anticipated to result in an increase in α/β combination rate, but rather a slow association rate was observed. Furthermore, only one double variant, β16Asp,β112Val, exhibited a rate reflective of an electrostatic role in meaningful α/β chain coupling.

Structural Consequences of β112 Substitutions

Macromolecular assembly and stability are attributed to the uniqueness of the physiochemical properties (size, shape, polarity, and hydrophobicity) of critical interface residues (26–28). Hydrophobicity (29) of side chains of amino acids at the β112 position appears to encourage meaningful assembly as documented from competition experiments (13) and from color yield of the present kinetic findings (and expected rates of association). The size and shape (bulkiness) of amino acid side chains is also a factor in these protein-protein interactions. When serine and threonine side chains are in the interior they must hydrogen bond to other groups; since the spatial arrangement of the G-helix is redesigned, the smaller serine side chain may find it difficult to participate in intrahelical hydrogen bonding and this may result in an unstable interface. Finally, it is apparent that introduction of the charged side chain of an aspartyl residue enhanced the rate of $\alpha\beta$ monomer association, but apparently disrupted and destabilized the intermolecular integrity of the dimer.

Differences in the structure of recombinant hemoglobin subunit interfaces have also evaluated by molecular modeling (21–23). Structural changes that occur upon binding of two stable monomers (assuming β monomers are stable) would be expected to include only rotations of certain side chains, and possibly only small amounts of change of the main chain at the site of interaction, the $\alpha_1\beta_1$ interface. This region of β112Cys and its β112 engineered amino acid counterparts are clearly depicted in Fig. 2 as well as a consensus of residues (α103His, α106Leu, α107Val, α117Phe and α122His) with which β112 interacts (20, 24, 25, 30). It appears that these α chain residues provide the molecular scaffolding of this intersubunit contact, and that the inclusion of a variety of rβ chain monomers can readily be accommodated within this structural framework. However, unless a spatial readjustment is induced, the α-subunit will not successfully couple with its β partner. The kinetic studies and molecular modeling investigations presented herein have provide unexpected and valuable information regarding the consequences of amino acid substitutions at positions 16 and 112 of the human hemoglobin β chain.

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