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Mutational analysis of phenylalanine β85 in the valine β6 acceptor pocket during hemoglobin S polymerization

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Abstract

Hemoglobin (Hb) S containing Leu, Ala, Thr, or Trp substitutions at β85 were made and expressed in yeast in an effort to evaluate the role of Phe-β85 in the acceptor pocket during polymerization of deoxy Hb S. The four Hb S variants have the same electrophoretic mobility as Hb S, and these β85 substitutions do not significantly affect heme–globin interactions and tetramer helix content. Hb S containing Trp-β85 had decreased oxygen affinity, whereas those with Leu-, Ala-, and Thr-β85 had increased oxygen affinity. All four supersaturated β85 variants polymerized with a delay time as does deoxy Hb S. This is in contrast to deoxy Hb S containing Phe-β88, Ala-β88, Glu-β88, or Glu-β85, which polymerized with no clear delay time (Adachi K, Konitzer P, Paulraj CG, Surrey S, 1994, *J Biol Chem 269*:17477–17480; Adachi K, Reddy LR, Surrey S, 1994, *J Biol Chem 269*:31563–31566). Leu substitution at β85 accelerated deoxy Hb S polymerization, whereas Ala, Thr, or Trp substitution inhibited polymerization. The length of the delay time and total polymer formed for these β85 Hb S variants depended on hemoglobin concentration in the same fashion as for deoxy Hb S: the higher the concentration, the shorter the delay time and the more polymer formed. Critical concentrations required for polymerization of deoxy Hb $S^{F\beta 85L}$, Hb $S^{F\beta 85A}$, Hb $S^{F\beta 85T}$, and Hb $S^{F\beta 85W}$ are 0.65-, 2.2-, 2.5- and 3-fold higher, respectively, than Hb S. These results suggest that the relative order for polymerization of β85 variants (Leu > Phe > Ala > Thr > Trp-β85) depends on amino acid hydrophobicity rather than stereospecificity of the side chain. These findings are in contrast to previous results for β88 variants. Trp-β85 in Hb S may affect Val-β6 acceptor pocket size, but may still accommodate insertion of Val-β6. These results also strengthen our previous conclusion that β88 amino acid stereospecificity is more critical than that of β85 for insertion of β6 Val.

Keywords: heme–globin interactions; hemoglobin S polymerization; mutational analysis

In addition to Val-β6, the key to polymerization of deoxy hemoglobin S is formation of a hydrophobic acceptor pocket between the E and F helices of the T-structure or deoxy form of hemoglobin (Hb). Important information on the structure of the deoxy Hb S molecule has come from single crystal X-ray diffraction studies (Wishner et al., 1975; Padlan & Love, 1985). A medium resolution map of deoxy Hb S crystals revealed several surface regions involved in intermolecular contacts between molecules on different strands in the paired-strand structure (Padlan & Love, 1985). The acceptor pocket for the Val-β6 is lined at the bottom with the side chains of Phe-β85 and Leu-β88, and has Asp-β73, Thr-β84, and Thr-β87 around the pocket perimeter. The hydrophobic Val-, Leu-, or Ile-β6 side chain appears to fit into the hydrophobic pocket between the E and F helices on an adjacent molecule, whereas Glu-β6 does not because it may interact with water molecules (Baudin-Chich et al., 1990; Adachi et al., 1993). Neither Phe- nor Trp-β6 appears to fit into the pocket because of their larger-sized side chains (Bihoreau et al., 1992; Adachi et al., 1993). Polymerization of deoxy Hb S is facilitated by Val-β6 and by formation of a hydrophobic acceptor pocket between the E and F helices that is present in deoxyhemoglobin (Wishner et al., 1975; Dickerson & Geis, 1983); therefore, characterization of the Val-β6 acceptor pocket, which includes Phe-β85 and Leu-β88, is important in order to understand hydrophobic interactions critical to polymerization.

Several β88 Hb S variants including Hb $S^{L\beta 88A}$, Hb $S^{L\beta 88F}$, and Hb $S^{L\beta 88E}$, in addition to the β85 variant, Hb $S^{F\beta 85E}$, have

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been engineered and expressed, and their polymerization properties have been characterized (Adachi et al., 1994a, 1994c; Martin de Llano & Manning, 1994). Both Ala- and Phe-β88 substitutions inhibited polymerization of deoxy Hb S (Adachi et al., 1994a). Supersaturated solutions of Hb $S^{L\beta 88F}$ polymerized without a delay time, similar to Trp- and Phe-β6 substituted hemoglobins, but kinetics of polymerization of Hb $S^{L\beta 88A}$ and Hb $S^{L\beta 88E}$ were biphasic (Adachi et al., 1994a, 1994c). Critical concentrations for polymer formation of Ala- and Phe-β88 Hb S variants were six- and ninefold higher, respectively, than that of deoxy Hb S in 1.8 M phosphate buffer, indicating that stereospecificity and hydrophobicity of the acceptor pocket are critical factors in understanding key hydrophobic interactions in deoxy Hb S polymerization.

Deoxy Hb S containing a hydrophilic Glu-β88 polymerized at a much higher hemoglobin concentration compared with deoxy Hb S having other β88 substitutions (Adachi et al., 1994a, 1994c). Furthermore, kinetics of polymerization for Hb $S^{L\beta 88E}$ were biphasic at lower hemoglobin concentrations, which is similar to Hb $S^{L\beta 88A}$ (Adachi et al., 1994a). In addition, deoxy Hb $S^{L\beta 85E}$ polymerized in the same way as deoxy Hb $S^{L\beta 88E}$; however, the concentration required for polymerization of Hb $S^{L\beta 85E}$ was about 1/3 that of Hb $S^{L\beta 88E}$, and the time required to complete polymerization was about three times longer than for deoxy Hb S (Adachi et al., 1994c). These differences in polymerization of Glu-β85 and β88 Hb S variants may be due to differences in location of β85 and β88 residues in the acceptor pocket. These results suggest that, although β85 and β88 amino acid side chains line the acceptor pocket, Phe-β85 may line the floor of the pocket, and Leu-β88 may be close to the pocket entrance (Adachi et al., 1994a). These differences in location could lead to different results when making the same Glu change at these two sites. In order to further understand the role of hydrophobicity and stereospecificity of Phe-β85 in the acceptor pocket for Val-β6 during deoxy Hb S polymerization, we have engineered and expressed four new Hb S variants at the β85 position and characterized their polymerization properties.

Results

Characterization of β85 Hb S variants

Purified recombinant Hb S variants containing Leu-, Ala-, Thr-, or Trp-β85 migrated as single bands following cellulose acetate electrophoresis at pH 8.6, with identical mobility to Hb S. It is interesting to note that Hb S containing Val-β85 was also engineered but did not result in measurable amounts of tetramer using the yeast expression system. It is not clear why we were unable to express this Val-β85 variant; DNA sequence analysis of the vector revealed no unanticipated PCR-induced mutations.

Mass spectral analysis of Leu-, Ala-, Thr-, and Trp-β85-containing Hb S variants and Hb $S^{F\beta 85E}$, which was previously reported (Adachi et al., 1994c), showed the expected β-globin chain molecular weights (Table 1). 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 (Adachi et al., 1993), except for Hb $S^{L\beta 85W}$. This variant showed a slightly higher absorbance in the UV range than the other β85 variants and native Hb S, no doubt caused by the additional tryptophan (Table 2). These results suggest that there are no major effects of these β85 substitutions on heme-globin interactions. Absorption maxima in the visible and UV ranges for the four β85 Hb S variants and Hb $S^{F\beta 85E}$ are shown in Table 2. CD spectra in the region from 190 nm to 290 nm for the Hb S variants were similar to that of native Hb S (Fig. 1), indicating that these substitutions do not significantly affect globin folding and/or overall secondary structure of hemoglobin tetramers (Martin de Llano & Manning, 1994).

Table 1. *Mass spectrometric analysis of β85 Hb S variants*

	β-Globin chain	Expected
Hb S	15,838.2	15,837.2
Hb $S^{F\beta 85L}$	15,803.2	15,803.3
Hb $S^{F\beta 85A}$	15,760.6	15,761.4
Hb $S^{F\beta 85T}$	15,791.4	15,791.2
Hb $S^{F\beta 85W}$	15,877.8	15,876.2
Hb $S^{F\beta 85E}$	15,818.0	15,819.2

Oxygen affinities were, however, different from Hb S and depended on the particular β85 substitution (Table 3). P_{50} values in 0.1 M phosphate buffer, pH 7.0, at 20 °C for Hb S containing Leu-, Ala-, Thr-, and Trp-β85 were 3.7, 2.1, 2.1, and 8.2, respectively, compared with 6.5 for recombinant Hb S and 2.0 for Glu-β85 Hb S, previously reported (Adachi et al., 1993, 1994c). Hill coefficients for these variants were near normal (Table 3).

Polymerization properties of β85 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 (Adachi & Asakura, 1979a; Eaton & Hofrichter, 1990). Polymerization of the deoxy forms of the β85 Hb S variants was studied in vitro by the temperature jump method employing 1.8 M phosphate buffer, pH 7.4, at 30 °C (Adachi & Asakura, 1979a). Polymerization of deoxy Hb S in 1.8 M phosphate buffer is similar to that under low phosphate conditions and occurs by a nucleation-controlled polymerization mechanism requiring much lower Hb S concentrations (Adachi & Asakura, 1979a, 1979b). Approximately 0.1 g/dL of deoxy Hb S is needed to initiate polymerization, whereas about 20 times

Table 2. *Spectral properties of β85 Hb S variants in the CO form*[a]

	539/568	419/538	275/538
Hb S	1.0	12.9	2.82
Hb $S^{F\beta 85L}$	1.0	13.0	2.65
Hb $S^{F\beta 85A}$	1.0	13.0	2.80
Hb $S^{F\beta 85T}$	1.0	13.0	2.80
Hb $S^{F\beta 85W}$	1.0	12.8	2.94
Hb $S^{F\beta 85E}$	1.0	13.6	2.87

[a] Absorption spectra of the β85 Hb S variants in the CO form were recorded in 0.1 M phosphate buffer, pH 7.0.

higher hemoglobin concentration is required under near physiological conditions.

The deoxy form of Hb $S^{F\beta85L}$ polymerized at a lower hemoglobin concentration than deoxy Hb S, whereas the other three variants required much higher hemoglobin concentrations for polymer formation (Fig. 2). Polymerization for all four variants was preceded by a delay time, similar to deoxy Hb S, which is in contrast to our previous results with several β88 Hb S variants and Hb $S^{F\beta85E}$, which showed biphasic and/or linear polymerization patterns (Adachi et al., 1994a). Logarithmic plots of

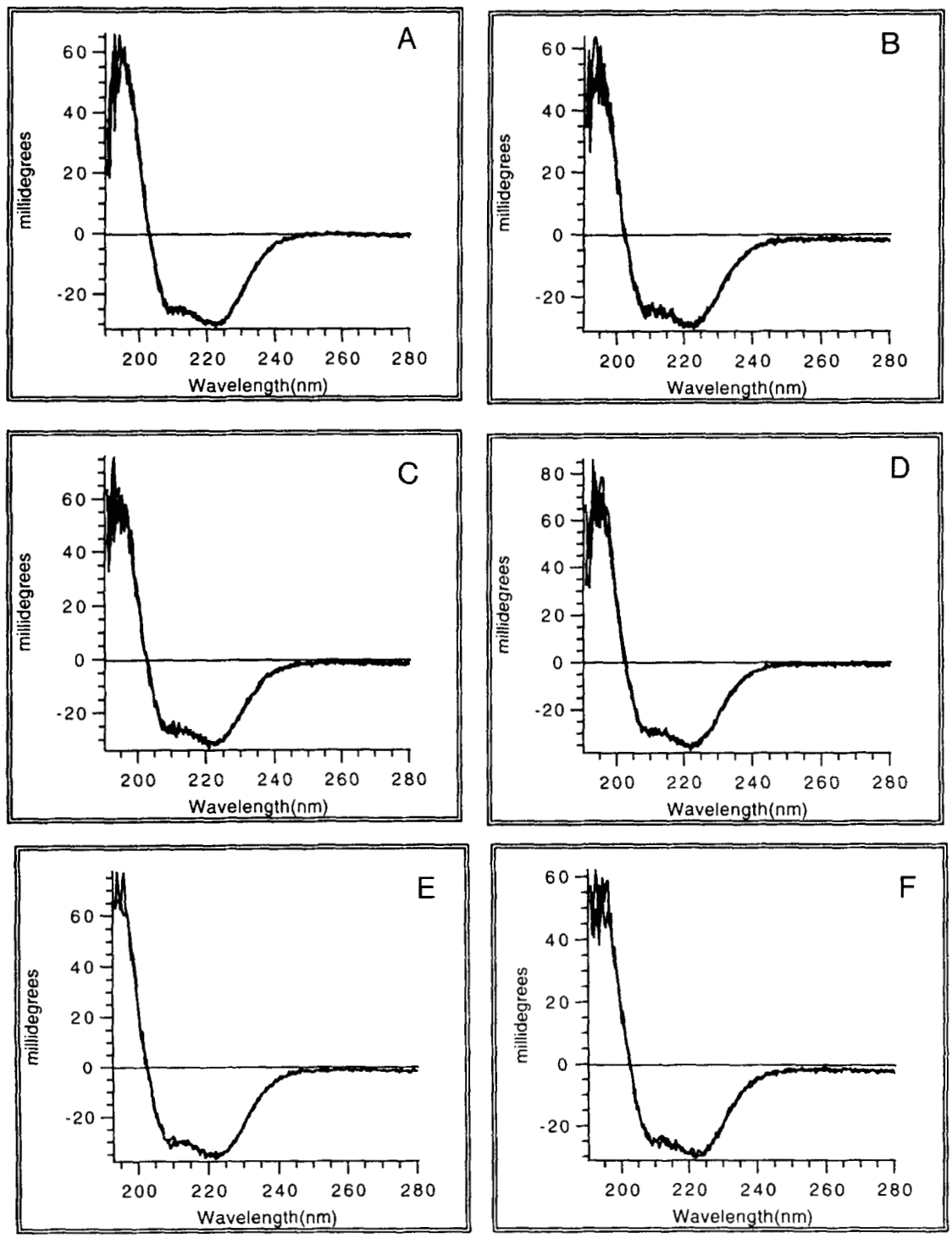

Fig. 1. CD spectra of β85 Hb S variants. Spectra were recorded for the CO forms of the β85 Hb S variants (~10 μM) in 0.1 M phosphate buffer, pH 7.0, at 5 °C and results were corrected for small differences in protein concentration. **A:** Hb S. **B:** Hb $S^{F\beta85E}$. **C:** Hb $S^{F\beta85T}$. **D:** Hb $S^{F\beta85A}$. **E:** Hb $S^{F\beta85L}$. **F:** Hb $S^{F\beta85W}$.

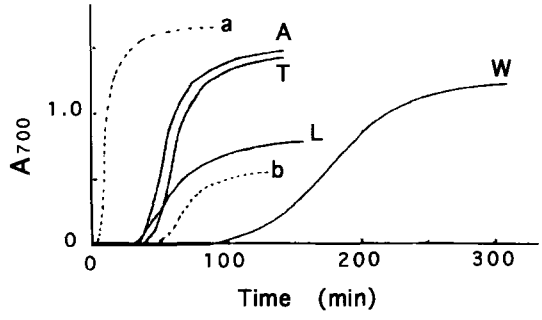

Fig. 2. Kinetics of polymerization for β85 Hb S variants. Time course of polymerization for deoxy forms of β85 Hb S variants was defined using 1.8 M phosphate buffer, pH 7.4, at 30 °C by the temperature jump method as described previously (Adachi & Asakura, 1979a). Dotted lines a and b represent 120 and 78 mg/dL of deoxy Hb S. Solid lines A, L, T, and W indicate Hb $S^{F\beta85A}$ (178 mg/dL), Hb $S^{F\beta85L}$ (69 mg/dL), Hb $S^{F\beta85T}$ (197 mg/dL), and Hb $SF^{\beta85W}$ (221 mg/dL), respectively.

delay time versus hemoglobin concentration for all four β85 Hb S variants showed straight lines like deoxy Hb S (Fig. 3). The line for Hb $S^{F\beta85L}$ shifted left 0.08 units on the *x*-axis, whereas the other variants shifted right from the line for Hb S: 0.35, 0.44, and 0.5 units for Hb $S^{F\beta85A}$, Hb $S^{F\beta85T}$, and Hb $S^{F\beta85W}$, respectively. These results show that, at equivalent initial hemoglobin concentrations, the time required to initiate polymerization for Hb $S^{F\beta85L}$ is 1.2 times shorter than Hb S, whereas times for Hb $S^{F\beta85A}$, Hb $S^{F\beta85T}$, and Hb $S^{F\beta85W}$ are 2.2, 2.7, and 3.1 times longer compared with Hb S.

Total polymer formed as a function of hemoglobin concentration was also determined in order to evaluate effects of the β85 substitutions on the critical concentration required for polymerization. Critical concentration depends on deoxyhemoglobin solubility: the higher the solubility, the higher the concentration required for polymerization. Polymer formation for the four variants increased linearly with increases in initial hemoglobin concentration (Fig. 4). Critical concentrations for polymer formation were then determined by extrapolation of the lines to zero turbidity (Adachi et al., 1993). Critical concentrations for Hb $S^{F\beta85L}$, Hb $S^{F\beta85A}$, Hb $S^{F\beta85T}$, and Hb $S^{F\beta85W}$ were 0.65-, 2.2-, 2.5-, and 3-fold higher, respectively, than Hb S. The value for Hb $S^{F\beta85E}$ was 2.4-fold higher than deoxy Hb S (Adachi et al., 1994c), and the kinetics of polymerization for this variant was different from the other four β85 variants. Furthermore, the slopes of the lines for the four variants were similar to that of Hb S but different from Hb $S^{F\beta85E}$, suggesting that Hb S polymer formation and/or structure containing neutral or hydrophobic amino acids at the β85 position is similar, whereas polymers containing hydrophilic amino acids, such as Glu, are different.

Table 3. *Oxygen-binding properties of β85 Hb S variants*[a]

	P_{50}	*n*-max[b]
Hb S	6.9	2.6
Hb $S^{F\beta85L}$	4.0	2.6
Hb $S^{F\beta85A}$	2.2	2.4
Hb $S^{F\beta85T}$	2.2	2.4
Hb $S^{F\beta85W}$	8.2	2.5
Hb $S^{F\beta85E}$	2.0	1.6

[a] Oxygen-dissociation curves of recombinant Hbs were determined in 0.1 M phosphate buffer, pH 7.0, at 20 °C. P_{50} is the partial oxygen pressure required to give 50% saturation of hemoglobin.

[b] *n*-max values were calculated from the Hill plot of oxygen equilibrium curves.

Discussion

Functional properties of β85 Hb S variants

X-ray crystallographic analysis showed that Phe-β85 (F1) is internally located, acts as a spacer between the F and H helical segments, and is important in maintaining the hydrophobic environment of the heme pocket (Perutz et al., 1968). Our previous studies with engineered hydrophilic substitutions, such as

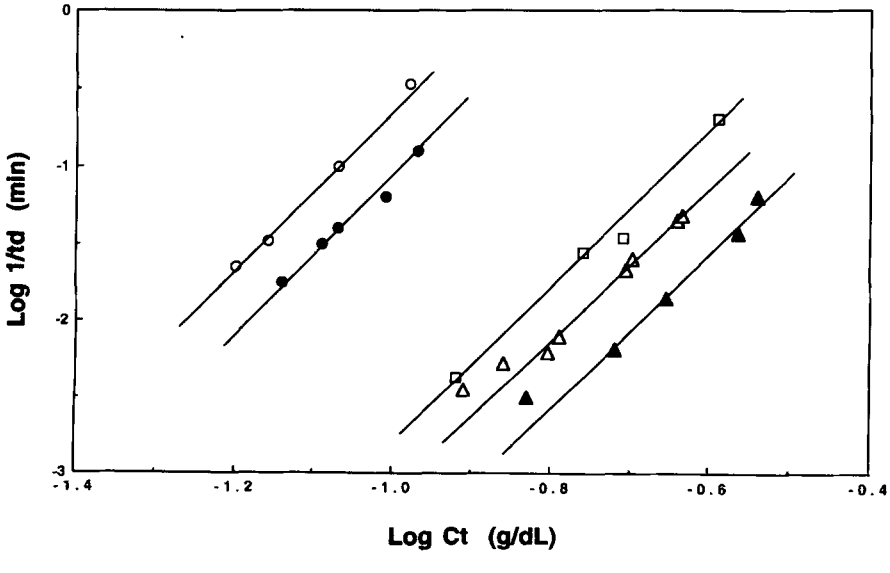

Fig. 3. Relationship between log of reciprocal delay time and hemoglobin concentration. Polymerization studies of hemoglobins at different concentrations were performed in 1.8 M phosphate buffer, pH 7.4, at 30 °C. ●, native Hb S; ○, Hb $S^{F\beta85L}$; □, Hb $SF^{\beta85A}$; △, Hb $S^{F\beta85T}$; ▲, Hb $S^{F\beta85W}$.

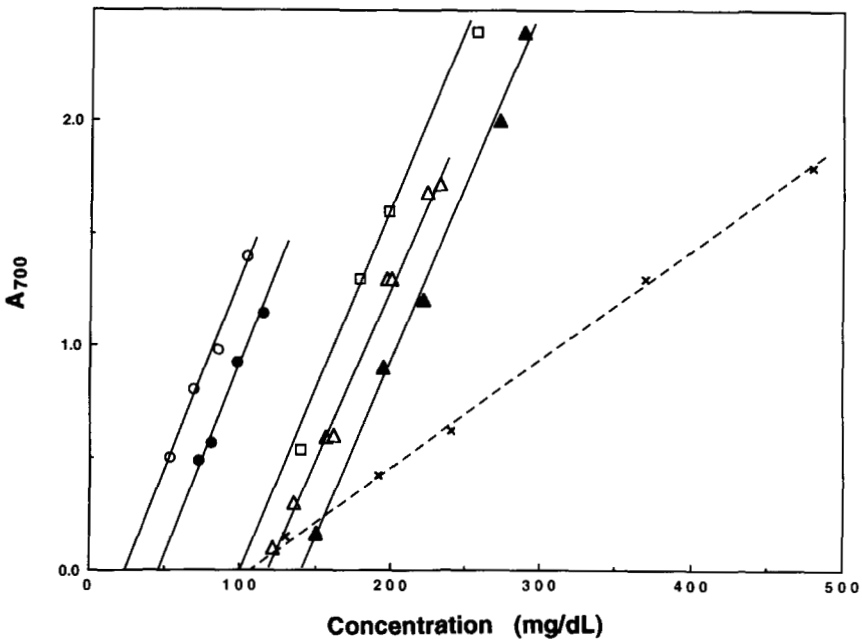

Fig. 4. Polymer formation as a function of hemoglobin concentration. Turbidity at the plateau of the polymerization curves in 1.8 M phosphate buffer at various hemoglobin concentrations was measured at 700 nm. Symbols are the same as those described in Figure 3; the dotted line shows our previously reported results for Hb $S^{F\beta 85E}$.

Glu for Phe at β85, showed increased oxygen affinity and decreased tetramer stability (Adachi et al., 1994c). A naturally occurring hemoglobin variant containing Ser at this position (Hb Buenos Aires [Hb $A^{F\beta 85S}$]) exhibits high oxygen affinity and is unstable (De Weinstin et al., 1973). Leu, Ala, and Thr substitutions at β85 also increased oxygen affinity, with Ala- and Thr-β85 substitutions resulting in higher oxygen affinities than Leu-β85, whereas Trp-β85 decreased oxygen affinity compared with Hb S. It is not surprising that stereochemical interference with F and H helical segments by these mutations causes changes in oxygen affinity. It is of interest that the larger side chain, such as Trp instead of Phe at β85, results in decreased oxygen affinity, whereas smaller amino acid side-chain spacers may have the opposite effect. Biophysical analyses of these hemoglobins are required to further understand how these β85 Hb S variants cause changes in oxygen affinity.

Polymerization properties of deoxy β85 Hb S variants

Our previous and present studies on the kinetics of polymerization of recombinant deoxy Hb S variants with second-site substitutions at β85 (Hb S [β85 Phe → Trp, Ala, or Glu]), β87 (Hb S [β87 Thr → Gln]) and β88 (Hb S [β88 Leu → Phe, Ala, or Glu]) show that all of these Hb S variants required higher critical concentrations for polymerization than deoxy Hb S (Adachi et al., 1994a, 1994b, 1994c). The critical concentrations for Hb $S^{L\beta 88E}$, Hb $S^{L\beta 88F}$, and Hb $S^{L\beta 88A}$ in the deoxy form are 7, 9, and 6 times higher, respectively, than that of deoxy Hb S. It is interesting to note that the critical concentration for Hb S with Glu- or Ala-β88 is lower than Phe-β88, even though Phe is more hydrophobic than Ala or Glu (Nozaki & Tanford, 1971; Kyte & Doolittle, 1982). Furthermore, critical concentrations for polymerization of Hb $S^{F\beta 85E}$ and Hb $S^{F\beta 88E}$ were 2.4- and 7-fold higher, respectively, than that of Hb S. In contrast, substitution of Leu for Phe at β85 accelerates polymerization. In addition, substitution of Ile for Thr at β87 also accelerates polymerization of Hb S (Witkowska et al., 1991), whereas substitution of Gln for Thr at β87 inhibits polymerization (Adachi et al., 1994b). These results indicate that increased hydrophobicity of the F helix accelerates polymerization. Our results also suggest that insertion of the bulky side chain of Phe instead of Leu at β88, but not at β85, which is Phe in native Hb S, disrupts the acceptor pocket and/or inhibits insertion of β6 Val into the pocket of an adjacent tetramer. In addition, inhibition of polymer formation by the Leu → Ala change at β88 in Hb S might be due to either decreased hydrophobicity and/or the smaller side chain of Ala compared to Leu (Adachi et al., 1994a).

The relative order of polymerization of the β85 Hb S variants (Leu > Phe > Ala > Thr > Trp-β85), which all polymerize after a distinct delay time, is different from results of the β88 variants (Adachi et al., 1994a, 1994c). Both aromatic rings of Phe and Trp exhibit strong multipoles, and their hydrophobicity values differ according to the methods for measurement (Nozaki & Tanford, 1971; Kyte & Doolittle, 1982). Hydropathy index calculated by Kyte and Doolittle (1982) shows the following order: Leu > Phe > Ala > Thr > Trp, which is consistent with the relative order of polymerization of the β85 Hb S variants. These results suggest that polymerization of the β85 Hb S variants depends on amino acid hydrophobicity rather than size of the side chain. The Trp-β85 substitution may affect pocket size, which can still accommodate insertion of Val-β6. Such flexibility does not appear to occur with bulky substitutions at β88. Furthermore, even though the Glu-β85 substitution may allow insertion of the donor Val-β6 in the pocket, this hydrophilic substitution may not facilitate interactions with Val-β6 that normally occur in Hb S. Structural studies of these β85 and β88 Hb S variants are now required to characterize polymer formation. These results collectively indicate that stereospecificity and hydrophobicity of the acceptor pocket and the donor site (β6) are critical factors in understanding key hydrophobic interactions of deoxy Hb S during polymerization. Furthermore, our present findings strengthen our previous conclusion that β88 amino acid

stereospecificity is more critical than that of β85 for insertion of β6 Val (Adachi et al., 1994c). These findings demonstrate that modified hemoglobins made using recombinant DNA technology are important tools for structure/function studies in defining parameters affecting polymerization of hemoglobin.

Kinetic studies of polymerization of deoxy Hb S have played a central role in understanding the pathophysiology of sickle cell disease and in the design of strategies for therapy (Eaton & Hofrichter, 1990). Polymerization of deoxy Hb S is characterized by a marked delay period prior to polymerization, which is followed by a dramatic and autocatalytic increase in the formation of polymers. Our previous studies using the deoxy form of Hb (β6 Glu → Trp) and Hb (β6 Glu → Phe) suggest that bulky hydrophobic amino acids, such as Phe and Trp at the β6 position, inhibit nuclei formation prior to polymerization, even though they may interact with the acceptor pocket on an adjacent Hb molecule (Adachi et al., 1993). In addition, a β88 Leu → Phe change in the β6 acceptor pocket in deoxy Hb S also prevents formation of nuclei prior to polymerization, similar to β6 Glu → Trp or Phe (Adachi et al., 1993). Kinetics of polymerization of these variants can be explained by a linear polymerization mechanism (Adachi et al., 1993). In contrast, substitution of the smaller size Ala for Leu at β88 showed a biphasic polymerization pattern characterized by a delay time prior to polymerization at lower hemoglobin concentrations. These results suggest that polymerization of Hb $S^{L\beta 88A}$ may occur by formation of nuclei prior to polymer formation at decreasing hemoglobin concentrations (Adachi et al., 1994a, 1994c). Hb $S^{F\beta 85E}$ and Hb $S^{L\beta 88E}$ also showed biphasic polymerization patterns similar to Hb $S^{L\beta 88A}$ (Adachi et al., 1994c). These results suggest that hydrophilic or neutral amino acid substitutions in the acceptor pocket do not completely inhibit the formation of nuclei prior to polymerization, but they may affect overall structure of the nuclei. Whether nuclei made by these substitutions are the same as those made by Hb S is not clear. In contrast, Hb S variants containing Phe-, Leu-, Ala-, Thr-, or Trp-β85 polymerized with a delay time like deoxy Hb S, although the relative order of polymerization appears to depend on amino acid hydrophobicity. These results show that increased hydrophobicity and appropriate interaction with Val-β6 are critical for inducing communication with other interaction sites that facilitate nuclei formation prior to polymerization. Characterization of nuclei prior to polymerization may be helpful in understanding hydrophobic interactions of proteins in general and may also hold promise for help in defining a rational strategy for inhibiting polymerization of deoxy Hb S in patients with sickle cell disease.

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 (Wagenbach et al., 1991; Adachi et al., 1993). 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 (Adachi et al., 1993). The basic strategy for site-specific mutagenesis at the β85 position involves recombination PCR as described previously (Adachi et al., 1993). β85 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 (Adachi et al., 1993). 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 (Adachi et al., 1993).

Yeast growth, plasmid transformation, induction, and purification of recombinant hemoglobin tetramers were described previously (Wagenbach et al., 1991; Adachi et al., 1993). Abnormal forms of recombinant hemoglobins, which may be sulphaem-containing hemoglobin and/or misfolded hemoglobin, were eliminated by chromatographic purification (Adachi et al., 1992; Hofmann et al., 1994). The purified β85 Hb S variants were subjected to electrospray mass analysis (Fisons Instruments, VG Biotech, Altricham, UK) using the multiply charged ion peaks from the α-globin chain (M_r = 15,126.4) as an external reference for mass scale calibrations (Shackleton & Witkowska, 1994). Val-β6 and N-terminal amino acid sequence of purified α and β chains were directly confirmed by Edman degradation employing a pulsed-liquid protein sequencer (ABI 477A, Applied Biosystems, Inc., Foster City, California). Absorption spectra of the purified Hb variants were recorded using a Hitachi U-2000 spectrophotometer. CD spectra of the variants were recorded using an Aviv-Model 62 DS instrument employing a 0.1-cm-lightpath cuvette at ~10 μM Hb concentrations. Methods for determination of oxygen-dissociation curves and kinetics of polymerization of deoxy hemoglobins were also as reported previously (Adachi & Asakura, 1979a; Adachi et al., 1993).

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