Polymerization of Recombinant Hemoglobin F γE6V and Hemoglobin F γE6V, γQ87T Alone, and in Mixtures With Hemoglobin S

By Kazuhiro Adachi, Jian Pang, Patrick Konitzer, and Saul Surrey

To further understand determinants for Hemoglobin (Hb) S polymerization, as well as the inhibitory mechanism of Hb F on Hb S polymerization, Hb F variants containing Val-γ6 (Hb F γE6V) or Val-γ6, Thr-γ87 (Hb F γE6V, γQ87T) were expressed in yeast. The oxy form of Hb F γE6V was about 10-fold less stable to mechanical agitation than native oxy Hb F, which is similar to stability differences comparing oxy Hb S and oxy Hb A. Deoxy Hb F γE6V showed ~20-fold decreased solubility compared with native deoxy Hb F in high phosphate buffer and formed gels like deoxy Hb S in low phosphate buffer, indicating that the Val-γ6 substitution decreases solubility of Hb F like Val-β6 in deoxy Hb S. Over-saturated deoxy Hb F γE6V polymerized without a delay time in low and high phosphate buffers, in contrast to deoxy Hb S, which is accompanied by a distinct delay time before polymerization. Deoxy Hb F γE6V, γQ87T also polymerized without a delay time like deoxy Hb F γE6V. These results suggest that deoxy Hb F γE6V/γQ87T polymers are different from those of deoxy Hb S, and that contact sites differ from those of deoxy Hb S, even though both have the same primary donor (A3) and acceptor sites in the EF helix. These results also suggest that other amino acids in addition to β6 Val and amino acids in the F helix are critical for nucleation-controlled polymerization of deoxy Hb S. 1:1 mixtures of deoxy Hb S and either Hb F variant polymerized with a delay time when the concentrations for the Hb S/Hb F γE6V and Hb S/Hb F γE6V, γQ87T mixtures were about 2- and 1.5-fold, respectively, higher than that for Hb S. Logarithmic plots of delay time versus concentration for Hb S/Hb F γE6V mixtures showed the same straight line as the line for Hb S/Hb S γT87Q mixtures, but values for Hb S/Hb F γE6V, γQ87T mixtures were intermediate between those for Hb S and Hb S/Hb F γE6V mixtures. A 1:1 mixture of deoxy Hb A and Hb F γE6V, γQ87T also polymerized, but exhibited bi-phasic kinetics, when the concentration was increased to more than 3.5-fold higher than that required for Hb S polymerization. These results suggest that Gln-γ6 is a critical amino acid for exclusion of F hybrids (αβγδ) from nucleation formation with Hb S. Our findings also show that Val-γ6 in hybrids that form in mixtures of the Hb F variants with either Hb S or Hb A interacts with the hydrophobic acceptor pocket on the EF helix of an adjacent tetramer containing Thr-β87.

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primers, 5'-ACACATAAAATACATGGGTCATtcacagaggag-3' and 5'-GGGAAACAAAGTCGACTcacgtgtacgagagagcAAC-3' were synthesized to facilitate isolation and insertion of full-length cDNA into yeast vectors. Capital letters indicate complementary overlapping regions with the yeast GGA promoter and the 3'-untranslated region from the yeast mating alpha factor (MFa3'-UN) region. The shuttle vector containing the full-length human 3-globin cDNA, was created from pGS189, which contains human beta-globin cDNA, by exchanging the XhoI 3' and 5' primers, 5'-GGGAACAAAGTCGACtcagtggtatctggaggacag-3' and 5'-ACACATAAATAAACCATGGGTCATttcacagaggag-3', which extension products were analyzed on an automated laser-pulsed-liquid, protein sequencer (ABI 477A, Applied Biosystems, Inc, Foster City, CA).

Cellulose acetate electrophoresis of Hb solutions was performed at pH 8.6 using Supre-Heme buffer (Helena Lab, Beaumont, TX), and Hb concentration was determined spectrophotometrically on a Hitachi U2000 spectrophotometer (Hitachi Instruments, Inc, Danbury, CT) using millimolar extinction coefficients of 13.5 at 541 nm for oxy Hb and 13.4 at 540 nm for carbonmonoxy Hb. Methods for determination of kinetics and solubility of polymerization of deoxyhemoglobin in high phosphate buffers using the temperature jump method, as well as mechanical stability of oxyhemoglobins, were as reported previously. Minimum gelling concentration of hemoglobins in 0.1 mol/L phosphate buffer, pH 7.0 was defined by slow evaporation using a stream of dry nitrogen until a nonflowing gel forms at room temperature as previously described. Time course for gelation of deoxy Hb S in 0.1 mol/L phosphate buffer containing sodium dithionite (∼50 mmol/L), pH 7.0 at 30°C was done by the temperature jump method by monitoring change in optical density at 800 nm using a MICROSLIDE (DYNANIC INC, Rockaway, NJ) with a 0.3 mm path length instead of an EPR-NMR tube. Solubility of deoxy Hbs after gelation in an anaerobic cylindrical cuvette (2 mm diameter) was measured by the determination of soluble deoxy Hb S concentration after centrifugation at 30°C for 3 hours at 30,000 rpm (110,000 g).

RESULTS

Characterization of the two Hb F variants. The wild type 3-globin cDNA was obtained from a cDNA library made from mRNA isolated from K562 cells and showed complete agreement with human 3-globin cDNA sequence. Plasmid containing Val-y6 globin cDNA or Val-y6, Thr-y87 globin cDNA was constructed by PCR mutagenesis and subcloning as described previously. The y-globin cDNA containing Val-y6 was used as a template to introduce the Thr-y87 change. The complete coding sequence of the wild type and mutated 3-globin cDNAs flanked by the GGAP promoter and functional yeast LEU2d gene and the URA3 gene for plasmid amplification and selection in yeast.

Expression in yeast and isolation of the Hb F variants were as described previously. The purified Hb F variants were subjected to electrospray mass analysis (Fisons, Instruments, VG Biotech, Altricham, UK) using the multiply charged ion peaks from the alpha-globin chain (Mr = 15,126.4 Da) as an external reference for mass scale calibrations. Val-y6 mutations and N-terminal amino acid sequence of purified alpha and gamma chains were directly confirmed by Edman degradation employing a pulsed-liquid, protein sequencer (ABI 477A, Applied Biosystems, Inc, Foster City, CA). Cellulose acetate electrophoresis of Hbs after gelation in an anaerobic cylindrical cuvette was measured by the determination of soluble deoxy Hb S concentration after centrifugation at 30°C for 3 hours at 30,000 rpm (110,000 g).

RESULTS

Characterization of the two Hb F variants. The wild type 3-globin cDNA was obtained from a cDNA library made from mRNA isolated from K562 cells and showed complete agreement with human 3-globin cDNA sequence. Plasmid containing Val-y6 globin cDNA or Val-y6, Thr-y87 globin cDNA was constructed by PCR mutagenesis and subjected to automated DNA sequence analysis for confirmation of the entire 3-globin cDNA. The PCRBased mutagenesis step created an unanticipated substitution of T for A at y143, which was located in one of the PCR primers, and results in a conservative substitution of Thr for Ser at y143. This mutation was corrected back to the wild type Ser-y143 by replacing this region with the Barn H1 fragment from wild type pGS-200-3. Variant cDNAs with and without the 143 change were made with either Val-y6 or Val-y6, Thr-y87 as the only other changes from the wild type 3-globin cDNA. The DNA sequence of mutated 3-globin cDNAs was confirmed by automated DNA sequence analysis, and cDNAs were subcloned into the expression vector and expressed in yeast.

Fast protein liquid chromatography (FPLC) chromatographic patterns of the Hb F variants were very similar, and the variants migrated as single bands with the same electrophoretic mobility on cellulose acetate electrophoresis at pH 8.6 (Fig 1). The absorption spectra for the variants in the CO form were virtually identical to those of native CO Hb F. Reverse phase FPLC analysis of denatured Hb F variant tetramers showed the same three peaks: heme, alpha- and 3-globin chains, which all coeluted with the corresponding chains of native Hb F (Fig 2). In addition, N-terminal sequence analysis to 25 residues for each chain showed identical results compared with native alpha and gamma globins, except for the change of Val at the y6 position.

Electrospray mass spectrometry analysis of alpha and gamma chains from the variants showed that the alpha chain had the same mass...
as native α globin (15,126 Da), while the γ-chain masses for Hb F γE6V and Hb F γE6V, γQ87T were 15,965.3 Da and 15,938.3 Da, respectively, which corresponded to the expected masses of 15,965 Da and 15,938 Da for mutagenized γ chains from Hb F γE6V and Hb F γE6V, γQ87T. The γ-chain masses for the two Hb F variants containing Thr-γ143 were, as expected, 14 Da more than those for the Ser-γ143 Hb F variants.

**Mechanical stability of the Val-γ6 Hb F variant.** The oxy form of Hb S denatures approximately 10-times faster than oxy Hb A during mechanical agitation, which may be related to differences in surface hydrophobicity of dimeric and tetrameric hemoglobins between Hb S and Hb A.\(^{21,22}\) The oxy form of Hb F is much more stable than Hb A during mechanical agitation.\(^{23}\) Furthermore, Hb F (α\(^{\gamma}\)γ) is more stable than Hb F (α\(^{\alpha}\)γ) during mechanical agitation, and the latter precipitates 1.6-times faster than the former, which may be due to increased hydrophobicity of Ala compared with Gly at γ136.\(^{24}\) Our present results show that native oxy Hb F, which contains 40% αγ and 60% γγ, was about three-fold more stable than oxy Hb A, while the oxy form of Hb F γE6V with 100% αγ was more unstable than oxy Hb A and about 10-fold less stable than native oxy Hb F during mechanical agitation (Fig 3). These results are consistent with our recent studies showing a direct relationship between mechanical instability and hydrophobicity at the β6 position.\(^{25,26}\)

**Polymerization properties of the Hb F variants.** Polymerization of deoxy Hb S in vitro using the temperature-jump method is characterized by a delay time before polymer formation\(^{20,21,22}\) whose length depends on hemoglobin concentration: the lower the concentration, the longer the delay time. Polymer formation in vitro can be assessed using low and high phosphate buffers, both of which result in nucleation-controlled formation of ordered polymers.\(^{20,23}\) This process clearly differs from a simple ‘salting out’ phenomenon in high phosphate (2.2 mol/L) in which hemoglobin sample is directly injected into deoxygenated high phosphate buffer and results in rapid formation of amorphous aggregates.\(^{26}\) Using the temperature-jump method deoxy Hb F γE6V polymerized in 1.8 mol/L phosphate buffer at a hemoglobin concentration >175 mg/dL compared with about 50 mg/dL for deoxy Hb A.\(^{21}\) Under these high phosphate buffer conditions, deoxy Hb A required more than 2 g/dL\(^{27}\) and Hb F required more than 4 g/dL for polymerization. It is known that deoxy Hb F solubility is about two times >deoxy Hb A in high phosphate buffers.\(^{28}\) Deoxy Hb F γE6V at a concentration of 26 g/dL also formed gels in low phosphate buffer (0.1 mol/L), pH 7.0, at room temperature compared with 24 g/dL for deoxy Hb S.\(^{21}\) Under these high phosphate buffer conditions, deoxy Hb A required more than 2 g/dL\(^{27}\) and Hb F required more than 4 g/dL for polymerization. It is known that deoxy Hb F solubility is about two times >deoxy Hb A in high phosphate buffers.\(^{28}\) Deoxy Hb F γE6V at a concentration of 26 g/dL also formed gels in low phosphate buffer (0.1 mol/L), pH 7.0, at room temperature compared with 24 g/dL for deoxy Hb S, indicating that the former requires slightly higher concentrations for gelation than the latter. Solubility of deoxy Hb F γE6V in 0.1 mol/L phosphate buffer, pH 7.0 at 30°C was 19.3 g/dL compared with 17 g/dL for deoxy Hb S. Polymerization of deoxy Hb F γE6V in both low and high phosphate buffers (Figs 4 and 5) was, however, not accompanied with a delay time, even at lower hemoglobin concentrations where total polymer formed decreased (Figs 4 and 5A). Deoxy Hb F γE6V containing Thr-γ87 (Hb F γE6V, γQ87T) also polymerized in 1.8 mol/L phosphate buffer without a delay time at a similar concentration to deoxy Hb F γE6V (Fig 5A).

Total polymer formed as a function of hemoglobin concentration was also determined to define critical concentra-

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**Fig 2.** Separation of α and γ chains of the Hb F variants. Purified Hb F γE6V (b), Hb F γE6V, γQ87T (c) or native Hb F (a) was fractionated on an FPLC-Pro RPC column and individual chains were eluted using a gradient from 0.3% trifluoroacetic acid (TFA) in 39% CH\(_3\)CN (A buffer) to 50% CH\(_3\)CN (B buffer). H, α, γγ and αγ in (a) represent hemes, αγ, γγ, and αγ-globin chains, respectively.

**Fig 3.** Mechanical stability of the oxy form of the Hb F-Val-γ6 variant. The oxy form of Hb F γE6V (A) in 0.1 mol/L phosphate buffer, pH 8.0 at room temperature was mechanically agitated for varying times, and % soluble hemoglobin determined after centrifugation and compared with native Hb F (Δ), Hb A (○) and Hb S (○).
Fig 4. Kinetics of polymerization of Hb F \( \gamma \text{E6V} \) in a low phosphate buffer. Time courses are shown for polymerization of deoxy Hb F \( \gamma \text{E6V} \) at 33 g/dL (a) and 29 g/dL (b) in 0.1 mol/L phosphate buffer, pH 7.0 at 30°C using the temperature jump method.

Polymization properties of mixtures of Hb S and Hb F variants. We next studied polymerization of Hb S/Hb F variant mixtures to assess inhibitory effects of the Hb F variants on Hb S polymerization and to compare those results with mixtures of Hb S and native Hb F. 1:1 mixtures of deoxy Hb S/ Hb F \( \gamma \text{E6V} \) or Hb S/ Hb F \( \gamma \text{E6V}, \gamma \text{Q87T} \), polymerized in 1.8 mol/L phosphate buffer with a delay time when the concentration was about 2- and 1.5-fold, respectively, more than that required for deoxy Hb S polymerization (Fig 5). Logarithmic plots of delay time versus concentrations for Hb S/ Hb F \( \gamma \text{E6V} \) or Hb S/ Hb F \( \gamma \text{E6V}, \gamma \text{Q87T} \) mixtures showed straight lines shifted right 0.3 and 0.17 units from the line for Hb S, respectively (Fig 6). Hb S/ native Hb F mixtures also polymerized with a delay time.
but the line for Hb S/native Hb F mixtures was right shifted about 0.55 units from Hb S (Fig 7), which can be explained by exclusion of Hb F and FS hybrid from Hb S nucleation.11,22 Exclusion of FS hybrids effectively decreases Hb S concentration, and therefore, shifts the line further to the right, indicating that a higher Hb S concentration is required for polymerization in FS mixtures than with Hb S alone.

Polymerization of 1:1 mixtures of Hb A/Hb F γE6V, γQ87T were performed under the same conditions to further clarify whether Val-γ6 in the hybrid could interact with the hydrophobic acceptor pocket of an adjacent tetramer containing Thr-γ143 instead of Ser (Hb F γE6V, γQ87T, γS143T) required much higher concentrations for polymerization (~1.2 g/dL) than Hb F γE6V, γQ87T and also polymerized linearly like Hb F γE6V, γQ87T. Polymerization of 1:1 mixtures of Hb A/Hb F γE6V, γQ87T, γS143T were also performed under the same conditions to evaluate whether Val-γ6 in this hybrid could participate in polymerization with a delay time. A 1:1 mixture of Hb A/Hb F γE6V, γQ87T, γS143T, in fact, polymerized with a clear delay time (Fig 5D) when the concentration was >400 mg/dL. Under the same conditions, Hb A and Hb F γE6V, γQ87T, γS143T alone do not polymerize unless the concentrations are increased to ~2 and 1.2 g/dL, respectively. Logarithmic plots of delay time versus concentration for the Hb A/Hb F γE6V, γQ87T, γS143T mixture showed a straight line shifted right 0.88 units on the x axis from the line for Hb S (Fig 6). Because neither Hb A nor Hb F γE6V, γQ87T, γS143T alone formed polymers under these conditions, copolymerization properties for both are zero. The difference between the lines for Hb S and the Hb A/Hb F γE6V, γQ87T, γS143T mixture in Fig 6 is ~0.9. Copolymerization probability compared with Hb S alone (1.0) for the α2β2γ6Valγ87Thr hybrid is, therefore, calculated to be 0.25, which is half the value for α2β2γValγ87Thr hybrids. These results
show that Val-γ6 can interact with the hydrophobic acceptor pocket of an adjacent tetramer containing Thr-β87 like Val-β6 of deoxy Hb S, and that this hybrid can polymerize by a nucleation-controlled mechanism that is accompanied with a delay time before polymerization.

Total polymer formed as a function of hemoglobin concentration was also determined to define critical concentrations required for polymerization of Hb S/Hb F γ6E6V, Hb S/Hb F γ6E6V, γQ87T, and Hb A/Hb F γ6E6V, γQ87T mixtures. These results were compared with those for Hb S and the Hb S/Hb S β87T87Q mixture. Polymer formation increased linearly with increases in hemoglobin concentration (Fig 7).

DISCUSSION

Polymerization properties of the Hb F variants. Although the amino acid sequence of the γ chain of Hb F is analogous to that of the β chain, the sequence of the two chains differs at 39 of 146 residues. Twenty-two of these differences are located on the external surface of the molecule, and these differences may affect surface structure.26 Higher solubility of deoxy Hb F than that of deoxy Hb A can be attributed to differences in surface structure. Our results show that substitution of Val for Glu at the 6th position of the N-terminus of deoxy Hb F decreases solubility to the same extent as that comparing deoxy Hb A and deoxy Hb S, even though solubility of Hb F γ6E6V is higher than that of deoxy Hb S. These results indicate that Val at the 6th position of the N-terminus of Hb A and Hb F plays a key role in decreasing solubility and facilitating polymerization of deoxyhemoglobin; however, polymerization of deoxy Hb F γ6E6V in 1.8 mol/L or in 0.1 mol/L phosphate buffers was not accompanied with a delay time like deoxy Hb S. Formation of deoxy Hb F γ6E6V polymers can be explained by polymerization of dispersed monomeric hemoglobins without formation of nuclei before polymerization when the solution becomes oversaturated. This model is consistent with a linear polymerization mechanism.27

There are, however, several limitations in extrapolating results from polymerization studies in high phosphate buffer to those in low phosphate buffer. Under certain well-defined conditions, behavior of some modified Hb S tetramers differs when comparing solubility in high phosphate to gelation in low phosphate buffers.30,31 Therefore, some solubility studies in high phosphate buffer involving, for example, chemically-modified Hb S and the evaluation of antisickling agents must be interpreted with extreme caution because of differences between "salting in" and "salting out" effects in low and high phosphate buffers, respectively. Although there are some limitations to interpreting studies of modified deoxy Hb S in high phosphate buffer, there are many compelling findings and parallelisms to suggest that studies in high phosphate buffer will continue to be of importance in furthering our basic understanding of deoxy Hb S polymerization. For example, contact sites in crystals of deoxy Hb S made in polyethylene glycol or high phosphate buffer correspond to those of polymers made in low phosphate.32 In addition, relative order of inhibition of Hb S polymerization by Hb A, Hb F and Hb A; in 1.8 mol/L phosphate buffer is also similar to that in low phosphate buffer, and FS hybrid in FS mixtures is excluded during the initiation of polymerization in both low and high phosphate buffers.25

We found previously that substitution of β87 Thr to Gln in Hb S did not dramatically inhibit polymerization of deoxy Hb S alone in 1.8 mol/L phosphate buffer1; and, in this study we found that substitution of γ87 Gln to Thr had no effect on polymerization of Hb F γ6E6V alone. Furthermore, deoxy Hb F γ6E6V, γQ87T also polymerized without a delay time like deoxy Hb F γ6E6V, even though substitution of Thr for Gln at γ87 affects interaction of the variant FS hybrids with deoxy Hb S as discussed later. Polymers of deoxy Hb F γ6E6V and Hb F γ6E6V, γQ87T are expected to be different from those made by a nucleation-controlled mechanism and from amorphous precipitates made by quick "salting out" in a high phosphate buffer (2.2 mol/L).20 Under quick "salting out" conditions, deoxy Hb S molecules associate immediately after addition into the deoxygenated high salt solution. In 1.8 mol/L phosphate buffer using the temperature-jump protocol for polymerization, deoxy Hb S molecules remain dispersed by incubation at 0°C and then the temperature is quickly raised to 30°C, which results in stepwise association of monomeric Hb S, formation of polymers when oversaturation occurs, and finally, ordered polymers at least in the case of deoxy Hb S polymer formation by a nucleation-controlled mechanism.29 Further studies are now required to address whether these Hb F variants form ordered polymers involving heterogeneous nuclei, even though absence of a delay time infers inability to form homogeneous nuclei during the initial phase just before measurable polymerization.33

Crystals of deoxy Hb F made in a high salt have been analyzed by x-ray diffraction.28 Comparison of Fourier electron-density maps of deoxy Hb F and deoxy Hb A show little, if any differences, except where γ- and β-globin chains differ in primary sequence. The only detectable differences are seen in the two N-terminal regions where the NA region of the helix is more distal to the EF region and the H helix in the γ chain, and the A helix is closer to the E helix than in the β chain.28 Our results on polymerization of 1:1 mixtures of Hb A/Hb F γ6E6V, γQ87T, γS143T suggest that Val-γ6 can interact with the hydrophobic acceptor pocket of an adjacent tetramer containing Thr-β87 like Val-β6 of deoxy Hb S, and that this hybrid can polymerize by a nucleation-controlled mechanism. Although differences between γ and β chains in amino acids located in the A helix (ie, Gly (NA1), Phe (NA3) Glu (A2), Asp (A4) Ala (A6), and Ser (A10) in γ versus Val, Leu, Pro, Glu, Ser, and Ala in β) do not appear to eliminate hydrophobic interactions, Val-γ6 can interact with the EF acceptor pocket of the β chain in mixtures of the Hb F variants with either Hb S or Hb A. These results suggest that although Val at A3 position of hemoglobin plays a key role in decreasing solubility, nuclei formation that occurs before polymerization of deoxy Hb S is not simply controlled only by Val-β6. Furthermore, these findings indicate that other critical amino acids in addition to the EF acceptor pocket containing Phe-β85, Leu-β88, and Thr-β87 contribute to nuclei formation and Hb S polymer-
compared with Hb between the line for Hb mixture is 0.17 units, which indicates that the probability which is similar to AS hybrids. In contrast, the difference Thr-87 in the line for Hb does not, suggesting that inhibition of polymerization po-
ticipation in Hb polymerization of Hb S/Hb F variant mixtures using 1.8 mol/L phosphate buffer employing temperature jump methods. In addition, the finding that Hb S/Hb F γE6V, γQ87T mixtures polymerize more readily than deoxy Hb F γE6V, γQ87T, which may be caused by perturbation of the R-state quaternary structure caused by the Thr-γ143 substitution even though the β143 position in Hb S is not a contact site. Understanding the basis for these differences may prove useful in deciphering protein-protein interactions that facilitate secondary tetramer communication, which results in formation of ordered polymers. It may also help in defining variant hemoglobin for use as antisickling agents with superior properties to Hb F for use in gene-therapy strategies for sickle cell disease.

Polymerization properties of mixtures of Hb S and the Hb F variants. Previous studies comparing naturally occurring variants indicated that γ- and δ-87 Gln are important sites for inhibition of Hb S polymerization by Hb F and Hb A₂, respectively. Our recent studies using mixtures of Hb S with the recombinant double mutant Hb S βT87Q (αβGlu-Val, 8T7Val-Glu) variant also suggest that the presence of Gln at γ87 in Hb F is important for exclusion of FS hybrids from Hb S polymers, which results in inhibition of Hb S polymerization in FS mixtures.11 Thr-β87 in 1-β₁ of deoxy Hb S is not a direct contact site for Val-β6, but is involved in lateral contacts with Ser-β9, Ala-β10, and Ala-β13 in 1-β₂ of Hb S polymers. Thr-β87 in Hb S is involved in interactions between parallel double strands in crystals or fibers and also forms strong hydrogen bonds with 1α₁, 139Lys and 2α₂, 81Ser. To further clarify the role of Gln-γ87 in the inhibition of deoxy Hb S polymerization by Hb F, we studied polymerization of Hb S/Hb F variant mixtures using 1 mol/L phosphate buffer.

1:1 mixtures of deoxy Hb S/Hb F γE6V or Hb S/Hb F γE6V, γQ87T polymerized with a delay time in 1.8 mol/L phosphate buffer employing temperature jump methods. In addition, the finding that Hb S/Hb F γE6V, γQ87T mixtures polymerize more readily than deoxy Hb F γE6V hybrids suggests that a higher proportion of αβγE6V,8Q7T hybrids participate in Hb S polymerization compared with αβγE6V hybrids. Half of the αβγE6V,8Q7T hybrids participate in nucleation and polymerization with Hb S, while the other half does not, suggesting that inhibition of polymerization potenti-ated by the γ-globin chain is primarily in trans to the Val-β6 contact and is caused mainly by the change from Thr to Gln at γ87 on the F-helix, as suggested previously.3 In these mixtures, αβγE6V,8Q7T hybrids participate in nucleation with Hb S only when Val-β6 or Val-γ6 appropriately contacts an acceptor pocket on an adjacent tetramer containing Thr-87 in the β₃ chain. Because the difference between the line for Hb S and the Hb S/Hb F γE6V mixture is ~0.3 units on the x axis in Fig 6, the probability for nuclei formation compared with Hb S (1.0) for αβγE6V,8Q7T hybrid is about 0.5, which is similar to AS hybrids. In contrast, the difference between the line for Hb S and the Hb S/Hb F γE6V, γQ87T mixture is 0.17 units, which indicates that the probability for nuclei formation for αβγE6V,8Q7T hybrids is 0.85. These results show that a higher proportion of αβγE6V,8Q7T hybrids participate in polymerization with Hb S compared with αβγE6V hybrids, and that other sequence differences in the γ chains besides Gln-γ87 contribute to inhibition of Hb S polymerization.

It is interesting to note that the lines for the Hb S/Hb F γE6V mixture and the recently reported Hb S/Hb S βT87Q mixture were similar.11 X-ray crystallographic studies show that only one Val-β6 in each Hb S tetramer is involved in forming an intermolecular bond in the polymer. Therefore, these results indicate that Gln-γ87 is critical for exclusion of αβγE6V hybrids in FS mixtures from nuclei formation with Hb S. These studies also show that Val-β6 can interact with the acceptor pocket formed by Phe at γ85, Thr at γ87, and Leu at γ88 in αβγE6V,8Q7T hybrids. These amino acids are identical to those that form the Val-β6 acceptor pocket in Hb S. These results show that the hydrophobic acceptor pocket involving β87-Gln in the Hb S/Hb S βT87Q hybrid also promotes exclusion of hybrid, and that Val-γ6 can participate as a donor site like Val-β6 in Hb S when the acceptor site is a β-chain containing Thr-β87. Further studies are now required to define polymer structure of the Hb F variants and to evaluate the role of other sites in promoting deoxy Hb S polymerization by a nucleation-controlled mechanism.

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