Role of Hydrophobic Amino Acids at β85 and β88 in Stabilizing F Helix Conformation of Hemoglobin S*

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Three Hb S variants containing Glu substitutions at Phe-β85 and/or Leu-β88 were expressed in yeast in an effort to evaluate the role of hydrophobic amino acids at these sites in stabilizing F helix conformation of Hb S. Helix stability of tetrameric Hb S βF85E,βL88E was measured by CD and compared with those of Hb S βF85E, Hb S βL88E, Hb A, and Hb S. The CD spectra of these Hb S variants were similar to those of Hb S and Hb A at 10 °C. However, changes in ellipticity at 222 nm for Hb S βF85E in the CO form at 60 °C were about 15-fold greater than that of Hb S, while those for Hb S βL88E and Hb S βF85E,βL88E were similar and about 30-fold greater than Hb S. Thermal stability measured by continuous scanning of spectral changes revealed the three Hb S variants were much more unstable than Hb S, and stability of Hb S βF85E,βL88E was similar to that of Hb S βL88E rather than Hb S βF85E. These results suggest that Glu insertion at both β85 and β88 makes heme insertion into the heme pocket more difficult; however, once inserted, stability of Hb S βF85E,βL88E is similar to Hb S βL88E rather than Hb S βF85E. Furthermore, these results suggest that both Phe-β85 and Leu-β88 are critical for F helix stabilization and that Glu insertion at β88 leads to more destabilization than insertion at β85.

In addition to Val-β6, the key to polymerization of deoxy-Hb S is formation of a Val-β6 hydrophobic acceptor pocket containing the hydrophobic amino acids Phe-β85 and Leu-β88 in the F helices of the T structure or deoxy form of Hb S which interacts with Val-β6 (1, 2). Therefore, characterization of the Val-β6 acceptor site, which includes Phe-β85 and Leu-β88, is important to understand hydrophobic interactions of deoxy-Hb S polymerization.

We previously engineered and expressed Hb S variants containing Glu at either β85 or β88, Hb S βF85E, and Hb S βL88E and characterized their polymerization properties in order to understand the role of hydrophobicity and stereospecificity of the acceptor pocket during deoxy-Hb S polymerization (3). The deoxy form of Hb S βL88E polymerized in vitro at a much higher hemoglobin concentration than deoxy-Hb S containing other amino acid substitutions at β85 and β88 such as Hb S βF85E and Hb S βL88F (3). Furthermore, kinetics of polymerization for Hb S βL88E were biphasic at lower hemoglobin concentrations. Deoxy-Hb S βF85E also polymerized like deoxy-Hb S βL88E; however, the concentration required for polymerization of Hb S βF85E was 3-fold less than that of Hb S βL88E.

These differences in polymerization between having Glu at β85 versus β88 may be due to residue location in the acceptor pocket. X-ray analysis of crystallized deoxy-Hb S shows that β85 and β88 amino acid side chains line the bottom of the acceptor pocket (1, 2), with Phe-β85 located internally at the floor of the pocket and Leu-β88 located near the pocket entrance. This difference in location might be expected to lead to different results when making the same Glu change at these two sites. Polymerization properties of several other recombinant Hb S variants containing changes at β85 and β88 have been studied, and those studies clarified the importance of stereospecificity of the acceptor pocket in interacting with Val-β6 in Hb S (4–6).

In order to further understand the role of hydrophobicity of the acceptor pocket, we prepared Hb S containing hydrophilic Glu residues at both β85 and β88, Hb S βF85E,βL88E. In this report we characterized F helix stability of 1) Hb S βF85E,βL88E, 2) Hb S βL88E, and 3) Hb S βF85E and compared the results with those of Hb A and Hb S.

MATERIALS AND METHODS

The plasmid pGS389 β6 contains the full-length human α- and β6-globin cDNAs under transcriptional control of dual GGAP promoters, as well as a partially functional yeast LEU2α gene and the URA3 gene for selection in yeast (7, 8). The plasmid pGS189 β6 contains a single GGAP promoter and β6-globin cDNA and was constructed by mutagenesis and subcloning as described previously (7). The basic strategy for site-specific mutagenesis at the β85 and β88 positions involves recombinase polymerase chain reaction as described previously (3, 7). βF85E cDNA was then used as a template to introduce the additional Glu-β88. The three Hb S variants 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 (7, 9). The mutated β-globin cDNA regions were then excised by XhoI digestion and subcloned back into the XhoI site of the expression vector pGS389 (4).

Yeast growth and plasmid transformation, induction, and purification of recombinant hemoglobin tetramers were described previously (3, 7, 8, 10). Abnormal forms of recombinant hemoglobins, which may include sulpham-containing and/or misfolded hemoglobins, were eliminated by chromatographic purification (10, 11). Hb S βF85E,βL88E was further purified using an FPLC1 Mono Q column with a linear gradient from 50 mM Tris-HCl, pH 8.5 (buffer A), to 50 mM Tris-HCl, pH 6.5, at 4°C to remove free α chains. Purified Hb S variants were subjected to electrospray mass analysis (Fisons Instruments, VG Bio- tech, Altrincham, UK) using the multiply charged ion peaks from the α-globin chain (Mα = 15,126.4) as an external reference for mass scale calibrations (12). The N-terminal amino acid sequence of purified α chain was directly confirmed by Edman degradation employing a pulsed-liquid, protein sequencer (ABI 477A, Applied Biosystems, Inc.,

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1 The abbreviation used is: FPLC, fast protein liquid chromatography.
Foster City, CA). 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 light path cuvette at \( \lambda = 10 \) m\( \text{M} \) Hb concentrations, and the temperature was controlled with a thermoelectric module. 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. Deoxyhemoglobin solutions were prepared by flushing with nitrogen and by adding a 2–3-fold molar excess of sodium dithionite.

**RESULTS**

**Characterization of Hb S \( \beta^85E,\beta^88E \)**—Recombinant Hb S tetramers containing Glu-\( \beta^85 \) or Glu-\( \beta^88 \) substitutions were purified on a Mono S column, and migrated as a single band following cellulose acetate electrophoresis, pH 8.6, with a more negative surface charge compared with normal human Hb S (3). It is interesting to note that FPLC Mono S column purification of Hb S \( \beta^85E,\beta^88E \) showed two peaks, while the other variants showed only a single peak of tetramer at this stage of purification (Fig. 1). Electrophoretic and amino acid sequence analysis indicated the major peak that eluted with the first buffer prior to gradient elution of the Hb S variant contained free \( \alpha \) chains. The ratio of free \( \alpha \) chains to this Hb S variant was more than 8:1. Purified Hb S \( \beta^85E,\beta^88E \) migrated as a single band following cellulose acetate electrophoresis, and its yield was very low (5–8% of that of the other two Hb S variants) probably because of precipitation of the \( \beta^S \) variant chain before assembly with \( \alpha \) chains in yeast.

Mass spectral analysis of the three \( \beta^S \) variants showed expected \( \beta \)-globin chain molecular weights (15,834 Da for \( \beta^85 \) Glu-\( \beta^85 \) and Glu-\( \beta^88 \); 15,818 Da for \( \beta^85 \) Glu-\( \beta^85 \); 15,853 Da for \( \beta^85 \) Glu-\( \beta^88 \); and 15,837 Da for \( \beta^S \)). Absorption spectra in the UV and visible ranges of the CO forms of these three Hb S variants were the same as those of native and recombinant Hb S.

The CD spectrum in the region from 190 to 290 nm for Hb S \( \beta^85E,\beta^88E \) at 10°C was similar to those of Hb S \( \beta^88E \) and Hb S \( \beta^85E \) as well as native Hb S and Hb A (Fig. 2). These results indicate that once the \( \beta^S \) chain variants assemble with \( \alpha \) chains, substitutions from hydrophobic to hydrophilic amino acids at \( \beta^85 \) and \( \beta^88 \) do not significantly affect globin folding and/or overall secondary structure of hemoglobin tetramers at low temperature (10°C). Initiation of polymer formation by deoxy-Hb S \( \beta^85E,\beta^88E \) required much higher hemoglobin concentrations than that of Hb S \( \beta^85E \) or Hb S \( \beta^88E \) even in high phosphate buffers, and due to its low yield (5–8% of that of the Hb S Glu-\( \beta^85 \) or Hb S Glu-\( \beta^88 \) variants), we were unable to characterize polymerization properties of the double Glu-containing Hb S variant.

**Effect of Hydrophilic Amino Acids in the \( \beta \) Acceptor Pocket on Instability of the Hb S Variants**—Helix thermal stability for...
FIG. 4. Thermal stability of Hb S variants in different liganded forms measured by repetitive scanning of absorption spectra. Thermal stability of oxy (A), CO (B), and deoxy (C) forms of hemoglobin (30 μM) was assessed using a Hitachi U3000 spectrophotometer. Results for Hb S βF85E, βL88E (e), Hb S βF85E (c), and Hb S βL88E (d) were compared with those of Hb A (a) and Hb S (b) using repetitive scanning (20
Hb S βF85E, βL88E in the CO form was measured by monitoring temperature-induced CD changes in ellipticity at 222 nm at 60 °C. These results were then compared with those of Hb S βF85E, Hb S βL88E, Hb S, and Hb A. Changes in ellipticity at 222 nm as a function of incubation time at 60 °C for Hb A and Hb S were minimal (Fig. 3). In contrast, signals for all three Hb S variants changed dramatically under the same conditions. The rate of change for Hb S βF85E was 15-fold greater than that of Hb S, while the rate of change for Hb S βL88E or Hb S βF85E, βL88E was 30-fold greater than that of Hb S. Changes for Hb S βF85E, βL88E were similar to Hb S βL88E rather than Hb S βF85E. These results suggest that both Phe-β85 and Leu-β88 are critical for stabilization and that a single Glu substitution at β85 in Hb S has less effect on pocket destabilization than that of a single Glu at β88. Furthermore, our results show that there was no further effect on destabilization of the pocket by insertion of Glu at β85 in the Glu-β85-substituted variant.

We also measured stability of the Glu-substituted Hb S variants at elevated temperatures in order to evaluate effects of β85 and β88 substitutions on globin stability as well as heme oxidation. Heat stability tests of Hb S βF85E, βL88E in different liganded forms were measured by repetitive scanning of their absorption spectra between 200 and 700 nm at 1-min intervals at constant temperature. These results were then compared with those for Hb S βF85E, Hb S βL88E, Hb A, and Hb S. Absorption spectra of the three variants, Hb A, and Hb S were measured at 45 °C for oxy and at 60 °C for CO and deoxy forms (Fig. 4). Stabilities of Hb S in the different liganded forms were similar to that for Hb A, and both showed minimal changes in absorbance at constant temperature after repetitive scans. In contrast, stabilities for all three Hb S variants changed dramatically under the same conditions. Hb S βF85E, βL88E was the most unstable with instabilities ordered as follows: Hb S βF85E, βL88E > Hb S βL88E > Hb S βF85E > Hb S and Hb A. Under these conditions, positions of absorption spectra peaks for all hemoglobin samples in the visible range were not significantly shifted even though their total peak heights and base-line values changed as a function of incubation time. Changes in base-line value as a function of time are caused by increased turbidity due to the precipitated hemoglobin. Soret band peaks (~410 nm) and visible range peak positions for all hemoglobins did not change, but absorption in the UV range for oxy and CO forms of the Hb S variants was significantly altered with increasing turbidity during incubation. These results suggest that precipitation of the Hb S variants occurred without heme oxidation and that unfolding of globins directly causes precipitation under these conditions. After 20 repetitive scans (20 min), precipitates were removed by centrifugation, and soluble hemoglobin concentration was determined from its absorption spectra (Fig. 5). The percentage of total hemoglobin in the oxy form, which precipitated after 20 min at 45 °C, for Hb A, Hb S, Hb S βF85E, Hb S βL88E, and Hb S βF85E, βL88E was 2.4, 16.1, 16.6, 27, and 30%, respectively. In contrast, when the temperature was raised to 60 °C, oxy-Hb S βF85E, βL88E underwent heme oxidation like Hb S βF85E, Hb S βL88E, and Hb S in the oxy form as reported previously (3), and all three Hb S variants quickly precipitated within 5 min.

CO and deoxy forms of the variant hemoglobins at 60 °C were more stable than oxy forms, and the heme moieties did not undergo oxidation under these conditions. Percentages of precipitated hemoglobins in the CO and deoxy forms after 20 min of incubation at 60 °C are shown in Fig. 5. Values for CO forms of Hb A, Hb S, Hb S βF85E, Hb S βL88E, and Hb S βF85E, βL88E were 16.4, 35.2, 54.1, 72.1, and 78.3%, respectively. In addition, deoxy forms were more stable than the corresponding CO forms. Values for deoxy forms of Hb A, Hb S, Hb S βF85E, Hb S βL88E, and Hb S βF85E, βL88E were 0.5, 1.3, 14, 26 and 27%, respectively.

**DISCUSSION**

X-ray crystallographic analysis showed that Phe-β85 and Leu-β88 are located on the F helix, which is also near the position of porphyrin in the heme pocket of the hemoglobin molecule. Heme is then inserted into a hydrophobic cleft on the surface subunit. The side chain of Leu-β88 (F4) contacts the heme side chain (13), and binding of heme to globin involves a specific stereochemical fit that helps stabilize tertiary conformation of the subunit. Phe-β85 (F1) is also near the heme pocket and acts as a spacer between the F and H helical segments (13). These two hydrophobic amino acids on the F helix are important in maintaining the hydrophobic environment of the heme pocket and in protecting against entrance of water molecules. Heme insertion can also stabilize globin chain structure (14, 15).

Our results showing large amounts of free α chains after cell lysis and low yield for only the double Glu-containing Hb S variant, Hb S βF85E, βL88E, suggest that substitution of hydrophilic amino acids such as Glu at both β85 and β88 possibly inhibits heme insertion into the pocket. In addition, our findings imply that apo-βF85E, βL88E chains precipitate before assembly with α chains; however, tetramers that do form, even though low yield, appear to fold like the single Glu-containing Hb S tetramers as well as like Hb A and Hb S, as assessed by CD studies.

Upon deoxygenation of Hb S, these hydrophobic sites on the F helix can form an acceptor pocket to contact with Val-β85 in a neighboring Hb S molecule, which promotes polymerization and sickling of red cells. Therefore, hydrophilic substitutions like Glu for Phe-β85 and Glu for Leu-β88 should reduce hydrophobic interactions and result in inhibition of deoxy-Hb S polymerization (3). X-ray analysis of crystallized deoxy-Hb S shows that β85 and β88 amino acid side chains line the bottom of the acceptor pocket, with Phe-β85 located internally at the pocket floor. The 3-fold lower critical concentration required for polymerization of deoxy-Hb S βF85E compared with deoxy-Hb S βL88E can be explained by the composition and

![Fig. 5. Comparison of thermal stability for Hb S βF85E, βL88E, Hb S βF85E, βL88E, and Hb S βF85E, βL88E. Percentage of denatured Hb S βF85E, βL88E (c), Hb S βL88E (d), and Hb S βF85E (c) in different liganded forms was determined by removal of precipitated hemoglobins after 20 repetitive scans and then measuring remaining soluble hemoglobin. Results were then compared with those of Hb A (a) and Hb S (b). Experimental conditions were the same as those described in Fig. 4 with A, B, and C indicating oxy, CO, and deoxy forms, respectively.](image-url)
geometry of the acceptor pocket (3). Although Phe-β85 and Leu-β88 line the floor of the pocket in the F helix, Leu-β88 is located near the pocket entrance, while Phe-β85 is located internally at the pocket floor (1, 2). Changing Leu to Glu at β88 might then be expected to have more influence on interaction with Val-β6 than changes at Phe-β88. In addition, changes at β88 are also expected to increase oxygen affinity and decrease tetramer stability. In fact, our previous studies with the hydrophilic substitutions of Glu for both Phe-β85 and Leu-β88 showed increased oxygen affinity and decreased tetramer stability (3, 6).

Our present results show that Hb S variants containing Glu substitutions at β85 or β88 as well as at both positions were less stable than Hb A and Hb S. Thermal stability of oxy-Hb S βL88E at 45 °C was slightly less than that of Hb S βF85E. Temperature-induced precipitation of these variants appears to occur by unfolding of chains in the absence of heme oxidation. In contrast, the oxy forms of the three Hb S variants as well as Hb A and Hb S all undergo ferric Hb formation upon incubation at 60 °C. The three Hb S variants containing Glu at β85 and/or β88 then quickly precipitate (3). Thermal stability of oxy-Hb S βL88E at 60 °C was similar to oxy-Hb S βF85E (3). In contrast, CO hemoglobin and deoxyhemoglobin are generally more unstable than Hb S βF85E, while thermal stability for Hb S βF85E, βL88E was similar to that for Hb S βL88E. These results with the three Hb S variants were the same as their relative stabilities as measured by CD temperature-induced changes in ellipticity at 222 nm and suggest that hydrophobicity at β85 and β88 is more critical in stabilizing globin chain structure than in maintaining heme stability. Furthermore, Glu insertion at β88 in the F helix appears to destabilize pocket structure much more than Glu insertion at β85. These results are also in agreement with our previous findings showing that Phe insertion at β85 inhibited Hb S polymerization less than Phe insertion at β88. In addition, oxy-Hb S βL88E was less stable than Hb S βF85E to mechanical agitation, which relates to surface hydrophobicity of dimeric hemoglobin (3). These results suggest that hydrophobic amino acids at β85 and β88 contribute to stabilization of globin structure and heme insertion as well as to stability of ββ assembly of hemoglobin. Detailed structural analysis of the acceptor sites in these different Hb S variants is now important to further our understanding of key hydrophobic interactions that promote polymerization of deoxy-Hb S.

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