Effects of Different β73 Amino Acids on Formation of 14-Stranded Fibers of Hb S versus Double-Stranded Crystals of Hb C-Harlem†

Kazuhiko Adachi,*,‡ Min Ding,‡ Suzanne Wehrli,‡ Konda S. Reddy,§ Saul Surrey,|| and Kazumi Horiuchi‡

Division of Hematology and NMR Core Facility, The Children's Hospital of Philadelphia, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, Department of Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104, and Cardeza Foundation for Hematologic Research, Department of Medicine, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Received August 27, 2002; Revised Manuscript Received December 13, 2002

ABSTRACT: Hb S ($\alpha_2\beta_2^{6Glu\rightarrow Val}$) forms polymers, while Hb C-Harlem ($\alpha_2\beta_2^{6Glu\rightarrow Val,73Asp\rightarrow Asn}$) forms crystals upon oversaturation. Since the only difference between the two is the β73 amino acid, it follows that this site is a critical determinant in promoting either polymerization or crystallization. β73 Asp in Hb S forms a hydrogen bond with β4 Thr, while β73 Asn in Hb C-Harlem may inhibit this interaction as well as increase the hydrophobicity at the EF helix β6 Val acceptor sites. Two new β73 Hb S variants (β73 His and Leu) were constructed and analyzed to define other amino acids facilitating formation of Hb S-like polymers versus Hb C-Harlem-like crystals. The two variants that were chosen were expected to either (1) enhance formation of the β73−β4 hydrogen bond (β73 His) or (2) inhibit it and increase the hydrophobicity of the EF helix β6 Val acceptor sites (β73 Leu). β73 His Hb S formed fibers but at a lower concentration than Hb S, while β73 Leu Hb S formed crystals but at a higher concentration than Hb C-Harlem. The solubility of β73 His Hb S was $^1/_7$ of that of Hb S, while the solubility of β73 Leu Hb S was similar to that of Hb C-Harlem. The delay time prior to polymer or crystal formation depended on Hb concentration. The delay time for β73 His Hb S was 10^5-fold shorter than that for Hb S, while that for β73 Leu Hb S was 10^5-fold longer in 1.0 M phosphate buffer. NMR results indicate β73 amino acid changes induce alteration in the β-chain heme pocket region, while CD results indicate no change in the helical content of the variants. These results suggest that enhancing the β73−β4 hydrogen bond and/or induced changes in the heme pocket by the β73 Asp to His change facilitate formation of Hb S-like fibers. Our results also suggest that removal of the β73−β4 hydrogen bond and enhancing the hydrophobicity of the EF helix β6 Val acceptor sites by the β73 Asp to Leu or Asn changes delay nuclei formation and facilitate formation of Hb C-Harlem-like crystals.

Replacement of β6 Glu with a hydrophobic Val in the β-chain of hemoglobin (Hb S) leads to formation of long, multistranded polymers when oversaturated Hb S is deoxygenated. The polymers form a gel, which is comprised of fibers. Intracellular polymers or fibers cause a significant reduction in red blood cell deformability (sickling), leading to obstruction of flow in the microcirculation. Each polymer of Hb S assembles into 14-stranded fibers, which then form a viscous gel (*1*, *2*). Further analysis of the fibers demonstrated that each is composed of seven half-staggered double strands (*1*), suggesting that the double-stranded structure of deoxy-Hb S crystals may be relevant components of fibers within cells.

An important feature of the double strand is the contact between two molecules in adjacent strands (the so-called "lateral" contact between adjacent members of the double strand, related by a diagonal displacement) and "axial" contact (along an individual strand, related by a vertical displacement). Structural analysis demonstrated that Hb S polymers have a packing arrangement very similar to that of deoxy-Hb S single crystals, which are composed of paired or double strands (*1*). The amino acid residues involved in these contacts are known from studies with other mutants to influence polymerization (*3*, *4*). The lateral contacts are more prominent because the mutant β6 Val appears in one of them and is thus a crucial feature in the Hb S polymerization process. This contact involves burying the β6 Val in a hydrophobic pocket formed by β88 Leu, β85 Phe, and several heme atoms. Within the hydrophobic pocket, although the β6 Val is buried, it is not fully in van del Waals contact with the receptor pocket, and this permits a network of water molecules to be located in the contact and facilitates formation of bridging hydrogen bonds between tetramers (*4*). Furthermore, β4 Thr and β73 Asp are expected to make contact and form a hydrogen bond in addition to influencing the hydrophobic interaction.

Hb C-Harlem has two amino acid changes, β6 Glu → Val and β73 Asp → Asn, which are located at interaction sites of deoxy-Hb S polymers (*5*). The crystal structure of the double strand of Hb S provides detailed atomic pictures of

† This research was supported in part by grants from the National Institutes of Health (HL58879 and HL38632), by the Cardeza Foundation for Hematological Research, and by Jefferson Medical College.

* To whom correspondence should be addressed. Division of Hematology, The Children's Hospital of Philadelphia, 34th St. & Civic Center Blvd., Philadelphia, PA 19104. Telephone: (215) 590-3576. Fax: (215) 590-4834. E-mail: adachi@email.chop.edu.

‡ University of Pennsylvania School of Medicine.

§ University of Pennsylvania.

|| Thomas Jefferson University.

10.1021/bi026740x CCC: $25.00
Published on Web 03/26/2003

the intermolecular contact involving β6 Val and β73 Asp in Hb S (*3*, *4*, *6*). The β4 and β73 positions are located near β6 Val and the EF helix acceptor sites in the β-subunit, respectively, which are critical lateral contact regions in Hb S polymers (*3*, *4*). Early studies of Hb C-Harlem showed a minimum gelling concentration (MGC), which was determined by concentrating hemoglobin using a dry inert gas until a gel is formed, ~1.5−2-fold higher than that of Hb S (*5*, *7*). In addition, employing kinetic studies with Hb C-Harlem in high-phosphate buffers (e.g., 1.5−1.8 M), we found that nucleation is controlled not only by interactions of β6 Val in the hydrophobic pocket formed by β88 Leu and β85 Phe but also by formation of a β73 Asp−β4 Thr hydrogen bond (*8*). Furthermore, the most convincing support for the existence of a double-stranded structure of Hb S polymers comes from extensive copolymerization studies of Hb S with other variants having an altered residue on the molecular surface such as Hb C-Harlem (*5*). These studies concluded that the β73 region of the β^S-chains *in trans* to the β6 Val acceptor site in Hb S polymers is involved in an intermolecular contact.

We recently reported nuclei and polymer formation of Hb C-Harlem using differential interference contrast (DIC) and electron microscopy in addition to presenting kinetic and thermodynamic studies of polymerization under near physiological conditions (*9*). DIC images showed that Hb S fibers grew with a wheat-sheaf pattern from spherulitic domains and made fibers with aligned bundles, while Hb C-Harlem grew straight fibers or crystals rather than bending fibers such as Hb S. Electron microscopy (EM) studies of Hb C-Harlem showed formation of only crystals with no fiber formation or any other Hb S polymerization characteristics such as macrofibers or bundles. Equilibrium studies of Hb C-Harlem polymers showed that its solubility was essentially the same as that of Hb S. In addition, a single amino acid change at β73 in Hb S, as in Hb C-Harlem, can increase the contact energy of the dominant hydrophobic interactions and decrease the vibrational entropy during assembly of Hb S monomers, which could explain the slow nucleation of Hb C-Harlem following crystal formation compared to the more rapid nucleation of the 14-stranded fibers such as Hb S. These results suggest that not only the shape but also the rate of fiber and nuclei formation of Hb S is influenced by the β73 amino acid. Furthermore, studies of prolonging the delay time of Hb C-Harlem polymerization can be used in identifying chemicals including peptides which might ameliorate sickle cell disease (*9*).

To understand further the determinants facilitating formation of 14-stranded Hb S fibers versus double-stranded crystals of Hb C-Harlem ($\alpha_2\beta_2^{6Glu\rightarrow Val,73Asp\rightarrow Asn}$) as well as help to explain the different equilibrium and kinetic properties of Hb C-Harlem and Hb S polymers, we made two new β73 Hb S variants, Hb S (β73 Asp → His) and Hb S (β73 Asp → Leu). The polymerization properties and kinetics of these variants then were characterized. We anticipated since crystallization of Hb C-Harlem is caused by mutation at β6 (Glu → Val) and β73 (Asp → Asn), and polymerization of Hb S is caused by the same mutation at β6, that probing of the β73 Asp−β4 Thr hydrogen bond in Hb S polymers would further our understanding of the role of this bond in promoting crystallization versus polymerization.

MATERIALS AND METHODS

Hb C-Harlem was purified from red blood cell hemolysates from an individual with Hb AC-Harlem by standard chromatography on a Source 15S FPLC column (Pharmacia Biotech, Inc., Piscataway, NJ) equilibrated with 40 mM Bis-Tris buffer (pH 5.8) and eluted using a linear gradient from 0 to 0.2 M NaCl in the same buffer. Hb S was purified from AS blood using the same chromatographic conditions (*7*).

Two recombinant β73 Hb S variants, Hb S (β73 Asp → His) and Hb S (β73 Asp → Leu), were expressed in bacteria after subcloning the corresponding cDNAs into pHE2 which was originally constructed to coexpress α- and β-globin chains with methionine aminopeptidase (MAP) under transcriptional control of a *p*tac promoter (*10*). The two β-globin chain variants (β6 Glu → Val,β73 Asp → His and β6 Glu → Val,β73 Asp → Leu) were constructed by site-specific mutagenesis of the normal β-chain using recombination and polymerase chain reaction (PCR) as described previously (*11*). The β-chain variants were expressed with α-chains to form tetrameric Hb S variants (*11*). These recombinant Hb S variants lack the chain-initiating N-terminal methionine due to cleavage by MAP which is also coexpressed. Clones of the engineered variants were subjected to DNA sequence analysis of the entire β-globin cDNA region using site-specific primers and fluorescently tagged terminators in a cycle sequencing reaction in which extension products were analyzed on an automated DNA sequencer. The plasmids were transfected into *Escherichia coli* (JM 109) (Promega Co., Madison, WI), and bacteria were grown at 30 °C with shaking at 225 rpm in 1 L of Terrific broth containing 10 μM ampicillin to a density of ~3 × 10^{10} bacteria/mL. Expression of the two Hb S variant tetramers was induced for 4 h at 30 °C by addition of 0.2 mM IPTG (Fisher Scientific, Fairlawn, NJ), and cultures then were supplemented with 30 μM hemin (Aldrich Chemical Co. Inc., Milwaukee, WI) and 1% (w/v) glucose. Purification of hemoglobins was carried out as described previously (*11*), and sample purity was assessed by cellulose acetate electrophoresis on Titan III membranes at pH 8.6 with Super-Heme buffer, by isoelectric focusing on the pH 3−10 and 5−8 Hb-resolve systems (Bio-Rad, Richmond, CA), and by SDS−PAGE.

Electrospray ionization mass spectrometry (ES-MS) was performed to confirm the mass of the two purified recombinant Hb S variants using a VG BioQ triple-quadrupole mass spectrometer (Micromass, Altrincham, Cheshire, U.K.). Data analysis employed the MassLynx software package (Micromass, Altrincham). Hemoglobin concentrations were determined spectrophotometically using a millimolar extinction coefficient mE_{555} of 50 for deoxyhemoglobin and an mE_{579} of 53.6 for carbon monoxyhemoglobin (on a tetramer basis). Circular dichroism (CD) spectra of hemoglobins were recorded using an Aviv model 62DS instrument employing a 0.1 cm light path cuvette at ~20 μM hemoglobin, and the temperature was controlled with a thermoelectric module. Purified hemoglobins were stored in the CO-liganded form at −80 °C until they were used. Hemoglobin solutions were concentrated using a Centricon centrifugal concentrator with a membrane cutoff of 30,000 Da (Centricon-30, Amicon, Inc.). NMR spectroscopy with recombinant Hb S variants, Hb A, Hb S, and Hb C-Harlem in the CO form in 0.1 M

Bis-Tris (pH 7.0) in 90% H_2O and 10% D_2O was performed using a DMX 400 NMR spectrophotometer (Bruker Instrument Inc., Billericia, MA) at 22 °C. Oxyhemoglobin was prepared by first blowing oxygen across the surface of the CO/Hb solution in a rotary evaporator under a 150 W floodlight bulb in an ice bath for ~1 h.

Hb S fiber formation was initiated on glass slides by first adding 100 mM sodium dithionite to oxy-Hb S solutions at 4 °C in 0.1 or 1.0 M phosphate buffer at pH 7.0 or 7.3, respectively. Approximately one microliter of solution was pipetted to a glass slide which then was sealed with an 18 mm square coverslip using Mount-Quick solution (Daido Sangyo Co., Ltd.) (*9*). Polymer formation (fiber and crystal) of deoxygenated hemoglobin was induced by the temperature-jump method from 0 °C on ice to room temperature (~22 °C). Polymers were analyzed by differential interference contrast (DIC) microscopy using an Olympus microscope equipped with DIC optics and a 40× oil (1.00 NA) or 60× oil (1.40 NA) immersion lens. The microscopic images were transferred to a personal computer via an image grabber board (Scion AG5, Scicon Corp., Frederick, MD) and a CCD (charge-coupled device) camera (Cohu Camera, Cohu Inc., San Diego, CA).

The kinetics of polymerization and solubility of Hb S, Hb C-Harlem, and the two β73 Hb S variants were evaluated in 1.0 M buffer at pH 7.3 and 30 °C, with solubility determined by centrifugation after completion of polymerization (*12*).

RESULTS

Expression and Purification of Soluble Hb S Variants Containing β73 His or Leu. To define further the role of β73 amino acids in polymerization of Hb S and crystallization of Hb C-Harlem, β^S-globin chain variant tetramers ($\alpha_2\beta_2^{6Glu\rightarrow Val,73Asp\rightarrow His}$ and $\alpha_2\beta_2^{6Glu\rightarrow Val,\beta73Asp\rightarrow Leu}$) were expressed in bacteria and characterized after purification. The two purified β73 Hb S variants migrated as single 16 kDa bands on SDS−PAGE (data not shown). The electrophoretic mobility on cellulose acetate membranes of purified β73 His Hb S and β73 Leu Hb S tetramers showed single bands whose migration was similar to that of Hb C ($\alpha_2\beta_2^{6Glu\rightarrow Lys}$) and Hb C-Harlem ($\alpha_2\beta_2^{6Glu\rightarrow Val,\beta73Asp\rightarrow Asn}$), respectively (data not shown). Each tetrameric hemoglobin variant eluted as a single main peak on FPLC using a Mono S column and a linear gradient from 0 to 0.2 M NaCl in 40 mM Bis-Tris buffer at pH 5.8 and 4 °C. It is noteworthy that the elution pattern of these recombinant hemoglobins showed only a single peak compared to the originally reported existence of two peaks for recombinant Hb A following cation-exchange column chromatography (*10*). The two peaks were presumed to be caused by different heme orientations in Hb A (*10*). We further confirmed the existence of a single component using isoelectric focusing at both pH 3−10 and 5−8 (data not shown), suggesting that no differently charged components are present in our purified fractions isolated by FPLC and CM chromatography.

Automated DNA sequence analysis of the entire β-globin cDNA region in the expression vectors using site-specific primers showed the expected sequence for the two variant cDNAs. Mass spectral analysis of the α- and β-chains of two β73 Hb S β-chain variants using electrospray ionization mass spectrometry (ES-MS) resulted in values of 15 859, 15,836, and 15,126 for the β73 His and β73 Leu β-chain variants and α-globin chains, respectively. Results of mass spectral analysis of the α- and β-chains of Hb C-Harlem were previously reported (*9*). In addition, no bands corresponding to an additional Met were detected in either the α- or β-chains, in agreement with expected masses, indicating complete cleavage of the N-terminal methionine by MAP and no post-translational modifications (data not shown) using this expression system (*10*). Carbon monoxy forms of the two variants as well as Hb S and Hb C-Harlem exhibited absorption spectral characteristics identical to those of human hemoglobin chains as shown for CO Hb A, including peaks at 569, 540, 344, 421.5, and 276 nm (*13*) except the Soret peak (421 nm) of β73 Leu Hb S. The Soret peak for β73 Leu Hb S shifted to the blue (~0.5 nm) even though its α-peak (569 nm) and β-peak (540 nm) regions of the spectra are identical to those of other hemoglobins (Figure 1A), indicating some alterations in the heme region of this variant. In addition, the CD spectra of these variants in the UV region

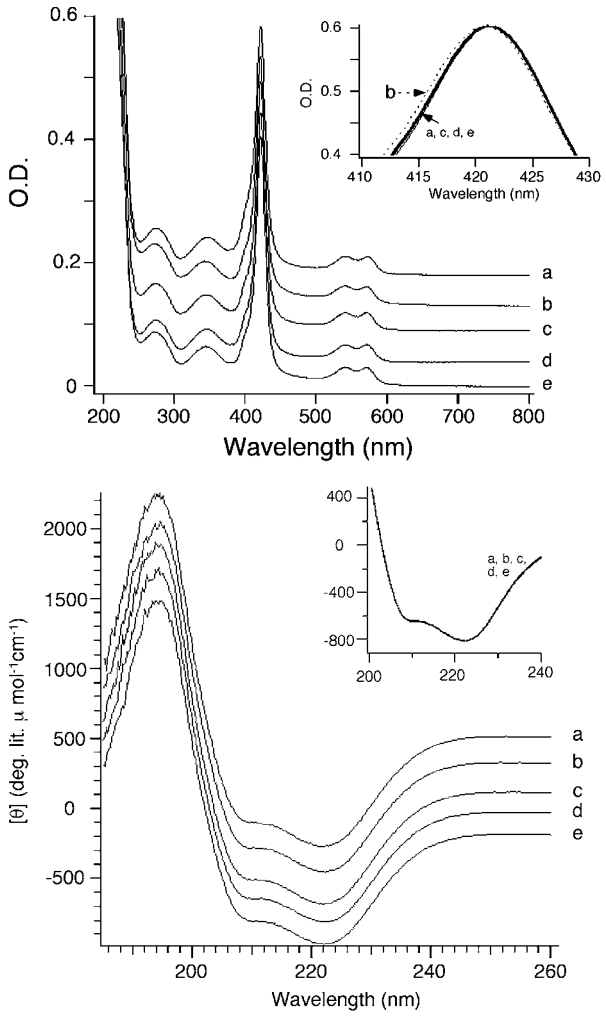

FIGURE 1: Absorption (A) and circular dichroism (B) spectra of purified β73 His, Leu, and C-Harlem compared to those of Hb A and Hb S. Absorption and circular dichroism (CD) spectra were determined for purified hemoglobin tetramers in the CO form in 10 mM phosphate buffer at pH 7.0 and 10 °C. Spectra are shown for β73 His Hb S (a), β73 Leu Hb S (b), Hb C-Harlem (c), Hb S (d), and Hb A (e). The inset represents the Soret region of absorption spectra between 410 and 430 nm (A) and CD spectra between 200 and 240 nm (B) for each hemoglobin tetramer normalized to values at 222 nn after subtraction of the buffer spectrum.

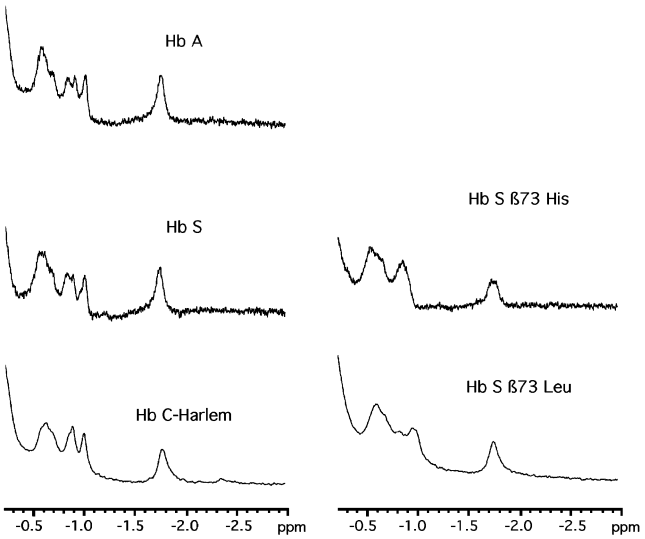

FIGURE 2: NMR ring current-shifted proton resonance spectra of purified recombinant β73 His Hb S and β73 Leu Hb S in the CO form were compared to those of purified human Hb A, Hb S, and Hb C-Harlem in the CO form in 0.1 M Bis-Tris buffer (pH 7.0) in 90% H_2O and 10% D_2O at 22 °C.

were superimposed on those of Hb C-Harlem and Hb S (Figure 1B).

We also compared NMR spectra of the two purified recombinant Hb S variants with those of Hb A, S, and C-Harlem in the CO form to monitor the existence of different heme orientations in the heme pocket of the β-chain in the Hb S variants (Figure 2). Alteration of heme structure in the heme pocket of β-chains in the CO−Hb A form causes a change in the NMR spectrum in the region from 0 to −2 ppm (*10*). The NMR spectra from 0 to −2 ppm for human hemoglobins A and S were the same, while the spectra for recombinant β73 Leu Hb S and C-Harlem were slightly different from that of Hb S. Furthermore, the region from 0 to −2 ppm for β73 His Hb S was different from that of Hb S. Most notable was the absence of a signal around −1.0 ppm in the spectrum of β73 His Hb S (Figure 2). However, the NMR spectrum of β73 His Hb S did not change significantly upon conversion of the CO form to the ferric form and then back to the CO form. These results and the existence of single components for the variants on electrophoretic and chromatographic analyses indicate that the different β73 amino acid substitutions do not result in different heme orientations in the β73 Hb S variants. The β73 amino acid change may, however, result in some alterations in the heme pocket region of the β-chains.

DIC Analysis of Oversaturated β73 Hb S Variants in 0.1 M Phosphate Buffer. Fibers of deoxy-Hb S in 0.1 M phosphate buffer grew rapidly into thick bundles at concentrations greater than its solubility (∼17 g/dL); fibers or bundles were observed within 1 min (*14*, *15*). A typical DIC image of deoxy-Hb S fibers (17 g/dL in 0.1 M phosphate buffer at pH 7.0 and room temperature) at the end of polymer formation is shown in Figure 3A. The rate of elongation and the size of deoxy-Hb S fibers depend on concentration: the higher the concentration, the faster the elongation rate. Under the same conditions, DIC images of fibers made from the oversaturated deoxy-β73 His Hb S were essentially the same as those of deoxy-Hb S (Figure 3B), while the concentration required to form fibers was much lower than that of Hb S (9 g/dL for β73 His Hb S compared to 17 g/dL for Hb S).

DIC microscopic studies of purified Hb C-Harlem ($\alpha_2\beta_2^{6Glu\rightarrow Val,73Asp\rightarrow Asn}$) and β73 Leu Hb S were also performed. Even though the solubility of Hb C-Harlem is the same as that of Hb S (*9*), the concentration required for formation of Hb C-Harlem polymers within 30 min was 2.5-fold higher than that of Hb S (42 g/dL for Hb C-Harlem vs 17 g/dL for Hb S in 0.1 M phosphate buffer at pH 7.0 and room temperature). DIC images of Hb C-Harlem polymers showed linearly shaped polymers with a wheat-sheaf-like pattern as shown in Figure 3C. These fibers grew from initially formed domains as seen for Hb S fibers; however, growth of Hb C-Harlem polymers was much slower than that of Hb S. These observations are consistent with the result that Hb C-Harlem polymers do not form 14-stranded fibers, but do form double-stranded crystals as shown by electron microscopy (*9*). The hydrophobic amino acid substitution of leucine for asparagine at position β73 enhances this property. In fact, at 42 g/dL for the β73 Leu Hb S, no polymer formed after incubation for 1 h, but polymers did form after overnight incubation as shown in Figure 3D.

Polymerization and Solubility of β73 Hb S Variants in 1.0 M Phosphate Buffer. We reported previously that with higher hemoglobin concentrations (42 g/dL) Hb C-Harlem polymerized with an ∼10 min delay time in 0.15 M phosphate buffer (*9*). As shown in Figure 3, polymerization of β73 Leu Hb S at this concentration required more than an overnight incubation. A concentration of 50 g/dL is required for β73 Leu Hb S to polymerize with a 10 min delay time like that of Hb C-Harlem at 42 g/dL. Since it is difficult to prepare solutions with such high concentrations, we characterized the kinetics and solubility and performed DIC analyses of the β73 Hb S variants in 1.0 M phosphate buffer to compare the results for these three β73 Hb S variants and Hb S under the same conditions. The hemoglobin solubility and concentration required for polymerization are affected by phosphate concentration: the higher the phosphate concentration, the lower the hemoglobin concentration required for polymerization (*11*, *12*). Therefore, increasing salt concentrations facilitate polymerization of hemoglobin variants with higher solubilities, thereby enabling kinetic analysis of polymerization at relatively lower hemoglobin concentrations. In addition, it is noteworthy that the structure of Hb S polymers made in high-phosphate buffer (1.5 M) is the same as that in low-phosphate buffer (0.05 M) using EM analysis (*16*). These findings indicate the structure of Hb S polymers at phosphate concentrations lower than 1.5 M is basically 14-stranded fibers (*16*).

In 1.0 M phosphate buffer, Hb S fibers were observed at 4 g/dL instead of at 17 g/dL which is required in 0.1 M phosphate buffer. The critical concentrations for observing fibers for β73 His Hb S, Hb C-Harlem, and β73 Leu Hb S after a 1 h incubation in 1.0 M phosphate buffer at room temperature were 0.7, 10, and 17 g/dL, respectively. DIC images of fiber or crystal formation for the β73 Hb S variants in comparison to those of Hb S and Hb C-Harlem are shown in Figure 4. Fibers of Hb S (Figure 4A) and β73 His Hb S (Figure 4B) formed quickly, and were the same, while both Hb S and the β73 Hb S variant fibers in 1.0 M phosphate buffer were shorter than those observed in 0.1 M phosphate buffer. Fiber formation for β73 His Hb S resulted in higher domain numbers with shorter fibers at the higher phosphate

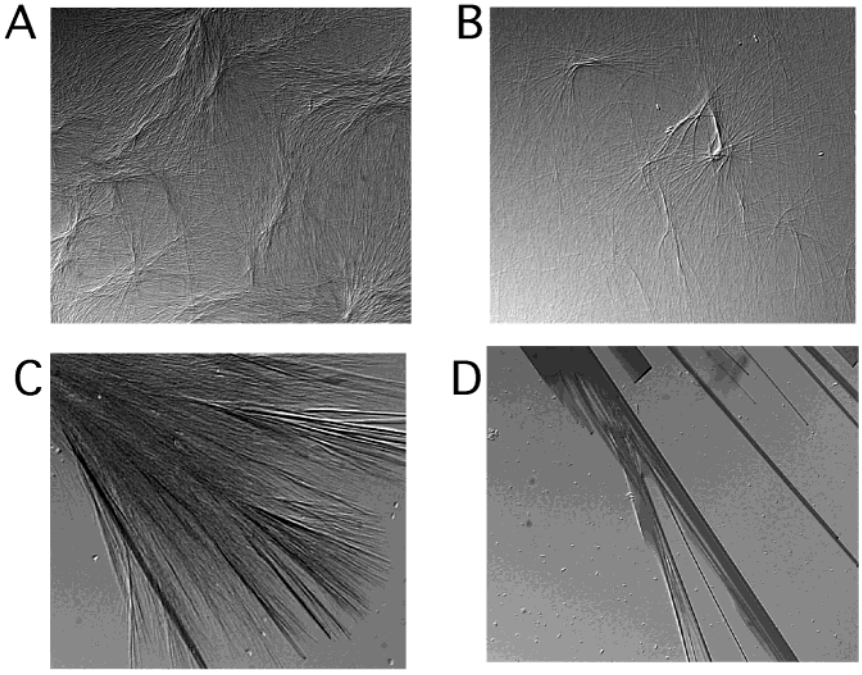

FIGURE 3: DIC images of deoxy-Hb S polymers formed in 0.1 M phosphate buffer containing different β73 amino acids. Panels A−D represent DIC images of polymerization of Hb S (17 g/dL after 30 min), β73 His Hb S (9 g/dL, after 20 min), Hb C-Harlem (β73 Asn) (42 g/dL after 30 min), and β73 Leu Hb S (42 g/dL after overnight incubation), respectively. Polymers were prepared in 0.1 M phosphate buffer (pH 7.0) after a temperature jump from 0 °C to room temperature. The length of the frame (*x*-axis) in the photograph represents 150 μm.

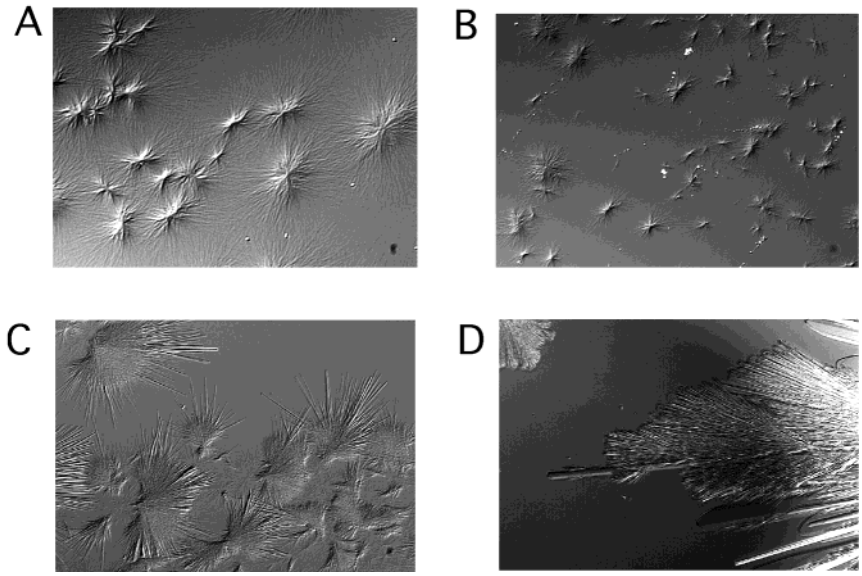

FIGURE 4: DIC images of deoxy-Hb S polymers formed in 1.0 M phosphate buffer containing different β73 amino acids. Panels A−D represent DIC images of polymerization of Hb S (3.8 g/dL after 4 min), β73 His Hb S (0.7 g/dL after 3 min), Hb C-Harlem (β73 Asn) (10 g/dL after 40 min), and β73 Leu Hb S (17 g/dL after 130 min), respectively. Polymers were prepared in 1.0 M phosphate buffer (pH 7.3) after a temperature jump from 0 °C to room temperature. The length of the frame (*x*-axis) in the photographs represents 150 μm.

concentration. DIC results for deoxy-Hb S polymerization above ~3 g/dL demonstrated formation of many domains followed by growth of fibers from the domains after a few minutes at room temperature (22 °C). Almost all of the polymers generated a pattern characteristically shaped like a cross, with two opposite quadrants of tightly packed polymers, some of which broadened outward to form a spherulite. The center of the cross, which appears to be a domain at the initial stage of polymer formation, exhibited the highest fiber density. Fibers were generated from the domains and elongated as a function of time. The rate of elongation of fibers and the number of domains were dependent on hemoglobin concentration: the higher the concentration, the greater the number of domains. Rapid domain formation followed by fiber growth of deoxy-Hb S in 1.0 M phosphate buffer was the same as that observed in low-phosphate buffer, as reported previously (*9*).

Polymers of Hb C-Harlem (β73 Asn) and β73 Leu Hb S also exhibited patterns similar to those observed in 0.1 M phosphate buffer and were characterized by formation of crystals rather than fibers. Hb C-Harlem polymers grew with domains and elongated without bending like Hb S polymers (Figure 4C), and were characterized by formation of needle-like crystals as seen in 0.1 M phosphate buffer. The β73

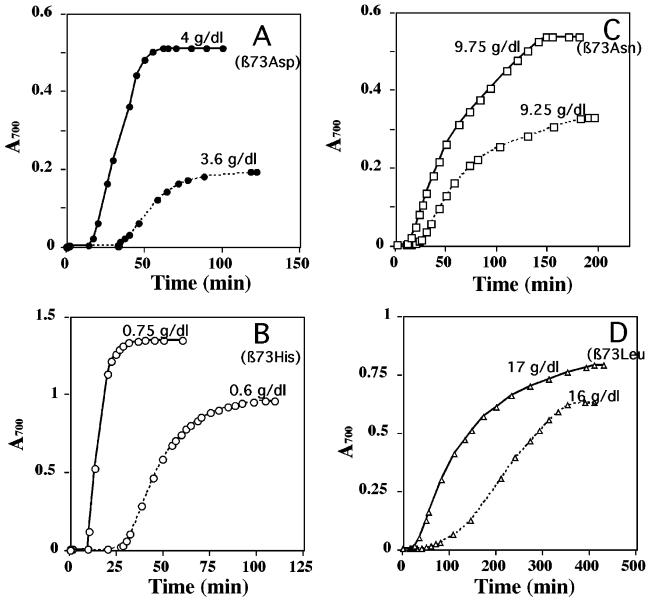

FIGURE 5: Polymerization of Hb S and β73 Hb S variants in 1.0 M phosphate buffer. Polymer formation was assessed by light scattering at 700 nm for (A) deoxy-Hb S (4 and 3.6 g/dL), (B) β73 His Hb S (0.75 and 0.6 g/dL), (C) Hb C-Harlem (β73 Asn) (9.75 and 9.25 g/dL), and (D) β73 Leu Hb S (17 and 16 g/dL). Experiments were performed using 1.0 M phosphate buffer (pH 7.3) with a temperature jump from 0 to 30 °C to initiate polymer formation.

Leu Hb S polymers (Figure 4D) also grew as crystals with a characteristic feathered shape.

The polymerization kinetics of the deoxy forms of these two β73 Hb S variants compared to Hb S and Hb C-Harlem were determined in 1.0 M phosphate buffer at 30 °C, and results are shown in Figure 5. Polymerization of deoxy-Hb S is characterized by a delay time prior to formation of the polymer, whose length depends on hemoglobin concentration: the lower the concentration, the longer the delay time (*1*). The presence of a delay time and the shape of the kinetic progress curves for β73 His Hb S (Figure 5B) are essentially the same as those of Hb S (Figure 5A). In contrast, the shape of the kinetic progress curve for polymerization of Hb C-Harlem (β73 Asn Hb S) (Figure 5C) differed from that of Hb S, even though there was a delay time prior to polymerization. The time required to reach a plateau in the progress curve for polymerization of Hb C-Harlem after a delay time was longer than that of Hb S. The shape of the kinetic progress curve for β73 Leu Hb S was similar to that of Hb C-Harlem. The ratio of polymerization time to delay time for Hb C-Harlem and β73 Leu Hb S was greater than 10-fold, while that for Hb S and β73 His Hb S was nearly 1. The relationship between logarithmic plots of delay time versus hemoglobin concentration of these four hemoglobins showed straight lines with similar slopes (~4) (Figure 6). The line for β73 His Hb S shifted 0.7 unit in log(grams per deciliter) to the left of the line for Hb S, while the line for β73 Leu Hb S shifted 0.7 unit in log(grams per deciliter) to the right of the line for Hb S. The line for Hb C-Harlem was intermediate between those of Hb S and β73 Leu Hb S. These results indicate that nuclei formation for oversaturated β73 His Hb S is 10^5-fold faster than that for Hb S, while that for β73 Leu Hb S is 10^5-fold slower than that for Hb S at the same Hb concentration. In addition, β73 Leu Hb S

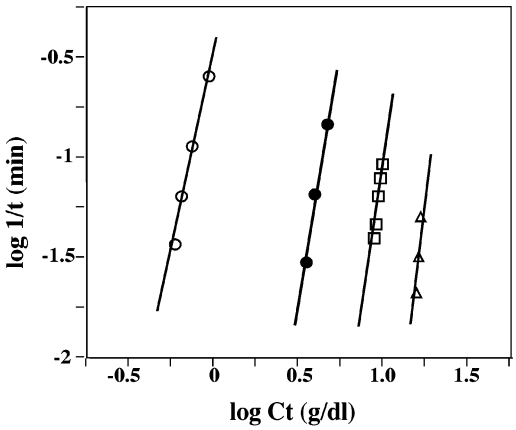

FIGURE 6: Relationship between the delay time prior to polymerization and Hb concentration. Logarithmic plots of the reciprocal delay time prior to polymerization vs Hb concentration are shown for Hb S (●), β73 His Hb S (○), Hb C-Harlem (□), and β73 Leu Hb S (△) in 1.0 M phosphate buffer (pH 7.3). Experimental conditions are the same as those described in the legend of Figure 4.

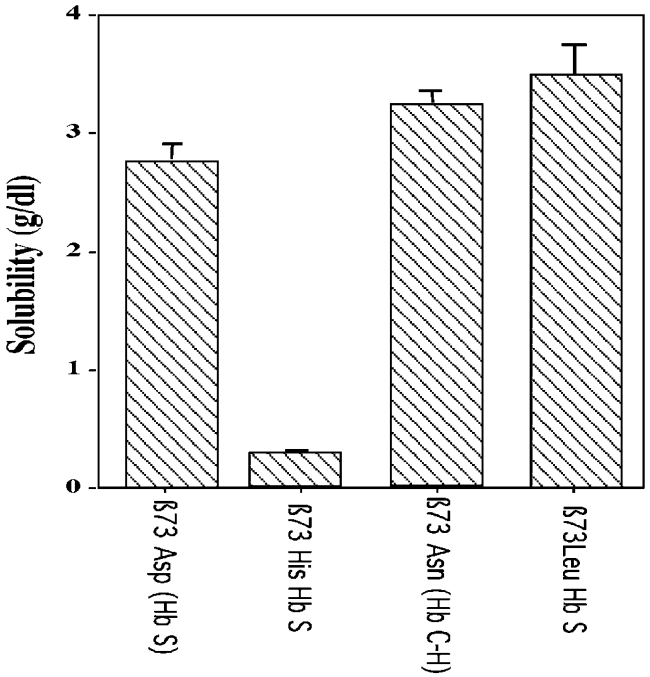

FIGURE 7: Solubility of Hb S (β73 Asp) and the β73 Hb S variants in 1.0 M phosphate buffer (pH 7.3) at 30 °C measured by centrifugation after completion of polymerization.

exhibited nucleation delayed by 10^3-fold compared to that of Hb C-Harlem in 1.0 M phosphate buffer.

We previously reported that the solubility of Hb C-Harlem was similar to that of Hb S in 0.15 M phosphate buffer, even though the MGC of Hb C-Harlem was ~2-fold higher than that of Hb S (*9*). The solubility of the Hb S variants after polymerization in 1.0 M phosphate buffer at 30 °C was measured after centrifugation (Figure 7). The solubility of β73 His Hb S under the same conditions was $\sim 1/7$ of that of Hb S. In contrast, the solubility of Hb C-Harlem was 1.2-fold higher than that of Hb S, while the solubility of β73 Leu Hb S was 1.06-fold higher than that of Hb C-Harlem. These results indicate that the inhibitory effect of β73 Leu on polymerization of β73 Leu Hb S compared to that of Hb S is not directly linked to its solubility, as shown in the studies of Hb C-Harlem (*9*).

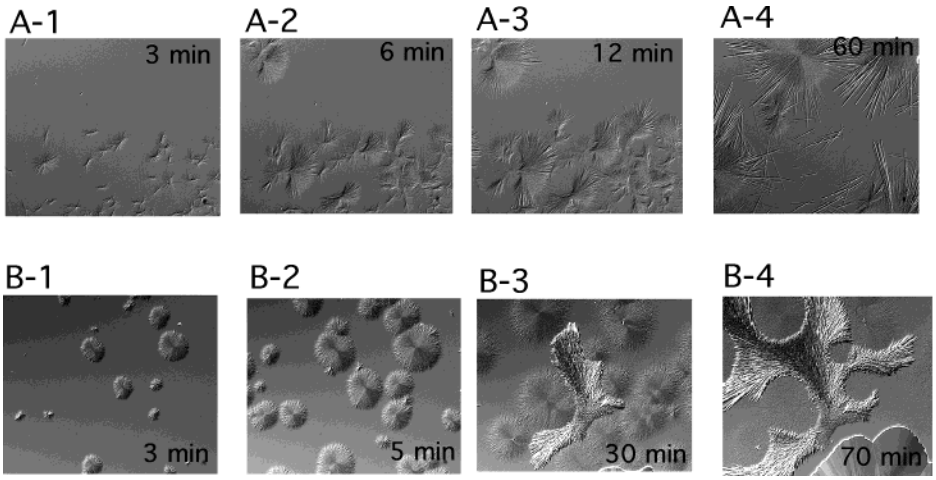

FIGURE 8: DIC images showing the time course of polymer formation for Hb C-Harlem and β73 Leu Hb S in 1.0 M phosphate buffer. DIC images of deoxy-Hb C-Harlem polymer formation (β73 Asn) (10 g/dL) after 3 (A-1), 6 (A-2), 12 (A-3), and 60 min (A-4) and DIC images of deoxy-β73 Leu Hb S polymer formation (19 g/dL) after 3 (B-1), 5 (B-2), 30 (B-3), and 70 min (B-4). Experiments were performed in 1.0 M phosphate buffer using a temperature jump from 0 °C to room temperature (~22 °C) to initiate polymerization. The length of the frame (*x*-axis) in the photographs represents 150 μm.

The time courses of polymer formation assessed by DIC microscopy for Hb C-Harlem and β73 Leu Hb S in 1.0 M phosphate buffer are shown in panels A and B of Figure 8, respectively. Hb C-Harlem (10 g/dL) polymers initially exhibited formation of domains such as Hb S (Figure 8A). Polymers with straight needle-type crystals elongated from domains, and growth of Hb C-Harlem polymers was slower than that of Hb S. Linear crystals with a radial shape pattern grew, and many needle-type crystals were produced at the final stage of Hb C-Harlem polymerization. The hydrophobic amino acid substitution of leucine for asparagine at position β73 in Hb C-Harlem enhanced this property. β73 Leu Hb S at 17 g/dL under the same conditions formed initial radial domains, but polymers formed with a spherulite shape (Figure 8, B-1 and B-2). Upon further incubation (30 min), straight, elongated, spiked crystals characteristic of a feathered shape formed from some of the initial spherulite-shaped polymers. After additional incubation, the initial spherulite-type polymer disappeared and elongated to form large spiked crystals with a feathered shape (Figure 8, B-4).

DISCUSSION

Promotion or Inhibition of Polymerization of Hb S by Changing the Amino Acid at Position β73. The similarly sized β73 Leu and His substitutions at the surface in Hb S may not change the stereochemical properties of the E-helix area like β73 Asn in Hb C-Harlem. Kinetics of polymerization of the two β73 Hb S variants were influenced by the amino acid at position β73. The kinetic progress curve for β73 His Hb S is similar to that of Hb S, while that for β73 Leu Hb S was similar to that of Hb C-Harlem. The progress curves of polymerization for Hb C-Harlem and β73 Leu Hb S after the initial delay time were much longer than that of Hb S. Furthermore, the initiation of polymerization or the minimum concentration required for polymerization of the β73 Hb S variants was affected by the type of amino acid (His $\ll$ Asp $\ll$ Asn $<$ Leu). Even though Leu is more hyrophobic than Asp, Asn, and His, this substitution inhibited polymerization of Hb S. Furthermore, promotion of polymerization and the decreased solubility of β73 His Hb S are consistent with the previous contention that polymerization of Hb S is controlled not only by hydrophobic interaction at the β6/EF hydrophobic pocket and several heme atoms but also by other amino acids, including those involved in hydrogen bond formation between β4 Thr and β73 Asp (*1*, *4*). These results suggest that inhibition of Hb C-Harlem polymerization compared to that of Hb S is due to removal of the hydrogen bond which is caused by substitution of Asn for Asp in addition to some influence on hydrophobic interactions of β6 Val, β88 Leu, and β85 Phe in Hb S polymers (*9*).

Table 1: Double-Strand Lateral Contacts within ~4.0 Å in the A-Helix (β_2) with EF Helices (β_1) of β-Globin Chains in Hb S Crystals[a]

	Thr4	Pro5	Val6	Ser9	Ala10	Ala13
Lys66		3.61				
Gly69		3.89				
Ala70		3.83				
Asp73	3.11		3.10			
Thr84			3.62			
Phe85			3.95			
Thr87					4.43	3.65
Leu88				3.50		

[a] Column 1 is for β_1 and row 1 for β_2. Lateral and axial contacts within the double strand were determined by X-ray crystallography at 2 Å resolution (*4*). Values are cited from lists of the unique $\beta_1-\beta_2$ lateral contacts in the double-strand structure of a deoxy-Hb S crystal (contact 1) (*4*).

An important feature of the double-strand model is that the contact between the two molecules in adjacent strands (i.e., so-called lateral contact) contains β6 Val and β4 Thr. Also, the contact between molecules in the same strand (the so-called axial contact) contains residues which are known from studies of other mutants to influence polymerization (*1*). Lateral contacts are critical since mutations at positions β6 and β73 affect polymerization (*5*). In fact, from the crystal structure of Hb S, the side chain of β73 Asp is located in the proximity of the side chains of β6 Val and β4 Thr (Table 1) (*6*). The δ-oxygen atom in the carboxyl group of β73 Asp is almost equidistant (~3 Å) from the β-carbon of β6 Val and the hydroxyl group in β4 Thr. It can therefore make hydrophobic contact with β6 Val, and possibly engage in a relatively weak hydrogen bond interaction with the hydroxyl

group of β4 Thr. The presence of a Leu or Asn at position β73 is likely to retain and/or enhance the hydrophobic interactions, and β73 Leu should abolish β4 Thr hydrogen bonding interactions. In addition, the presence of β73 Asn should allow β73−β4 hydrogen bond interaction at a level reduced compared to that with β73 Asp.

Furthermore, polymerization properties of Hb C-Harlem/Hb A mixtures were similar to those of Hb A/Hb S mixtures. These results suggest that only β73 Asp *in trans* to the β6 Val acceptor site of Hb S forms a hydrogen bond with β4 Thr as a lateral contact (*5*). Our previous polymerization studies of various mixtures using Hb A, Hb S, Hb C-Harlem, and Hb Korle Bu ($\alpha_2\beta_2^{73Asp\rightarrow Asn}$) also suggested that reducing the extent of hydrogen bond formation by β73 Asn inhibits nucleation and polymerization (*17*). Therefore, substitution of Asn or Leu for β73 Asp in Hb S should reduce or abolish the interaction energy of the β4−β73 hydrogen bond *in trans* to the β6 Val acceptor site as well as increase the contact energy of the β6 Val−EF pocket hydrophobic interaction (*9*). Furthermore, we believe that disruption of the hydrogen bond and enhancement of the hydrophobicity of β6 interaction sites by the Asp → Asn or Leu change may make the A-helix more flexible. This could result in new interactions with an adjacent amino acid, resulting in disruption of fiber formation and promotion of crystallization of Hb S. This would result also in increasing the critical concentration required for polymerization.

In contrast, promotion of polymerization by β73 His Hb S may not be related to altered hydrophobic contact with β6 Val, but may be influenced by formation of a hydrogen bond with β4 Thr. The change may also induce some conformational changes in the heme area of β-chains which should increase the stability of interactions of Hb S polymers and result in facilitating formation of 14-stranded fibers. We are now attempting to clarify the role of β73 amino acids in formation of fiber and crystal polymers in the *cis*−*trans* position of β-chains using mixtures of these β73 Hb S and β73 Hb A variants with Hb S.

Crystal Formation for Hb C-Harlem and β73 Leu Hb S versus Fiber Formation for Hb S and β73 His Hb S. We previously reported that Hb C-Harlem formed straight fibers followed by formation of needle-like crystals (*8*, *9*). β73 Leu Hb S also crystallized like Hb C-Harlem in <1.0 M phosphate buffers, which is in contrast to fiber formation of Hb S and β73 His Hb S. It is also known that when 14-stranded bundled polymers are stirred for many hours that the fiber bundles are replaced by macrofibers, followed by formation of double-stranded crystals (*4*, *18*). In the assembly process in the formation of 14-stranded Hb S fibers, a significant entropic penalty (∼35.5 kcal/mol) is incurred by the removal of hemoglobin monomers from solution in which Hb S monomers can rotate and translate in the fibers (*19*). Polymer stability arises from the additive contributions of contact energy and the free energy due to recovered vibrational motion (*1*, *9*). We reported previously that crystal rather than fiber formation for Hb C-Harlem may be attributed to increases in contact energy coupled with decreases in vibrational entropy (*9*). The change in interaction energies and surface lateral contact area around the β6 Val−EF helix and β4 Thr−β73 interaction sites of β-chains in the polymers by amino acid substitution also may cause alterations in strand structure of Hb S polymers. Promotion of crystal formation in β73 Leu Hb S also may be caused by decreasing vibrational entropy in addition to disruption of formation of a hydrogen bond with β4 Thr. This may in turn lead to different interactions in addition to enhancement of the β6 hydrophobic interactions.

These results clearly indicate that this hydrogen bond plays a critical role in Hb S polymerization. X-ray analysis also indicated that only one of the two β6 Val residues in Hb S tetramers ($\alpha_2\beta_2$) is involved in an intermolecular contact. In contrast, the results presented here suggest that the two β73 Asp residues may be involved in polymerization; the one near the β6 Val interaction site may affect hydrophobicity, while the other *in trans* to this site may play a critical role in polymerization by forming a hydrogen bond. These results suggest that altering the formation of a hydrogen bond between β73 Asp and β4 Thr produces Hb S molecules with different properties, and that decreasing the level of hydrogen bond formation inhibits polymerization without significantly changing tetramer solubility. However, enhancing the hydrogen bond promotes nucleation and changes the solubility of Hb S. Elucidation of the properties of Hb S-containing β73 variants, including Hb C-Harlem, will provide clues to finding chemicals and/or peptides that interact with sites other than the β6 Val donor site or the β85 Phe/β88 Leu hydrophobic acceptor site of Hb S. These sites, therefore, may be better targets for ameliorating Hb S polymer formation in the future. Detailed X-ray crystallographic studies of the crystals of the two new variants and Hb C-Harlem compared to Hb S are now required to address critically the structure and function consequences of these β73 changes in Hb S.

ACKNOWLEDGMENT

We thank Dr. Frank Ferrone for helpful discussions and critical reading of the manuscript. We are grateful to Drs. E. Daikhin and M. Yudkoff for mass analysis of β- and α-globin chains performed at the Children's Hospital of Philadelphia Mass Spectrometry Research Core Facilities.

REFERENCES

1. Eaton, W. A., and Hofrichter, J. (1990) Sickle cell hemoglobin polymerization, *Adv. Protein Chem. 40*, 63−279.
2. Dykes, G. W., Crepeau, R. H., and Edelstein, S. J. (1979) Three-dimensional reconstruction of the 14-filament fibers of hemoglobin S, *J. Mol. Biol. 130*, 451−472.
3. Padlan, E. A., and Love, W. E. (1985) Refined crystal structure of deoxyhemoglobin S. II. Molecular interactions in the crystal, *J. Biol. Chem. 260*, 8280−8291.
4. Harrington, D. J., Adachi, K., and Royer, W. E., Jr. (1997) The high-resolution crystal structure of deoxyhemoglobin S, *J. Mol. Biol. 272*, 398−407.
5. Bookchin, R. M., Nagel, R. L., and Ranney, H. (1967) Structure and properties of hemoglobin C Harlem, *J. Biol. Chem. 242*, 248−255.
6. Harrington, D. J., Adachi, K., and Royer, W. E., Jr. (1998) Crystal structure of deoxy-human hemoglobin β6 Glu→Trp, *J. Biol. Chem. 273*, 32690−32696.
7. Adachi, K., Kinney, T. R., Schwartz, E., and Asakura, T. (1980) Molecular stability and function of Hb C-Harlem, *Hemoglobin 4*, 1−26.
8. Adachi, K., and Asakura, T. (1980) Polymerization of deoxyhemoglobin C Harlem (β6Glu→Val, β73Asp→Asn), *J. Mol. Biol. 144*, 467−480.
9. Ivanova, M., Jasuja, R., Kransnosselskaia, L., Josephs, R., Wang, Z., Ding, M., Horiuchi, K., Adachi, K., and Ferrone, F. (2001) *J. Mol. Biol. 314*, 851−861.

10. Shen, T., Ho, N. T., Simplaceanu, V., Zou, M., Green, B. N., Tam, M. F., and Ho, C. (1993) Production of unmodified human adult hemoglobin in *Escherichia coli*, *Proc. Natl. Acad. Sci. U.S.A. 90*, 8108−8112.
11. Adachi, K., Konitzer, P., Kim, J., Welch, N., and Surrey, S. (1993) Effects of β6 aromatic amino acids on polymerization and solubility of recombinant hemoglobins made in yeast, *J. Biol. Chem. 268*, 21650−21658.
12. Adachi, K., and Asakura, T. (1979) Nucleation-controlled aggregation of deoxyhemoglobin S. Possible difference in the size of nuclei in different phosphate concentrations, *J. Biol. Chem. 254*, 7765−7771.
13. Yamaguchi, T., Pang, J., Reddy, K. S., Witkowska, H. E., Surrey, S., and Adachi, K. (1996) Expression of soluble human β-globin chains in bacteria and assembly in vitro with α-globin chains, *J. Biol. Chem. 271*, 26677−26683.
14. Samuel, R. E., Salmon, E. D., and Briehl, R. W. (1990) Nucleation and growth of fibers and gel formation in sickle cell hemoglobin, *Nature 345*, 833−835.
15. Briehl, R. W., and Guzman, A. E. (1994) Fragility and structure of hemoglobin S fibers and gels and their consequences for gelation kinetics and rheology, *Blood 83*, 573−579.
16. Wang, Z., Kischenko, G., Chen, Y., and Josephs, R. (2000) Polymerization of deoxy-sickle cell hemoglobin in high phosphate buffer, *J. Struct. Biol. 131*, 197−209.
17. Adachi, K., Kim, J., Kinney, T. R., and Asakura, T. (1987) Effect of β73 amino acid on the hydrophobicity, and the kinetics of polymerization of deoxyhemoglobin S, *J. Biol. Chem. 262*, 10470−10474.
18. Josephs, R., Jarosch, H. S., and Edelstein, S. J. (1976) Polymorphism of sickle cell hemoglobin fibers, *J. Mol. Biol. 102*, 409−426.
19. Ferrone, F. A., Hofrichter, J., and Eaton, W. A. (1985) Kinetics of sickle hemoglobin polymerization I: Studies using temperature-jump and laser photolysis techniques, *J. Mol. Biol. 183*, 611−631.

BI026740X