Role of β87 Thr in the β6 Val Acceptor Site during Deoxy Hb S Polymerization†

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ABSTRACT: Three new Hb S variants containing β87 Leu, Trp, or Asp instead of Thr were expressed in yeast in order to further define the role of the β87 position in stability and polymerization of deoxy Hb S. Previous studies showed that hydrophobicity at β85 Phe and β88 Leu is critical for stabilization of hemoglobin. Results with the three Hb S β87 variants, however, showed minimal differences in stability, suggesting that β87 amino acid hydrophobicity is not critical for stabilization of hemoglobin. Polymerization properties of the variants in the deoxy form, however, were affected by the β87 amino acid. Polymerization of Hb S β87 Thr → Leu and Hb S β87 Thr → Trp was preceded by a delay time like Hb S, while Hb S β87 Thr → Asp did not show a delay time. In addition, changes in time required for half polymer formation ($T_{1/2}$) as a function of hemoglobin concentration for Hb S β87 Thr → Asp were similar to that for β87 Thr → Gln. Hb S β87 Thr → Leu polymerized at a lower hemoglobin concentration than Hb S while β87 Thr → Trp and Hb S β87 Thr → Asp required much higher hemoglobin concentrations for polymer formation. Critical concentration required for deoxy Hb S β87 Thr → Asp polymerization was 6- and 2.3-fold greater than that for Hb S β85 Phe → Glu and Hb S β88 Leu → Glu, respectively. These results suggest that even though β87 Thr is not a direct interaction site for β6 Val in deoxy Hb S polymers, it does play a critical role in formation of the hydrophobic acceptor pocket which then promotes protein−protein interactions facilitating formation of stable nuclei and polymers of deoxy Hb S.

Hydrophobic acceptor pocket formation between the E and F helices which is present in deoxyhemoglobin but absent in oxyhemoglobin is in addition to β6Val, critical to induce Hb S polymerization (*1*). The acceptor pocket is lined at the bottom with the side chains of β85 Phe and β88 Leu and has β73 Asp, β84 Thr, and β87 Thr located around the pocket perimeter (*2*, *3*). β87 Thr is located on the F helix (F3) and does not interact directly with the donor β6 Val in deoxy Hb S polymers. Elucidation of the role of the pocket perimeter in the acceptor pocket is critical to understand the hydrophobic interactions of deoxy Hb S. β87 Thr in 1-β_1 is also involved in a lateral contact with β9 Ser, β10 Ala, β13 Ala, and β126 Val in 1-β_2 in Hb S fibers.

Our previous results suggested that hydrophobic amino acids like Phe and Leu at β85 and β88, respectively, are critical for maintaining the hydrophobic acceptor pocket for β6 Val in deoxy Hb S polymers and for promoting hemoglobin stability (*4*−*6*). The role of hydrophobicity at the β87 position in promoting stability and polymerization of Hb S is, however, not clear. Furthermore, differences at residue non-α 87 comparing Thr versus Gln in Hb A and Hb A_2 or Hb F, respectively, appear responsible for inclusion of AS versus exclusion of A_2S or FS hybrids in deoxy Hb S polymers (*7*−*10*).

Polymerization of deoxy Hb S is characterized by a marked delay period which is followed by a dramatic and autocatalytic increase in the formation of polymers which can be explained by a nucleation-controlled polymerization mechanism (*11*, *12*). Our recent studies with deoxy Hb A_2 Eδ6V, which contains δ87 Gln, showed decreased solubility like deoxy Hb S (*10*). Polymer formation occurred *in vitro* with this variant, suggesting that Val at the 6th position in non-α-chains is critical for decreasing tetramer solubility; however, polymerization was not accompanied with a delay time like Hb S. The critical concentration required for polymerization of Hb A_2 δE6V was slightly higher than deoxy Hb S (1.5-fold) and less than half of that for Hb F γE6V (*9*, *10*). Engineering an additional change of Gln to Thr at δ87 in Hb A_2 δE6V (Hb A_2 δE6V, δQ87T) promoted polymerization in the deoxy form after a clear delay time like deoxy Hb S. Other studies show that β87 Thr → Ile of Hb S accelerates nuclei formation while β87 Thr → Gln inhibits nuclei formation (*8*, *13*). Furthermore, recent studies showed that β87 Thr → Lys in Hb S inhibited polymerization, suggesting an important role for β87 Thr in Hb S polymerization (*14*). In addition, the critical concentration required for deoxy Hb S βT87Q polymerization was only 1.4-fold higher than that of deoxy Hb S, and mixtures of Hb S βT87Q and Hb S, like mixtures of Hb S and Hb F or Hb A_2, did not copolymerize (*8*). These results suggest that even though β87 Thr in deoxy Hb S is not a direct interaction site with β6 Val, β87 Thr is a critical amino acid which helps facilitate nuclei formation prior to polymerization. However,

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details of the role of β87 Thr in the acceptor pocket of deoxy Hb S and of β87 amino acid specificity for nucleation prior to polymerization as well as effects on stability are not clear. In this report, we assess overall stability and polymerization properties of three new β87 Hb S variants in order to further define the role of the β87 amino acid in Hb S stability and polymerization.

MATERIALS AND METHODS

The plasmid pGS389 β^s contains the full-length human α- and β^s-globin cDNAs under transcriptional control of dual GGAP promoters, as well as a partially functional yeast LEU2d gene and the URA3 gene for selection in yeast (*15, 16*). The plasmid pGS189 β^s contains a single GGAP promoter and β^s-globin cDNA and was constructed by mutagenesis and subcloning as described previously (*16*). The basic strategy for site-specific mutagenesis at β87 involves recombination/polymerase chain reaction as described previously (*8, 16*). β87 mutants were subjected to DNA sequence analysis of the entire β-globin cDNA using site-specific primers and fluorescently-tagged terminators in a cycle sequencing reaction in which extension products were analyzed on an automated DNA sequencer (*17*). The mutated β-globin cDNA region was then excised by *Xho*I digestion and subcloned back into the *Xho*I site of the expression vector pGS389 (*16*).

Yeast growth, plasmid transformation, induction, and purification of recombinant hemoglobin tetramers were described previously (*16, 18*). The β87 Hb S variants were purified using FPLC with a Mono S column (*18*). In order to identify the molecular masses of β-chain variants, electrospray ionization mass spectrometry (ES/MS) was performed on a VG BioQ triple quadrapole mass spectrometer (Micromass, Altrincham, Cheshire, U.K.) (*19*). The multiply charged ions derived from α globin (M_r = 15 126.4) served as internal and external standards for mass scale calibration. Data analysis employed the MassLynx software package (Micromass, Altrincham, Cheshire, U.K.). Absorption spectra of the variants were recorded using a Hitachi U-2000 spectrophotometer (Hitachi Instruments Inc. Danbury, CT).

Cellulose acetate electrophoresis of hemoglobin solutions was performed at pH 8.6 using Super-Heme buffer (Helena Lab., Beaumont, TX), and hemoglobin concentration was determined spectrophotometrically on a Hitachi U2000 spectrophotometer using millimolar extinction coefficients of 13.5 at 541 nm for oxyhemoglobin and 13.4 at 540 nm for carbonmonoxy hemoglobin (*20*). Oxygen dissociation curves were determined in 0.1 M phosphate buffer, pH 7.0, at 20 °C using a Hemox Analyzer (TCS Med. Co., Huntington Valley, PA) (*21*). Circular dichroism (CD) spectra of the variants were recorded using an Aviv-Model 62 DS instrument (Varian Analytical Instruments, San Fernando, CA) with a thermoelectric module. Spectra from 190 to 280 nm were recorded for the CO forms of β87 Hb S variants (∼10 μM; 1 cm light path cuvette) in 0.01 M phosphate buffer, pH 7.0, at 10 °C. CD spectra in the near-UV (260−340 nm using ∼30 μM; 1 cm light path cuvette) and visible regions (340−440 nm using ∼30 μM; 1 mm light path cuvette) were recorded for the CO forms of the Hb S variants. Studies of kinetics of polymerization of deoxy Hb S were performed in 1.8 M phosphate buffer, pH 7.4, at 30 °C as described previously by the temperature-jump method (*12*). Thermal denaturation of hemoglobins was evaluated by monitoring temperature-induced changes in ellipticity at 222 nm using a temperature control unit and by repetitive scanning of the absorption spectra of hemoglobins in 10 mM phosphate buffer at constant temperature using a Hitachi U-3000 absorption spectrophotometer (*6*).

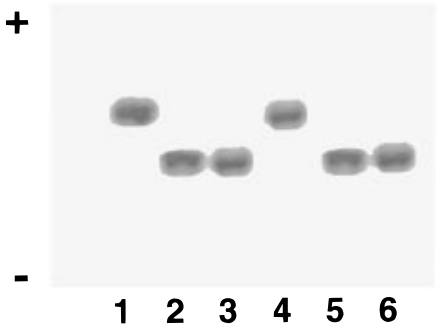

FIGURE 1: Cellulose acetate electrophoresis of β87 Hb S variants. Mobilities of β87 Hb S variants [Gln-β87 (lane 3), Asp-β87 (lane 4), Trp-β87 (lane 5), and Leu-β87 (lane 6)] were compared with native Hb A (lane 1) and Hb S (lane 2) following cellulose acetate electrophoresis.

RESULTS

Characterization of β87 Hb S Variants. We selected three amino acids, (e.g., Leu, Trp, and Asp) for substitution at β87 Thr in Hb S in order to further define the role of the β87 amino acid in stability and polymerization of Hb S. Leu is more hydrophobic and Trp is more bulky and larger than the normal β87 Thr, while Asp is negatively charged, hydrophilic, and similar in size to Thr. Purified recombinant Hb S variants containing β87 Leu, Trp, or Asp migrated as single bands following cellulose acetate electrophoresis at pH 8.6. Mobilities of Hb S βT87L and Hb S βT87W were identical to those of Hb S and Hb S βT87Q (*8, 16*), while surface charge of Hb S βT87D was more negative than that of Hb S and similar to that of Hb A (Figure 1). Mass spectral analysis showed expected β-globin chain molecular masses of 15 849, 15 922, and 15 850 Da for β^s 87 Leu, Trp, and Asp, respectively. Absorption spectra of the CO forms of these Hb S variants in the visible range were the same as those of native and recombinant Hb S (*16*), except for Hb S βT87W, which showed a slightly higher absorbance in the UV range, no doubt caused by the additional tryptophan. These results suggest that there are no major effects of these β87 substitutions on heme−globin interactions. Circular dichroism spectra in the region from 190 to 440 nm for the β87 Hb S variants were similar to that of native Hb S (data not shown), indicating that these substitutions do not significantly affect globin folding and/or overall secondary/tertiary structure of hemoglobin tetramers.

Oxygen affinity of Hb S βT87W was slightly increased in comparison with that of Hb S, while affinities of the other two β87 Hb S variants, Hb S βT87L and Hb S βT87D, were similar to that of Hb S. P_{50} values in 0.1 M phosphate buffer, pH 7.0, at 20 °C for Hb S containing β87 Leu, Trp, or Asp were 6.7, 4.5, and 6.0, respectively, compared with 6.5 for recombinant Hb S[1] (*8*). Hill coefficients for these variants were similar to Hb S (2.8−2.9).

[1] The range of the P_{50} values for Hb S is ±0.2 under these conditions and the values for the variants represent the mean of two measurements.

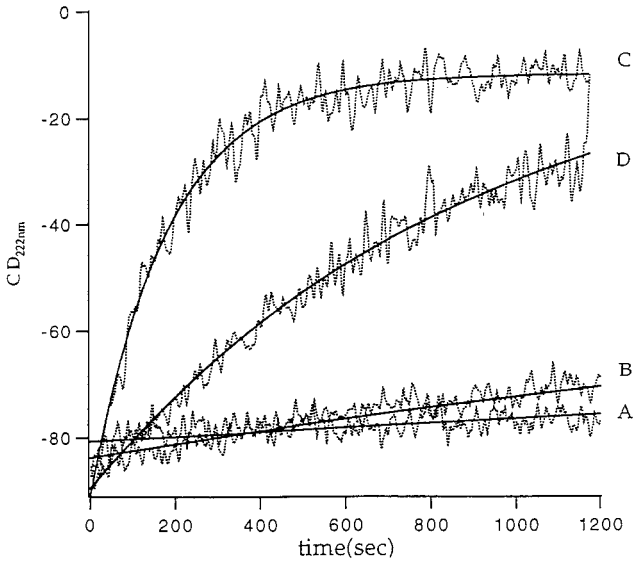

FIGURE 2: Thermal stability of Hb S, Hb S βT87D, Hb S βF85E, and Hb S βL88E measured by temperature-induced changes in ellipticity at 222 nm as a function of time at 60 °C. CD spectral changes at 60 °C for the CO form of Hb S βT87D (10 μM) (B) at 222 nm were compared with those of Hb S (A), Hb S βL88E (C), and Hb S βF85E (D) in 10 mM phosphate buffer, pH 7.4.

Table 1: Thermal Denaturation Rate Constants of β87 Hb S Variants at 60 °C[a]

	rate constant, k (1/s) (10^{-5})
Hb A	9.3
Hb S	16.1
Hb S βT87Q	37.7
Hb S βT87W	64.7
Hb S βT87L	37.9
Hb S βT87D	33.3

[a] Spectral changes in CD ellipticity at 222 nm for each sample of hemoglobin at 10 °C were evaluated as a function of time of incubation at 60 °C. Changes in the CD spectra under these experimental conditions follow first-order kinetics and rate constants were calculated as described previously (*6*).

Effect of β87 Amino Acid on Stability of Hb S. Thermal stability of the three Hb S β87 variants in the CO form was measured by monitoring temperature-induced CD changes in ellipticity at 222 nm as a function of time at 60 °C (*6*). These results were then compared with our previous results for Hb S βF85E, Hb S βL88E, and Hb S (*6*). Our previous findings showed that substitution of the hydrophobic amino acids β85 Phe or β88 Leu for a hydrophilic Glu at β85 and/or β88 resulted in dramatic decreases in tetramer stability (*6*). Changes in ellipticity at 222 nm as a function of incubation time at 60 °C for CO-Hb S Tβ87D were minimal like Hb S (Figure 2). Even though the hydrophilic amino acid β87 Asp is present on the F helix, this tetramer in the CO form was as stable as Hb S (*6*). CD changes were more dramatic for Hb S Fβ85E than for Hb S and Hb S Tβ87D and plateaued after about 400−600 s at 60 °C. These results suggest rapid temperature-induced (4 °C → 60 °C) unfolding for Hb S βF85E, while Hb S Tβ87D and Hb S remain in the CO form relatively stable under these conditions (Figure 2) (*6*). In addition, CD changes follow first-order kinetics. Results of thermal denaturation for the other β87 Hb S variants are shown in Table 1. The relative ratios standardized to Hb A of the initial rates of change for Hb S βF85E, Hb S βL88E, Hb S βT87D, Hb S, and Hb A were 12.3:60.2:3.5:1.7:1, respectively. Furthermore, CD changes of the four β87 Hb S variants were similar and the relative ratios of Hb S βT87D:Hb S βT87Q:Hb S βT87L:Hb S βT87W were 1:1.1:1.1:1.9, respectively. These results suggest that hydrophobic residues at both the β85 and β88 positions but not at β87 are critical for stabilization of hemoglobin.

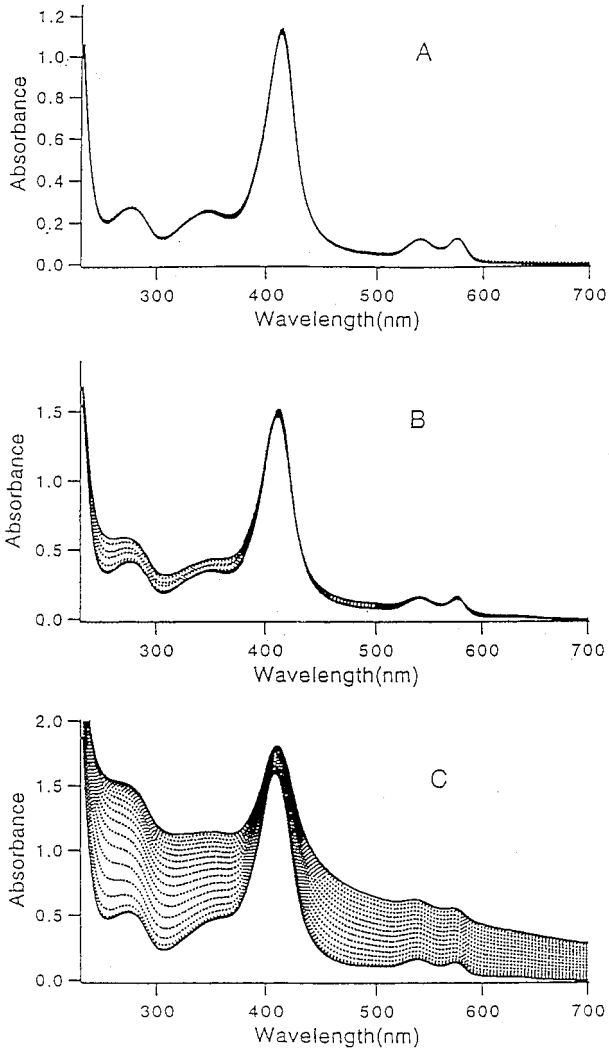

FIGURE 3: Thermal stability of oxy Hb S βT87D. Thermal stability of oxy Hb S βT87D in 10 mM phosphate buffer pH 7.4 was assessed at 45 °C using a Hitachi U3000 spectrophotometer using repetitive scanning (20 times) of absorption spectra from 220 to 700 nm. Results for oxy Hb S βT87D (B) were compared to those of oxy Hb S (A) and Hb S βF85E (C).

We also measured stability of the oxy form of Hb S βT87D at constant temperature (45 °C) by repetitive scanning of the absorption spectra between 200 and 700 nm at 1 min intervals and compared those results with our previous results for negatively charged β85 and β88 Glu substitutions (Figure 3) (*6*). Our previous results showed that Hb S βF85E and Hb S βL88E were very unstable compared with Hb S and Hb A (*6*). The total peak heights and baseline values of the absorption spectra of these β85 abd β88 Hb S variants changed as a function of time of incubation (*6*). Under these conditions positions of absorption spectra peaks for all hemoglobin samples in the visible range were not significantly different, even though total peak heights and baseline values changed as a function of incubation time. Changes in baseline for Hb S Fβ85E and Hb S βL88E as a function of time are caused by increased turbidity due to oxydation of heme and quick formation of precipitated methemoglobin

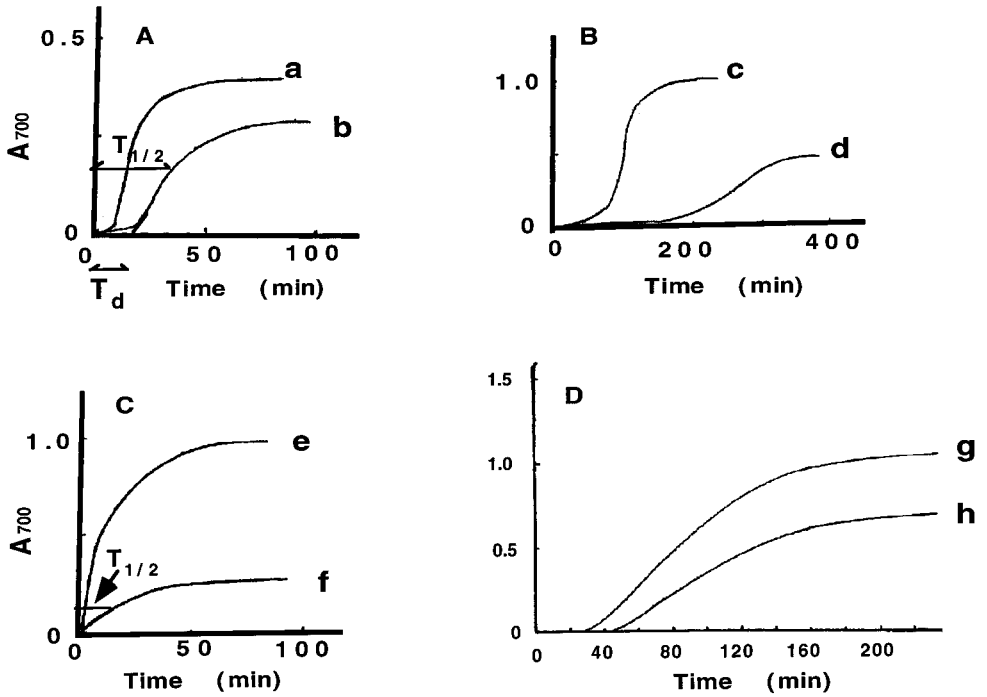

FIGURE 4: Kinetics of polymerization of β87 Hb S variants. Time course of polymerization for deoxy forms of β87 Hb S variants was defined using 1.8 M phosphate buffer, pH 7.4 at 30 °C by the temperature-jump method as described previously (*10*). Results: (A) 54.6 (a) and 46.5 (b) mg/dL of deoxy Hb S Tβ87L; (B) 374 (c) and 307 (d) mg/dL of deoxy Hb S Tβ87W; (C) 1143 (e) and 762 (f) mg/dL of deoxy Hb S Tβ87D. These results were then compared with our previous results: (D) 165 (g) and 115 (h) mg/dL of deoxy Hb S Tβ87Q (*8*).

(*6*). Soret band peaks (~410 nm) and visible range peak positions for the oxy form of Hb S βF85E and Hb S βL88E were significantly altered with increasing turbidity during incubation (*6*). However, results for oxy Hb S βT87D only slightly changed, possibly by oxidation of heme but this change was much less than that of Hb S βL88E, compared with oxy Hb S which was quite stable under these conditions. These results also suggest that hydrophobicities at both the β85 and β88 positions but not at β87 are critical for stabilization and oxidization of hemoglobin.

Polymerization Properties of β87 Hb S Variants. Polymerization of deoxy Hb S is characterized by a delay time prior to polymer formation whose length depends on hemoglobin concentration: the lower the concentration, the longer the delay time (*11*, *12*). Presence of a delay time as well as the shape of the kinetic progress curve were dependent on the type of β87 amino acid (Figure 4). Polymerization of Hb S βT87L and Hb S βT87W was preceded by a delay time like deoxy Hb S, while polymerization of Hb S βT87D showed no delay time (Figure 4C). In addition, Hb S βT87L polymerized at a lower hemoglobin concentration than Hb S, while Hb S βT87W and Hb S βT87D required much higher concentrations for polymer formation. It is noteworthy that a recent study showed a delay time for polymerization of Hb S βT87K even though this substitution inhibited polymerization (*14*). In contrast, our previous studies showed that Hb S βT87Q polymer formation was preceded by a delay time, but the kinetic progress curve for polymer formation was linear and not sigmoidal like deoxy Hb S (Figure 4D) (*8*).

It is also informative to compare times required for half polymer formation ($T_{1/2}$) when assessing polymerization properties of variants like Hb S βT87D or Hb S βT87Q, which either do not exhibit a delay time or do not show sigmoidal kinetic progress curves for polymer formation. In this case, $T_{1/2}$ represents the time required to form one half of the total amount of polymer (see panels A and C in Figure 4) and is related to hemoglobin concentration and critical concentration required for polymerization just like the delay time prior to polymerization of Hb S. Logarithmic plots of $T_{1/2}$ versus hemoglobin concentration showed straight lines (Figure 5) with slopes of 3.2, 3.2, 0.45, 6.5, and 0.45 for Hb S βT87L, Hb S, Hb S βT87Q, Hb S βT87W, and Hb S βT87D, respectively. The line for Hb S βT87L shifted left from the Hb S line, while those for Hb S βT87Q, Hb S βT87W, and Hb S βT87D shifted right from the Hb S line. Interestingly, slopes of the lines for Hb S βT87Q and Hb S

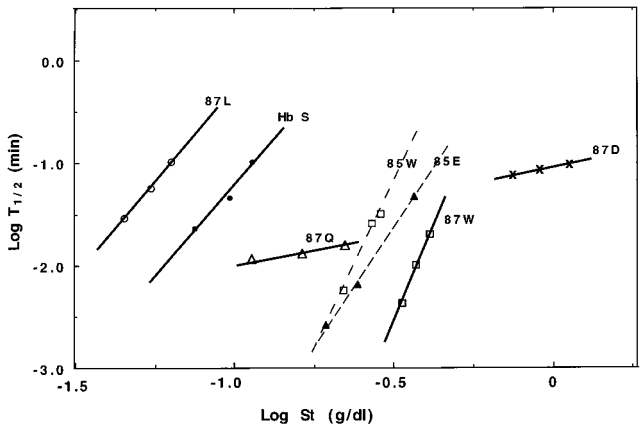

FIGURE 5: Relationship between log of reciprocal $T_{1/2}$ for polymerization and Hb concentration for the β87 Hb S variants. Logarithmic plots of half time for polymerization (*y*-axis) versus hemoglobin concentration (*x*-axis) for the deoxy forms of β87 Leu (○, 87L), β87 Trp (□, 87W), β87 Asp (×, 87D) and β87 Gln (△, 87Q) Hb S variants (solid lines) were compared with native Hb S (●, Hb S). Our previous results for variants containing β85 Trp (□, 85W), β85 Glu, and (▲, 85E) are also shown (dotted lines from refs 4 and 5).

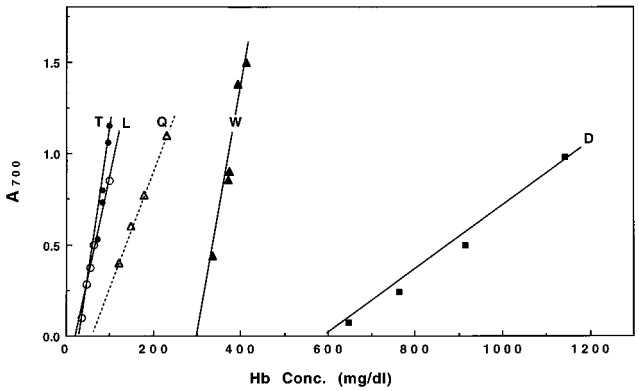

FIGURE 6: Polymer formation as a function of hemoglobin concentration for β87 Hb S variants. Polymer formation in 1.8 M phosphate buffer as measured by light scattering 700 nm at the plateau of the polymerization curve is shown (*y*-axis) as a function of hemoglobin concentration for the deoxy forms of the different β87 Hb S variants (*x*-axis). T (Hb S), L, Q, and D represent Thr, Leu, Gln, and Asp at β87 in Hb S, respectively.

βT87D were the same, indicating that changes in $T_{1/2}$ as a function of hemoglobin concentration for Hb S βT87Q and Hb S βT87D are the same. These results suggest that the inhibitory effects on polymer formation by substitution of Gln for Thr is similar to that for Asp even though the critical concentrations required for polymer formation of these two Hb S β87 variants differ.

Rates of polymerization for Hb S βF85W and Hb S βT87W were also analyzed by measurement of $T_{1/2}$ as a function of hemoglobin concentration, and those results were compared with our previous findings for Hb S βL88A (Figure 5) (*5*, *22*). Logarithmic plots of $T_{1/2}$ versus hemoglobin concentration for Hb S βF85W and Hb S βT87W were right-shifted from Hb S and had similar slopes, with the line for Hb S βT87W being more right-shifted than that for Hb S βF85W. Interestingly, the line for Hb S βT87W was slightly more right-shifted compared to the Hb S βL88A line (*22*).

Total polymer formed as a function of hemoglobin concentration was also determined in order to evaluate effects of the β87 substitutions on the critical concentration required for polymerization (Figure 6). Critical concentrations required for polymerization depend on solubility: the higher the solubility, the higher the concentration required for polymerization. Polymer formation of the β87 Hb S variants in the deoxy form in 1.8 M phosphate buffer was determined at the plateau of the polymerization curves and increased linearly with increases in initial hemoglobin concentration (*4*, *5*, *12*). Critical concentrations are then determined by extrapolation of the lines to zero turbidity (Figure 6). Critical concentrations for Hb S βT87L, Hb S βT87W, and Hb S βT87D were 0.8-, 6.3- and 13-fold higher, respectively, than that of Hb S (Figure 7). We previously showed that the value for Hb S βT87Q was 1.4-fold higher than deoxy Hb S (*8*). It is also noteworthy that critical concentration required for Hb S βT87K polymerization was intermediate between those for Hb S βT87Q and Hb S βT87W (*14*). Furthermore, the critical concentration required for deoxy Hb S βT87D polymerization was 6.2- and 2.3-fold greater than that for Hb S βF85E and Hb S βL88E, respectively (see Figure 7, and ref 4). In addition, critical concentrations for Hb S βF85W and Hb S βT87W were 3- and 6.3-fold higher than that of deoxy Hb S, respectively (*5*).

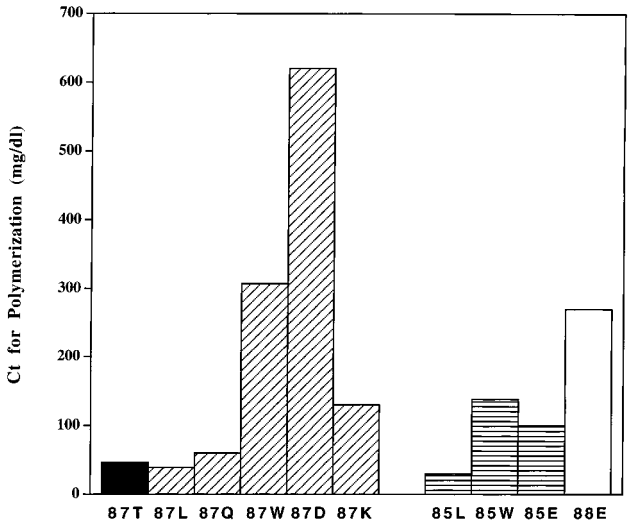

FIGURE 7: Critical concentration required for polymerization of the β87 Hb S variants in comparison to that of the β85 and β88 Hb S variants. Critical concentrations required for polymerization of the β87 Hb S variants [β87 Leu (87L), β87 Trp (87W), β87 Gln (87Q), and β87 Asp(87D)] were compared to those for Hb S (87T) and our previous results for β85 and β88 Hb S variants (*4*, *5*). 85L, 85W, and 85E represent results for Hb S variants containing β85 Leu, β85 Trp, and β85 Glu, respectively, while 88E represents results for the β88 Glu Hb S variant (*4*). Critical concentration required for polymerization of Hb S Tβ87K (87K) was also calculated from results of Ho *et al.* (*14*).

DISCUSSION

β87 Thr is an external residue located on the F helix (F3) of hemoglobin and is not close to the heme area compared with β85 Phe and β88 Leu. In contrast, β85 Phe (F1) and β88 Leu (F4) are also located on the F helix but are near porphyrin in the heme pocket of the molecule (*23*). Heme is stabilized by the hydrophobic cleft made by these hydrophobic amino acids. Furthermore, the side chain of β88 Leu (F4) contacts the heme side chain, and binding of heme to globin involves a specific stereochemical fit that helps stabilize the tertiary conformation of the subunit. β85 Phe (F1) acts as a spacer between the F and H helical segments to stabilize β-chain subunits (*23*). These two hydrophobic amino acids, β85 Phe and β88 Leu, on the F helix play an important role in maintaining the hydrophobic environment of the heme pocket and in protecting against entrance of water molecules, while β87 Thr exists externally and can interact with water molecules and may not contribute to formation of the hydrophobic cleft for heme. Therefore, it is not surprising that even though previous studies showed that changes from β85 Phe and/or β88 Leu to β88 Glu promote instability (*6*), changes from β87 Thr to Asp show minimal differences in stability. However, since β87 Thr is located on the perimeter of the hydrophobic acceptor pocket for β6 Val and is involved in a lateral contact with 1-β_2 9 Ser, 1-β_2 10 Ala, β 1-β_2 13 Ala, 1-β_2 126 Val, 1α_1 139 Lys, and 2-α_2 81 Ser in Hb S fibers, amino acid substitutions at this position might be expected to influence polymerization of Hb S (*2*, *3*).

Polymerization of deoxy Hb S is characterized by a delay time prior to polymer formation, which facilitates formation of ordered polymers (*11*, *12*). Polymerization of Hb S βT87L and Hb S βT87W was accompanied with a delay time and their polymerization curves were sigmoidal like Hb S, which can be explained by a nucleation-controlled

polymerization mechanism like deoxy Hb S (*1, 11, 12, 24*). In addition, Ho *et al.* recently reported that Hb S βT87K polymerized with a delay time even though the critical concentration required for polymerization was much higher than that of Hb S (*14*). The logarithmic plots of $T_{1/2}$ versus hemoglobin concentration for Hb S βT87L and Hb S βT87W shifted left and right, respectively, from the Hb S line (Figure 5). These results are a reflection of differences in critical concentrations required for polymerization of Hb S and the β87 Hb S variants. Ratios of critical concentrations required for polymerization of Hb S βT87L/Hb S and Hb S βT87W/Hb S were 0.8 and 6.3, respectively. These results indicate that the β87 Thr → Leu change in deoxy Hb S accelerates nucleation and polymerization by promoting protein−protein interactions, which may be caused by the increased hydrophobicity of Leu. In contrast, the β87 Thr → Trp as well as β87 Thr → Lys changes inhibit protein−protein interactions in addition to changing the hydrophobic environment of the acceptor pocket, resulting in a requirement for higher hemoglobin concentrations for polymerization.

Our previous results showed that the kinetic progress curve of polymer formation for Hb S βT87Q was linear and not sigmoidal like deoxy Hb S, even though their solubilties were similar (*8*). In the current study, the kinetic progress of polymerization for Hb S βT87D was also linear. However, critical concentration required for Hb S βT87D polymerization was 13 times higher than that for deoxy Hb S compared to only 1.4-fold for Hb S βT87Q. The slopes of the lines for logarithmic plots of $T_{1/2}$ versus hemoglobin concentration for Hb S βT87Q and Hb S βT87D were very similar, but differed from those for Hb S βF85E and Hb S βL88E (Figure 5). These results suggest that the β87 Thr → Gln and β87 Thr → Asp changes cause similar inhibitory effects on polymer formation as reflected in the nonsigmoidal kinetic progress curves. Our previous results also showed that Hb A_2 δE6V, which contains δ87 Gln, polymerized linearly without a delay time (*10*). Furthermore, our results of Hb S/Hb A_2 δE6V and Hb S/Hb S βT87Q mixtures showed little participation of Hb A_2 δE6V or Hb S βT87Q in formation of nuclei with Hb S (*10*). These findings indicate that β87 Gln or δ87 Gln does not interact with an adjacent Hb S molecule like β87 Thr and that Gln at position 87 results in exclusion of $\alpha_2\beta^S\beta^{87Q}$ and $A_2S(\alpha_2\beta^S\delta)$ hybrids from Hb S nuclei and/or polymers. Inhibitory effects of Hb A_2 and Hb F on Hb S polymerization can be attributed to this property (*9, 10*). Our results also demonstrate that determination of critical concentrations required for polymerization or solubility are not sufficient in order to evaluate inhibitory effects on deoxy Hb S polymerization by amino acid substitutions or antisickling agents. Analysis of the kinetic progress curves for polymer formation are also important.

Our recent structural analysis of deoxy Hb S crystals at 2.0 Å resolution revealed some marked differences from the previous 3.0 Å resolution structure, including several residues in the lateral contact which have shifted by as much as 3.5 Å (*2, 3, 25*). The double strand is stabilized by lateral contacts involving the β6 Val interacting with an acceptor pocket between EF helices on another tetramer. The lateral contacts include, in addition to the hydrophobic interactions involving Val β6, bridging water molecules at the periphery of the acceptor pocket involving β87 Thr. Most of these water molecules are well-ordered molecules arranged in a cluster and associated with the atoms of the acceptor pocket. These interactions may promote stabilization of the hydrophobic interactions between Val β6 and EF acceptor pocket. Thus, water molecules form bridging hydrogen bonds, which are clustered in the bend between F′ and F helix regions between tetramers in the lateral contacts. In addition, recently, Wang *et al.* have compared deoxy Hb S polymer structure made in high phosphate buffer with that made in low phosphate buffer by electron microscopy (*26*). They found that oversaturated deoxy Hb S in high phosphate buffer first formed fibers, which then began to form bundles, macrofibers and crystals; all having the same appearance in both low and high phosphate buffers. They concluded that deoxy Hb S fibers formed in high phosphate buffer have the same structure as those formed in low phosphate buffer.

β87 Thr in Hb S is involved in interactions in lateral contacts between parallel double strands in crystals or fibers, and also forms strong hydrogen bonds with $1\alpha_1$ 139 Lys and $2\alpha_2$ 81 Ser (*3, 11*). Ile and Leu substitutions at β87 in Hb S may strengthen the hydrophobic environment in the pocket and accelerate nucleation and polymerization, while Asp and Gln substitutions at this position inhibit nucleation and polymerization. Asp at this position may therefore weaken the hydrophobic environment of the pocket much more than the Glu substitution at β85 and/or β88. Critical concentrations for polymerization of Hb S β87 Asp variant might then be increased much more than that of the Glu-substituted Hb S variants. In addition, our previous studies on polymer formation of deoxy Hb βE6F, Hb βE6W, and Hb S βL88F suggest that bulky and larger hydrophobic amino acids like Phe and Trp at the β6 or β88 position may not insert correctly into the hydrophobic pocket or may inhibit insertion of β6 Val. Such changes then result in inhibition of nuclei formation prior to polymerization, and tetramers containing these changes polymerize linearly without a delay time (*16, 22*). Furthermore, kinetic patterns of polymerization for Hb S βT87W and Hb S βT87K are also different from those of the hemoglobin variants which contain bulky amino acids at β87. These results suggest that β87 Thr → Trp and β87 Thr → Lys may change shape, size, and/or hydrophobicity of the EF pocket in addition to promoting disruption of additional protein−protein interactions. These changes, however, may not completely inhibit insertion of β6 Val and interactions with other amino acids such as $1\text{-}\beta_2$ 9 Ser, $1\text{-}\beta_2$ 10 Ala, $1\text{-}\beta_2$ 13 Ala, $1\text{-}\beta_2$ 126 Val, $1\alpha_1$ 139 Lys, and $2\alpha_2$ 81 Ser.

From these results, we propose that the β87 amino acid strongly influences hydrophobicity or shape of the β6 acceptor pocket and that β87 Thr in Hb S plays a major role in formation of nuclei and polymers. β6 Val first directly interacts with β85 Phe and β88 Leu and also communicates with β87 Thr through ordered water molecules in the pocket. Insertion of the hydrophilic amino acid Asp for Thr at β87 in Hb S may therefore influence hydrophobicity of the acceptor site and modify normal interactions of β87 Thr with other amino acids, thereby inhibiting nucleation and polymerization. This would result in an increase in critical concentration required for polymerization and loss of a delay time prior to polymerization. In contrast, insertion of Gln at β87 would be expected to have minimal effect on hydrophobicity of the EF acceptor pocket, but would affect significantly subsequent protein−protein interactions which are involved in formation of stable nuclei and polymers.

In conclusion, our results as well as those of Ho *et al.* (*14*) on kinetics of polymerization of β87 Hb S variants suggest that changes in hydrophobicity and polarity of the pocket perimeter in the EF acceptor site play a critical role in modifying protein−protein interactions between the hydrophobic acceptor pocket and the β6 site. β87 Thr in Hb S is located on the hydrophobic pocket perimeter and induces communication with other sites which then facilitates stable nuclei formation prior to polymerization. These results reinforce our contention of the importance of β87 Thr in the nucleation of deoxy Hb S prior to polymerization and provide a basis for understanding how Gln at F3 (δ and γ 87 positions) in Hb A_2 and Hb F inhibits Hb S polymerization (*7*, *9*, *10*). X-ray analysis of these β87 Hb S variants is now in progress in an attempt to evaluate structural effects of the β87 changes on β6 Val interaction with the pocket and to monitor β87 interactions with other sites in Hb S tetramers.

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