Inhibition of Hemoglobin S Polymerization in Vitro by a Novel 15-mer EF-Helix β73 Histidine-Containing Peptide†

Mohammed G. K. Akbar,‡ Yutaka Tamura,§ Min Ding,‡ Hua Ding,|| Michael M. Rosenblatt,|| Konda S. Reddy,⊥ Saul Surrey,# and Kazuhiko Adachi*,‡

Division of Hematology, The Children's Hospital of Philadelphia, and University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, Joseph Stokes, Jr., Research Institute, Protein Core Facility, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104, Department of Bioinformatics, Chiba University Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan, 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 19104

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ABSTRACT: Our mutational studies on Hb S showed that the Hb S β73His variant (β6Val and β73His) promoted polymerization, while Hb S β73Leu (β6Val and β73Leu) inhibited polymerization. On the basis of these results, we speculated that EF-helix peptides containing β73His interact with β4Thr in Hb S and compete with Hb S, resulting in inhibition of Hb S polymerization. We, therefore, studied inhibitory effects of 15-, 11-, 7-, and 3-mer EF-helix peptides containing β73His on Hb S polymerization. The delay time prior to Hb S polymerization increased only in the presence of the 15-mer His peptide; the higher the amount, the longer the delay time. DIC image analysis also showed that the fiber elongation rate for Hb S polymers decreased with increasing concentration of the 15-mer His peptide. In contrast, the same 15-mer peptide containing β73Leu instead of His and peptides shorter than 11 amino acids containing β73His including His alone showed little effect on the kinetics of polymerization and elongation of polymers. Analysis by protein-chip arrays showed that only the 15-mer β73His peptide interacted with Hb S. CD spectra of the 15-mer β73His peptide did not show a specific helical structure; however, computer docking analysis suggested a lower energy for interaction of Hb S with the 15-mer β73His peptide compared to peptides containing other amino acids at this position. These results suggest that the 15-mer β73His peptide interacts with Hb S via the β4Thr in the β^S-globin chain in Hb S. This interaction may influence hydrogen bond interaction between β73Asp and β4Thr in Hb S polymers and interfere in hydrophobic interactions of β6Val, leading to inhibition of Hb S polymerization.

Hb S[1] is a naturally occurring mutation of human tetrameric hemoglobin in which the β subunits have a hydrophobic Val in place of a negatively charged Glu at the β6 position. The consequence of this mutation is that solubility of Hb S decreases when oxy-Hb S loses oxygen. When deoxy-Hb S becomes oversaturated, deoxy-Hb S assembles into long, multistranded fibers under physiological conditions (*1*, *2*). Fiber formation is characterized by a delay time prior to polymerization, which is explained by homogeneous and heterogeneous nucleation. During oversaturated Hb S conditions deoxy-Hb S monomers form very small polymers by homogeneous nucleation, and these polymers grow by the end addition of hemoglobin molecules in solution. The surface of these growing fibers also can serve as heterogeneous nucleation sites for further growth of additional polymers (*1*, *3*). The polymer then assembles into 14-stranded fibers, which finally form a viscous gel. Intracellular polymers or fibers cause reduction in red blood cell deformability (sickling), leading to obstruction of flow in the microcirculation, thus creating vasoocclusion and a wide array of physiological problems including episodes of painful crises (*1*, *4*).

Computational refinements of X-ray-determined crystal structures clarified the details of many of the axial and lateral contacts in Hb S polymers (*5*). These results and properties of the β^S chain and of β^S peptides (*6*, *7*) show a slight hinge-like motion of the A-helix in the β^S-globin subunits that makes intermolecular contact with the adjacent Hb S tetramer. Crystal structural analysis of Hb S in the deoxy form also showed not only β6Val in a largely hydrophobic acceptor pocket but other contact sites involving a hydrogen bond between β4Thr and β73Asp in Hb S which play a critical role in protein interactions. The β4 and β73 positions

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* Address correspondence to this author. Tel: 215-590-3576. Fax: 215-590-4834. E-mail: adachi@email.chop.edu.

‡ Division of Hematology, The Children's Hospital of Philadelphia, and University of Pennsylvania School of Medicine.

§ Chiba University Graduate School of Medicine.

|| Joseph Stokes, Jr., Research Institute, Protein Core Facility, The Children's Hospital of Philadelphia.

⊥ University of Pennsylvania.

Thomas Jefferson University.

[1] Abbreviations: Hb S, sickle hemoglobin; Hb A, normal hemoglobin A; Hb F, fetal hemoglobin; DIC, differential interference contrast.

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are located near β6Val and the EF-helix acceptor sites in the β subunit, respectively, which are critical lateral contact regions in Hb S polymerization (*1*, *5*, *8*). In addition, recent advances in understanding the molecular and cellular pathophysiology of sickle cell disease, coupled with new insights into the developmental regulation of human globin-gene expression as well as characterization of Hb S polymerization, have provided the scientific impetus and clinical rationale to attempt augmentation of the production of Hb F (*4*). Furthermore, rational drug design (also known as structure-based drug design) through the use of computer modeling and results of X-ray diffraction is an emerging approach that is revolutionizing the practice of drug discovery. The first antisickling molecules designed from receptor-based molecular modeling, the substituted benzaldehyde BW12C (now referred to as 12C79), were described by Beddell et al. (*9*). This drug has reached clinical trials, but analysis of the covalent complex of 12C79 and Hb A showed that this agent binds to the N-terminal amino group rather than the direct interaction sites of Hb S polymers including the β6Val donor site, β85Phe and β88Leu hydrophobic acceptor sites, or the β4Thr−β73Asp hydrogen bond.

Our mutational studies on Hb S polymerization showed that the Hb S β73His variant (β6Val and β73His) promoted polymerization compared to deoxy-Hb S, while Hb S β73Leu (β6Val and β73Leu) inhibited polymerization like naturally occurring deoxy-Hb C-Harlem (β6Val and β73Asn) (*10*). These results suggest that the β73 position (Asp in Hb S) serves as a unique site to promote or inhibit polymerization by amino acid replacement (*10*). Kinetics of polymerization, solubility, and minimum concentration required for polymerization of the Hb S β73 variants were affected by the β73 amino acid (inhibition of polymerization: His ≪ Asp < Asn < Leu). Inhibition of Hb S β73Leu polymerization compared to Hb S may be caused by weakening of the hydrogen bond interaction between the β4Thr hydroxyl group and the β73 amino acid-like Hb C-Harlem (*10*). Furthermore, kinetics of polymerization of 1:1 Hb S/Hb A β73His mixtures were enhanced like Hb S β73His compared to AS mixtures. These results suggest that the Hb A β73His variant promotes Hb S polymerization almost as efficiently as Hb S β73His and that β73His in Hb A and Hb S variants strengthens the hydrogen bond with β4Thr, which facilitates formation of domains and 14-stranded fibers. In addition, our results suggested that altering hydrogen bond formation between β73Asp and β4Thr produces Hb S molecules with different properties and that decreasing hydrogen bond formation inhibited polymerization without changing significantly tetramer solubility (*11*). On the basis of these results, we hypothesized that EF-helix peptides containing β73His would interact with β4Thr in Hb S and disturb protein-protein interactions of Hb S tetramers, resulting in inhibition of Hb S polymerization. We, therefore, synthesized several EF-helix peptides containing β73His of different lengths and then evaluated their effects on Hb S polymerization. We also studied the effects of these peptides on domain formation and elongation of Hb S polymers using differential interference contrast (DIC) microscopy and performed kinetics and thermodynamic and computer docking studies, comparing and contrasting results of the β73His peptide to those of the β73Leu peptide.

MATERIALS AND METHODS

Hb S was purified from AS blood as previously described (*12*). Hemoglobin concentrations were determined spectrophotometically using a millimolar extinction coefficient of $mE_{555} = 50$ for deoxyhemoglobin and $mE_{579} = 53.6$ for carbonmonoxyhemoglobin (on a tetramer basis). Sample purity was assessed by cellulose acetate electrophoresis and HPLC (*13*). Hemoglobin solutions were concentrated using a Centricon centrifugal concentrator with a membrane cutoff of 30000 Da (Centricon-30; Amicon, Inc.). Oxy-Hb S, which was stored as CO-Hb S, 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 about 1 h. EF-helix peptides [15-mer (Lys-Lys-Val-Leu-Gly-Ala-Phe-Ser-**His**-Gly-Leu-Ala-His-Leu-Asp), 11-mer (Gly-Ala-Phe-Ser-**His**-Gly-Leu-Ala-His-Leu-Asp), 7-mer (Ala-Phe-Ser-**His**-Gly-Leu-Ala), and 3-mer (Ser-**His**-Gly) as well as the 15-mer β73Leu peptide (Lys-Lys-Val-Leu-Gly-Ala-Phe-Ser-**Leu**-Gly-Leu-Ala-His-Leu-Asp)] were synthesized (Syn Pep Corp., Dublin, CA), solubilized in 0.5% (v/v) acetonitrile, and purified by HPLC (∼98% purity), and molecular weights were confirmed by electrospray ionization mass spectrometry (ESI-MS).

Kinetics of polymerization and solubility of Hb S in the presence and absence of the peptides were evaluated in 1.0 and 1.8 M buffers, pH 7.3 at 30 °C, with solubility determined by centrifugation after completion of polymerization (*12*). The time courses of Hb S polymer elongation in the presence and absence of peptide in 1.0 M phosphate buffer were analyzed by DIC microscopy using an Olympus microscope equipped with optics employing a 40× oil (1.00 NA) immersion lens (*10*). For microscopy, approximately 1 μL of solution was pipetted to a glass slide which then was sealed with a 18 mm square cover slip using Mount-Quick solution (Daido Sangyo Co., Ltd., Japan) (*10*). Polymer formation of deoxygenated Hb S in the presence and absence of peptides was induced by temperature jump from 0 °C on ice to room temperature (∼22 °C). DIC polymer images were captured from a 150 μm square area through a CCD (charge-coupled devise) camera (Cohu Camera; Cohu Inc., San Diego, CA), transferred to a PC via an image grabber board (Scion AG5; Scicon Corp., Frederick, MD), and processed by an automated image analysis system (Universal Image Co., Downingtown, PA). Still images were sent to a computer for every second of video footage. To measure the length of Hb S polymer fibers, images were taken of a hemocytometer with 50 μm divisions (American Optical Corp., Buffalo, NY) at 400× magnification. Using the line measurement tool in Universal Image analysis software, we determined the length of a 50 μm division to be 206 pixels, which is the smallest division of an image. The length of one pixel was determined to be 0.243 μm, and images of Hb S fibers then were measured in pixels and converted to micrometers. The total length of fibers in a single domain as a function of time was measured by counting the number of pixels in each image using NIH Image Analysis (version 1.63) and Universal Image analysis software.

Peptide binding to Hb S was assessed using SELDI (surface-enhanced laser desorption/ionization) mass spectrometry as follows (*14*). Desalted Hb S in the oxy form (8 pM) was bound to a RS100 chip (Ciphergen Biosystems Inc.,

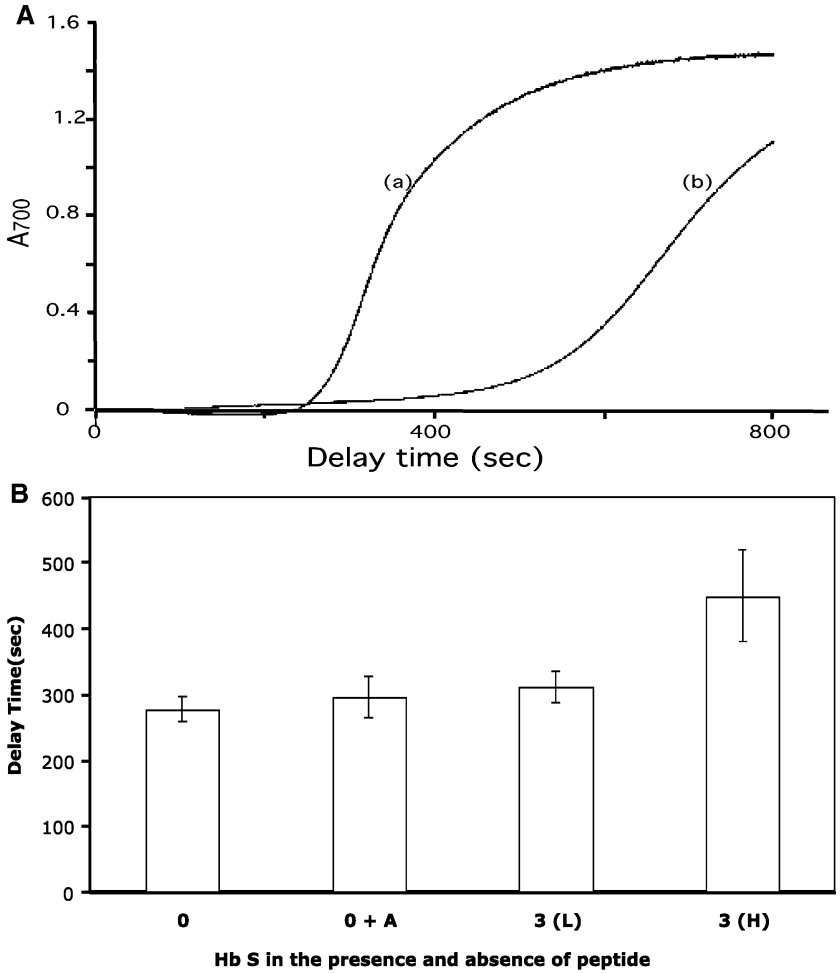

FIGURE 1: Kinetics of polymerization of Hb S in the presence and absence of the β73His- or β73Leu-containing peptides. The kinetics of deoxy-Hb S (4 g/dL) polymerization were measured in the presence (b) or absence of a 3-fold molar excess of the β73His peptide (a) (3 mol of peptide/mol of hemoglobin tetramer) to Hb S dissolved in 0.5% (v/v) acetonitrile in 1.0 M phosphate buffer at 30 °C by the temperature-jump method (panel A). Delay times prior to polymerization of Hb S (4 g/dL) also were measured in the presence of a 3-fold molar excess of the 15-mer β73His [3 (H)] or β73Leu peptides [3 (L)], and mean values were compared to Hb S alone (0) or Hb S in the presence of 0.5% (v/v) acetonitrile (0 + A) (panel B).

Fremont, CA) surface in coupling buffer (50 mM sodium carbonate, pH 9.0) at room temperature. The unbound parts on a chip surface then were blocked using 1 M Tris buffer, pH 8.0. The β73His peptides including 15-, 11-, 7-, and 3-mers and 15-mer β73Leu peptide (4 and 8 pM) in PBS buffer containing 0.5% (v/v) acetonitrile (pH 7.1) were incubated with the chips containing immobilized Hb S for 2.5 h at room temperature. Unbound peptides were removed using a PBS buffer wash followed by a second wash using PBS buffer containing 0.5 M NaCl. Ionization of the chip surface was enhanced using α-hydroxy-4-cinnamic acid after binding of peptides to Hb S. The mass of bound peptide then was measured using the mass spectrometer-based SELDI protein-chip analysis system (Ciphergen Biosystems Inc., Fremont, CA).

Circular dichroism (CD) spectra of the 15-mer peptides solubilized in 0.5% (v/v) acetonitrile were analyzed at room temperature using an Aviv model 62 DS instrument employing a 1 mm light path cuvette equipped with a thermoelectric module.

Three-dimensional structures of the 15-mer EF-helix peptides containing β73His or β73Leu were constructed using MOE (version 2004.03; CCG Inc., Montreal, Canada), while the structure of the sickle β-globin chain (β^S) was from 2HBS, which is deposited in the Brookhaven Protein Data Bank. Docking simulations of the 15-mer peptides containing β73His or β73Leu with β4Thr in the β^S-globin chain were performed using BioMedCAChe (version 6; Fujitsu, Tokyo, Japan), and results were displayed using MolFeat (version 2; FiatLux, Tokyo, Japan). Algorithm parameters for docking simulations using BioMedCAChe were as follows: (1) pop size 100, crossover rate 0.8, (2) elitism 20, (3) max generation 30000, (4) mutation rate 0.2, and (5) convergence (kcal) 1.0.

RESULTS

Kinetics of Polymerization of Hb S and Solubility in the Presence and Absence of the β73His- or β73Leu-Containing Peptides. Polymerization and solubility of Hb S in the presence of the 15-mer β73His peptide were studied in 1.0 M phosphate buffer. Deoxy-Hb S (4 g/dL) polymerized in 1.0 M phosphate buffer, pH 7.4, at 30 °C with a 275 s delay time (Figure 1A). We reported previously that Hb S β73His and β73Leu variants polymerized in 1.0 M phosphate buffer with significantly shorter and longer delay times, respectively, than Hb S (*10*). In contrast, the delay time for Hb S polymerization was much longer (~450 s) in the presence

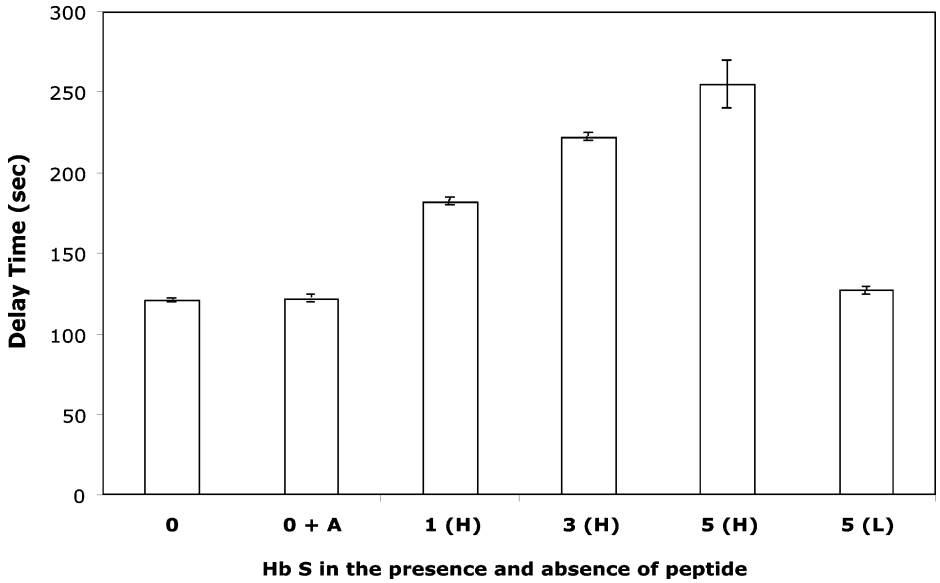

FIGURE 2: Effect of the β73His peptide concentration on delay time prior to polymerization of Hb S in 1.8 M phosphate buffer. Delay times prior to Hb S (0.18 g/dL) polymerization were measured in 1.8 M phosphate buffer, pH 7.3, at 30 °C at increasing amounts of the β73His peptide, and results were compared to those following addition of the 15-mer β73Leu peptide. 0, 0 + A, 1 (H), 3 (H), 5 (H), and 5 (L) on the *X*-axis represent Hb S alone, Hb S in 0.5% (v/v) acetonitrile, and Hb S in the presence of a 1-, 3-, or 5-fold molar excess of the β73His peptide, respectively. Results also are shown using a 5-fold molar excess of the β73Leu peptide [5 (L)].

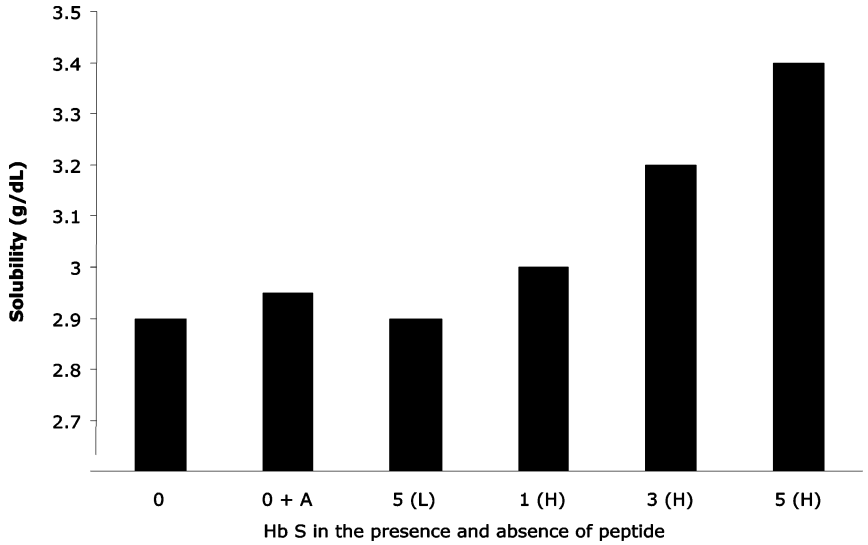

FIGURE 3: Effect of the β73His peptide on Hb S solubility in 1.0 M phosphate buffer. The solubility of Hb S in 1.0 M phosphate buffer was measured in the presence of varying amounts of the 15-mer β73His peptide [1- [1 (H)], 3- [3 (H)], and 5- [5 (H)] fold molar ratio] in the presence of 0.5% (v/v) acetonitrile, and results were compared to those using the 15-mer β73Leu peptide [5-fold molar excess, 5 (L)] in the presence of 0.5% (v/v) acetonitrile. Solubility following completion of polymerization was assessed after centrifugation. Solubilities of Hb S alone (0) and in the presence of 0.5% (v/v) acetonitrile (0 + A) also were measured with values representing the mean of two measurements (maximum different ranges of the two values are within 3.3%).

of a 3-fold molar excess (e.g., 3 mol of peptide/mol of hemoglobin tetramer) of the 15-mer β73His peptide to Hb S solubilized in 0.5% (v/v) acetonitrile in 1.0 M phosphate buffer at 30 °C (line b in Figure 1A). Acetonitrile at the same final concentration in the absence of peptide had no effect on polymerization of Hb S. In addition, the 15-mer β73Leu peptide at the same concentration as the 15-mer β73His peptide only slightly increased the delay time (e.g., from 280 to 311 s). Delay times for Hb S (4 g/dL) polymerization in the presence and absence of the 15-mer β73His or β73Leu peptide are shown in Figure 1B. Delay times in the presence of a 3-fold molar excess of the 15-mer β73His peptide to Hb S increased 1.15- and 1.5-fold for the β73Leu and β73His peptides, respectively, compared to Hb S alone. In addition, 5-fold molar excess additions of 3-, 7-, and 11-mer His-containing peptides showed no significant difference from results of Hb S without peptides and His alone. These results indicate that the 15-mer β73His peptide specifically inhibits nucleation prior to polymerization of Hb S. The length of the delay time in 1.8 M phosphate buffer increased with increasing amounts of the 15-mer β73His peptide (Figure 2). These results indicate that the apparent association constant of this 15-mer His peptide with deoxy-Hb S is in the range of 30 μM under these experimental conditions.

Solubility of Hb S in 1.0 M phosphate buffer at 30 °C in the presence and absence of the 15-mer β73His or β73Leu peptides was measured after polymerization and compared

to that of Hb S (Figure 3). The solubility of Hb S increased with increasing 15-mer β73His peptide. In the presence of a 5-fold molar excess of the 15-mer β73His peptide to Hb S the solubility of Hb S increased 1.17-fold compared to Hb S. In contrast, a 5-fold molar excess of the 15-mer β73Leu peptide had no effect on Hb S solubility. These results indicate that the critical concentration for Hb S polymerization increased in the presence of the 15-mer β73His peptide.

DIC Image Analysis of Hb S Polymerization in the Presence of the β73His Peptides. We reported previously that Hb S fibers were observed by DIC analysis using ~4 g/dL deoxy-Hb S in 1.0 M phosphate buffer employing the temperature-jump method. These images were similar to those using 0.1 M phosphate buffer (*10*). DIC image analysis showed that deoxy-Hb S fibers formed from many domains and elongated from each domain after a few minute delay time, which depended on Hb S concentration. In the presence of a 5-fold molar excess of the 15-mer β73His peptide to Hb S, deoxy-Hb S (4 g/dL) showed fiber formation from many domains (Figure 4B), but the domain size and final length of Hb S fibers were different from those of Hb S alone (Figure 4A). Growth rates of Hb S fibers (4 g/dL) in the presence and absence of the 15-mer β73His peptide in 1.0 M phosphate buffer were measured by calculation of the length of the longest fibers in a single domain as a function of time (Figure 5). Elongation rates in a single domain are initially high and then become low, corresponding to the initial and final stages of Hb S polymer growth, respectively (Figure 5A). The fiber elongation rate of Hb S in the presence of peptide was much lower than that of Hb S alone (Figure 5B). The elongation rate of Hb S fibers in the presence of a 5-fold molar excess of the 15-mer β73His peptide to Hb S was ~6-fold lower compared to those of Hb S alone or in the presence of the β73Leu peptide (Figure 6A). The effect of the 15-mer β73His peptide on elongation rate of Hb S fibers depends on peptide concentration; the higher the concentration, the lower the elongation rate (Figure 6B). It also is noteworthy that the Hb S elongation rate in the presence of a 5-fold molar excess of peptides containing β73His to Hb S which were smaller than 11 amino acids including 7- and 3-mers as well as His alone was not significantly affected (Figure 6C). These results indicate that the 15-mer β73His peptide specifically inhibits not only nucleation but also elongation of Hb S polymers.

Peptide Binding to Hb S. Mass spectrometric-based SELDI protein-chip technology with solid-state, time of flight (TOF) was employed to detect interactions between the β73His peptides and Hb S (Figure 7). A mass signal for the chip-bound 15-mer β73His peptide at *m*/*z* 1598 was detected when the peptide was incubated at 4 pM with the chip containing bound Hb S in the oxy form (Figure 7, panel A). Signal intensity doubled at 8 pM 15-mer β73His peptide (Figure 7, panel B), while the 15-mer β73Leu peptide (*m*/*z* 1572) (Figure 7, panels A′ and B′) and the 11-mer β73His peptide (*m*/*z* 1128) showed little or no binding under the same conditions (Figure 7, panels C and C′). In addition, signal intensities for the β73Leu peptide and the 11-, and 7-mer peptides did not increase going from 4 to 8 pM peptide concentration (data not shown). Furthermore, there was no signal for the 15-mer β73His peptide following incubation with chip-bound lysozyme instead of Hb S. These results indicate that the 15-mer β73His peptide interacts selectively in a concentration-dependent manner with oxy-Hb S prior to deoxy-Hb S polymerization.

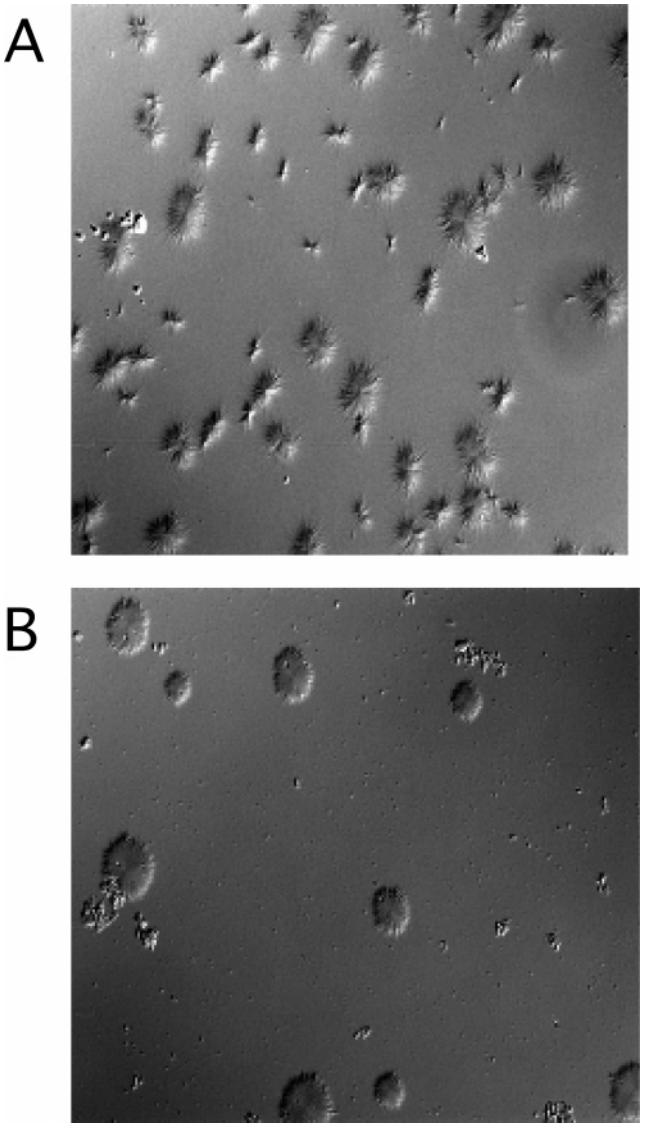

FIGURE 4: DIC images of deoxy-Hb S polymers in the presence of the β73His peptide. DIC images of Hb S (4 g/dL) polymers in 1.0 M phosphate buffer (pH 7.3) at room temperature after 4.30 min (A) and 9 min (B) following initiation of polymerization by temperature jump are shown in the absence (panel A) and presence (panel B) of a 5-fold molar excess of the β73His peptide.

CD Spectra of the β73His and β73Leu Peptides. The helix content of the 15-mer β73His and β73Leu peptides solubilized in 0.5% (v/v) acetonitrile was assessed by circular dichroism (CD) between 190 and 260 nm at room temperature using an Aviv model 62 DS instrument equipped with a thermoelectric module (Figure 8). The shapes of the two spectra were similar and did not show any specific helical structure, indicating random coil structures for both peptides in this solution.

Computer Docking Analysis of β73His and β73Leu Peptides with the β^S-Globin Chain in Hb S. Results from DIC images and SELDI-TOF MS suggested that the 15-mer β73His peptide selectively interacted with Hb S, presumably via the β4Thr in the β^S-globin chain in Hb S prior to Hb S polymerization. We therefore speculated that the random coil structure of these peptides might assume some structure after

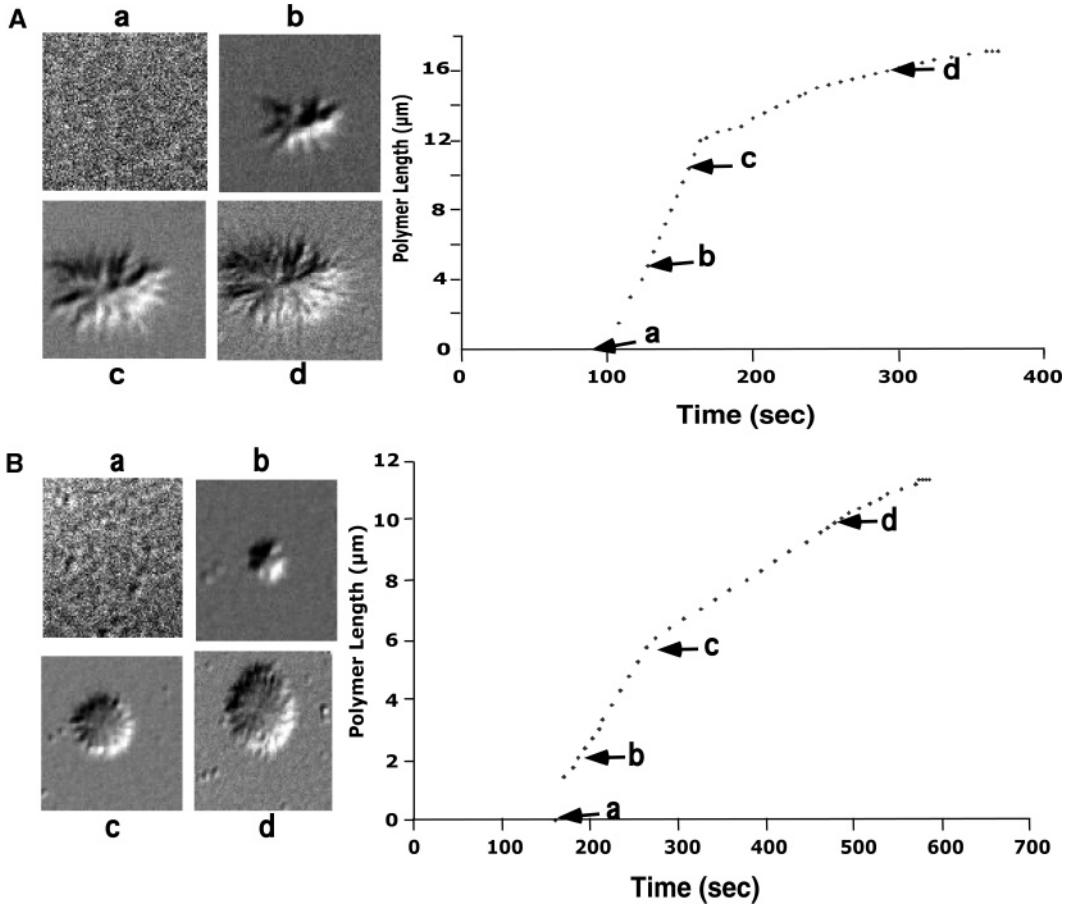

FIGURE 5: DIC images of Hb S fiber growth in a single domain in the presence and absence of the β73His peptide as a function of time. DIC images of Hb S (4 g/dL) polymer growth from a single domain in the presence (B) and absence (A) of a 5-fold molar excess of the 15-mer β73His peptide as a function of time were measured in 1.0 M phosphate buffer (pH 7.3). Frames a, b, c, and d in panel A represent images at 100, 128, 156, and 290 s, respectively, while frames a, b, c, and d in panel B represent images at 160, 186, 274, and 486 s, respectively. Experimental conditions are the same as those of Figure 4.

interaction with a specific area of β4Thr in Hb S. We next evaluated docking energies of the 15-mer β73His and β73Leu peptides with Hb S using conformation analysis simulation (Figure 9). Our results indicate that His in the 15-mer β73His peptide is much closer to β4Thr in the β^S-globin chain than Leu in the 15-mer β73Leu peptide (compare panels a and b in Figure 9). Docking energy differences between the 15-mer β73His peptide and Hb S (−181.162 kcal/mol) versus the β73Leu peptide and Hb S (−138.835 kcal/mol) probably arise from differences in interaction between the two different β73 amino acids and β4Thr in Hb S. It is also noteworthy that the order of energy required for interaction of amino acids at β73 in helical 15-mer EF-helix peptides with β4Thr in the β^S chain increases in the order of His ≪ Asp < Asn < Leu, which is consistent with ease of polymerization of Hb S with the β73 variants. These results suggest that the 15-mer β73His peptide selectively interacts with Hb S when the peptide length is ≥15 amino acids, presumably via the β4Thr in the β^S-globin chain in Hb S. Furthermore, we propose that this interaction preferentially influences the hydrogen bond interaction between β73Asp and β4Thr in Hb S polymers and may induce structural changes in the 15-mer His peptide after its interaction with oxy- Hb S, leading to inhibition of deoxy-Hb S polymerization. This interaction also may interfere with hydrophobic interactions of β6Val during Hb S polymerization.

DISCUSSION

Our results show that the 15-mer EF-helix peptide containing β73His inhibits Hb S polymerization while the 15-mer β73Leu peptide and His-containing peptides smaller than 11 amino acids have no effect. This can be explained from our previous results of polymerization of recombinant Hb S β73His and Hb S β73Leu as well as our findings using S/A β73His and S/A β73 mixtures compared to AS mixtures (*10*). Namely, β73His in deoxy-Hb S promotes while β73Leu inhibits Hb S polymerization. These findings indicate the β73−β4 hydrogen bond in Hb S polymers influences rates of nucleation and polymerization of Hb S. This supports our hypotheses regarding the basis for inhibition of polymerization by specific interaction of EF-helix peptides containing β73His with Hb S. In fact, using various engineered Hb S variants, we showed that changing the amino acid side chains at the β73 position at Hb S polymer interaction sites alters contact energy to stabilize polymers (*11*). This energy change affects vibrational entropy of Hb S molecules upon polymer assembly (*11*). Mutation at Hb S polymer contact regions generally affects kinetic and thermodynamic properties of Hb S polymerization.

Furthermore, polymerization of mixtures of Hb S and the recombinant variant Hb A β73Leu produced morphological changes in polymer domains that are characteristic of polymer formation. We speculated nonproductive binding

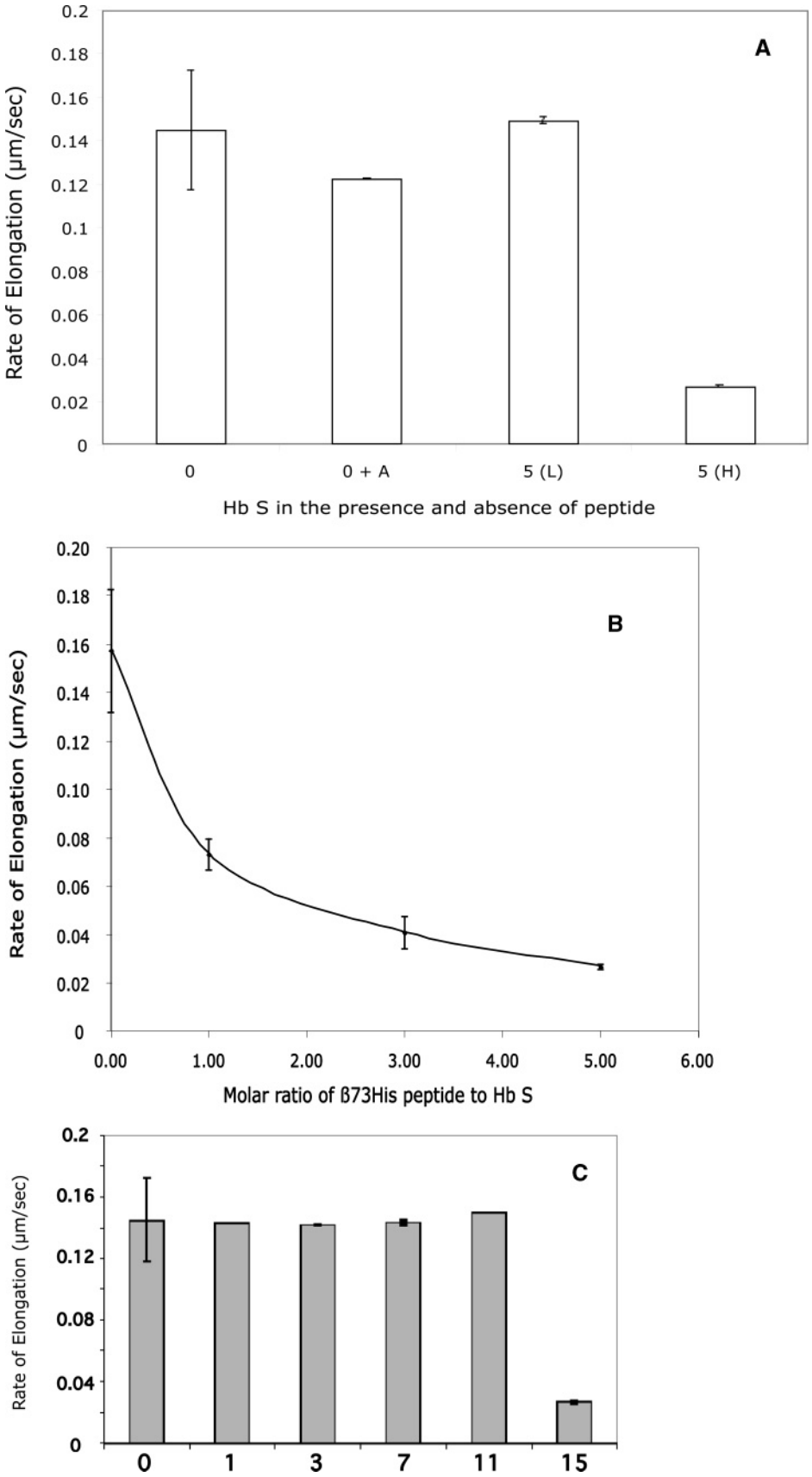

FIGURE 6: Effect of the β73His peptide on Hb S polymer elongation rate. The elongation rate of Hb S polymers in 1.0 M phosphate buffer at room temperature in the absence (0) and presence of a 5-fold molar excess of the β73His-containing [5 (H)] or β73Leu-containing [5 (L)] peptides in the presence of 0.5% (v/v) acetonitrile was determined by DIC (A). Results were compared to Hb S alone (0) and Hb S in 0.5% (v/v) acetonitrile (0 + A). Effects of the 15-mer β73His peptide concentration on elongation rates (B) and a 5-fold molar excess of shorter peptides (11-, 7-, and 3-mer) as well as His alone (C) also were calculated by DIC image analysis. The numbers on the *X*-axes in panel C represent no peptides (0) and His alone (1) while 3, 7, 11, and 15 represent 3-mer, 7-mer, 11-mer, and 15-mer peptides, respectively. Experimental conditions and rate calculations are the same as those in Figure 5.

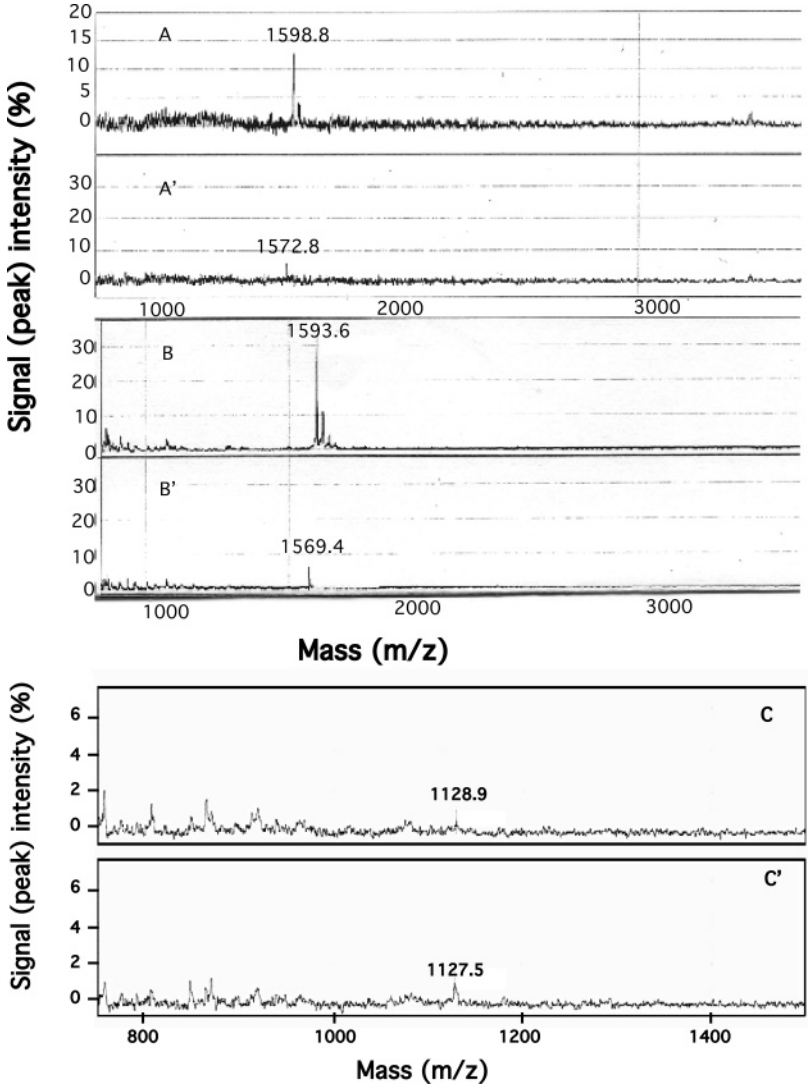

FIGURE 7: Detection of β73His peptide binding to Hb S using SELDI-TOF MS. The amine groups on the surface of Hb S (8 pM) in the oxy form were coupled to a RS100 chip, and peptide bound to Hb S was measured by a SELDI-TOF MS system. A and A′ are mass traces of the 15-mer β73His and β73Leu Hb S-bound peptides, respectively, using 4 pM initial peptide concentration, while B and B′ show results using 8 pM peptide concentrations, respectively. C and C′ are mass traces of 4 and 8 pM 11-mer His peptide. The *X*- and *Y*-axes represent mass (*m*/*z*) and signal intensity of bound peptide, respectively.

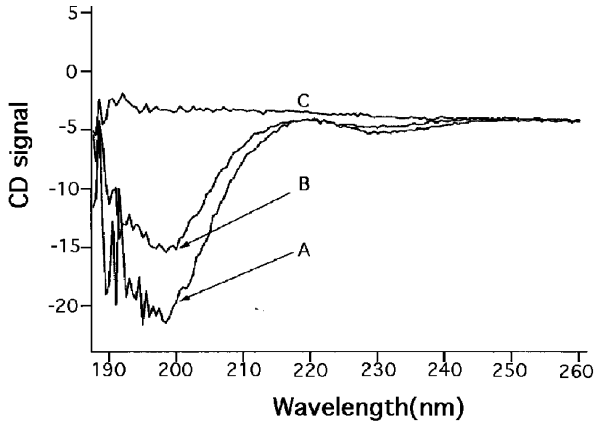

FIGURE 8: Circular dichroism spectra of β73His and β73Leu peptides. Circular dichroism (CD) spectra of the 15-mer β73His (A) and β73Leu peptides (B) solubilized in 0.5% (v/v) acetonitrile compared to acetonitrile alone (C) were analyzed at room temperature using an Aviv model 62 DS instrument employing a 1 mm light path cuvette equipped with a thermoelectric module.

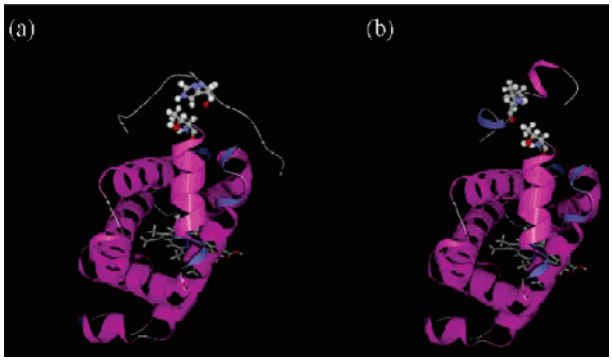

FIGURE 9: Molecular docking simulations of 15-mer β73 peptides with β4Thr in the β^S-globin chain. Computer docking simulations of the 15-mer β73His-containing (a) and β73Leu-containing (b) peptides with β4Thr in the β^S-globin chain were compared using BioMedCAChe software (version 6; Fujitsu, Tokyo). β73His and β73Leu positions relative to β4Thr in the β^S-globin chain are from lowest energy values for interactions based on energy calculations using computer simulations.

of SA β73Leu hybrids to the ends of growing Hb S polymers. This "cap" then would prohibit growth of polymers and produce smaller domains than those of Hb S, AS, or FS mixtures. In fact, Hb A β73Leu is the first Hb variant with

antipolymerization properties that exceed those of Hb F. We also speculate that interactions of deoxy-Hb S with specific peptides such as the 15-mer β73His peptide at the EF-helix of β chains may play a capping-like role similar to Hb SA β73Leu hybrids in Hb S fibers, even though inhibition by the peptide is less than that of Hb SA β73Leu hybrids. An interesting aspect of this study is that the 15-mer β73His peptide not only showed inhibition of the delay time and enhancement in solubility but also resulted in a delay of elongation rates of deoxy-Hb S fibers. DIC microscopic results further indicate inhibition of elongation of domains and formation of smaller domains in the presence of the β73His peptides. Furthermore, the β73His peptide can bind to both the deoxy and oxy forms of Hb S. Even though interactions of the β73His peptide with oxy-Hb S presumably around β4Thr at the A-helix appear weakened upon deoxygenation, deoxy-Hb S still interacted with the β73His peptide, possibly influencing the critical concentration for polymerization and inhibiting the rate of nucleation. This interaction may not only influence hydrogen bond interaction between β73Asp and β4Thr in formation of deoxy-Hb S nuclei and polymers but also interfere with hydrophobic interactions of β6Val leading to inhibition of Hb S polymerization, since β4Thr is so close to β6Val. In addition, the 15-mer peptides contain two His residues which might form a salt bridge with β7Glu (*7*). Formation of this salt bridge may disrupt the first turn of the α-helix in β^S chains and could therefore impact on Hb S polymerization. These results suggest that the 15-mer His peptide inhibits not only homogeneous but also heterogeneous nucleation by interaction of the peptides with Hb S, resulting in a delay in the assembly of monomers in addition to a delay in the elongation rate of fibers. We speculate that peptides larger than 15-mers may require specific interaction with β4Thr in the A-helix of β^S chains in order to inhibit Hb S polymerization. In fact, it was reported that the β^S (1−55) peptide showed a higher content of β-sheet and a lower amount of α-helix compared to the β^A (1−55) peptide, while there was very little secondary structural differences comparing shorter β^S versus β^A peptides (1−30) (*6*). Therefore, peptides longer than 15 amino acids may require additional structural conformation to promote specific interactions involving His in the peptide with β4Thr in the A-helix of β^S chains in order to inhibit Hb S fiber growth like Hb SA β73Leu hybrids.

Several small molecules, including phenylalanine and tryptophan derivatives, also were designed to interact with Hb S at the β6 donor and its acceptor pocket (*15*−*17*). However, none showed much promise as a therapeutic agent (*18*), partly because of the large dose of drug required to interact with circulating hemoglobin and the structural change of the EF-helix in the β6Val donor area that occurs during oxygenation and deoxygenation. Furthermore, bioavailability of the drugs was low due to serum protein binding. Our results on the inhibitory effects of the 15-mer β73His peptide were more compelling than that of single amino acids or peptides shorter than 11 amino acids. Furthermore, results of energy calculations using computer simulations as well as results of polymerization of Hb S β73 variants and mixtures of Hb S with Hb A containing the β73 variants suggest potential design of more efficient inhibitory peptides. It may be possible to express soluble peptides in erythroid cells that inhibit Hb S polymerization more than the 15-mer β73His peptide like Hb A β73Leu or Hb F after additional consideration of the specificity of the A-helix of β^S chains in the deoxy form. Such peptides would not require consideration of partner chains to form hemoglobin tetramers or heme insertion into globin chains and could possibly be introduced or expressed in an erythroid-specific fashion for gene therapy of sickle cell disease. In addition, it appears that neither Hb F nor its hybrids with Hb S enter the polymer, so Hb F and its variants would seem to represent the limit of maximum inhibition. Therefore, Hb F plus such peptides might exhibit antisickling effects exceeding those of Hb F alone. This has important implications for gene therapy for sickle cell disease, since antipolymerization induced by these peptides and Hb F may possibly be accomplished by completely different mechanisms: inhibition by the former via direct interaction with Hb S and the latter by reduction in Hb S concentration via formation of hybrids which are excluded from Hb S polymers (*19*). We are screening for such peptides and will evaluate their antipolymerization properties under near physiological conditions.

REFERENCES

1. Eaton, W. A., and Hofrichter, J. (1990) Sickle cell hemoglobin polymerization, *Adv. Protein Chem. 40*, 63−279.
2. Bunn, H. F., and Forget, B. G. (1996) *Hemoglobin: Molecular and Clinical Aspects*, Sanders, Philadelphia, PA.
3. Ferrone, F. A., Hofrichter, J., and Eaton, W. A. (1985) Kinetics of sickle hemoglobin polymerization. II. A double nucleation mechanism, *J. Mol. Biol. 183*, 611−631.
4. Embury, S. H., Hebbel, R. P., Mohandus, N., and Steinberg, M. H. (1994) *Sickle Cell Disease: Basic Principles and Clinical Practice*, Raven Press, New York.
5. 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.
6. Fronticelli, C., and Gold, R. (1976) Conformational relevance of the beta6Glu replaced by Val mutation in the beta subunits and in the beta(1−55) and beta(1−30) peptides of hemoglobin S, *J. Biol. Chem. 251*, 4968−4972.
7. Scholberg, H. P., Fronticelli, C., and Bucci, E. (1980) Conformational changes in the hemoglobin S system as seen by proton binding, *J. Biol. Chem. 255*, 8592−8598.
8. 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.
9. Beddell, C. R., Goodford, P. J., Norrington, F. E., Wilkinson, S., and Wootton, R. (1976) Compounds designed to fit a site of known structure in human haemoglobin, *Br. J. Pharmacol. 57*, 201−209.
10. Adachi, K., Ding, M., Wehrli, S., Reddy, K. S., Surrey, S., and Horiuchi, K. (2003) Effects of different beta73 amino acids on formation of 14-stranded fibers of Hb S versus double-stranded crystals of Hb C-Harlem, *Biochemistry 42*, 4476−4484.
11. Ivanova, M., Jasuja, R., Krasnosselskaia, L., Josephs, R., Wang, Z., Ding, M., Horiuchi, K., Adachi, K., and Ferrone, F. A. (2001) Flexibility and nucleation in sickle hemoglobin, *J. Mol. Biol. 314*, 851−861.
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. Haewon, C. K., Adachi, K., and Schwartz, E. (1995) Separation of hemoglobin, in *Williams Hematology* (Beutler, E., Lichtman, M. A., Coller, B. S., and Kippes, T. J., Eds.) 5th ed., pp L35−L42, McGraw-Hill, New York.
14. Tolson, J., Bogumil, R., Brunst, E., Beck, H., Elsner, R., Humeny, A., Kratzin, H., Deeg, M., Kuczyk, M., Mueller, G. A., Mueller, C. A., and Flad, T. (2004) Serum protein profiling by SELDI mass spectrometry: detection of multiple variants of serum amyloid alpha in renal cancer patients, *Lab. Invest. 84*, 845−856.
15. Noguchi, C. T., Torchia, D. A., and Schechter, A. N. (1982) Determination of sickle hemoglobin polymer in SS and AS erythrocytes, *Blood Cells 8*, 225−235.

16. Noguchi, C. T., Ackerman, S., DiMaio, J., Schiller, P. W., and Schechter, A. N. (1983) The effect of phenylalanine derivatives on the solubility of deoxyhemoglobin S. A model class of gelation inhibitors, *Mol. Pharmacol. 23*, 100−103.
17. Kumpati, J. (1987) Effect of phenylalanine- or tryptophan-loaded liposomes on the rheological properties of AA and SS erythrocytes, *Biochem. Med. Metab. Biol. 38*, 246−251.
18. Orringer, E. P., Abraham, D. J., and Parker, J. C. (1994) Sickle Cell Disease: Basic Principles and Clinical Practice, in *Development of Drug Therapy* (Embury, S. H., Hebbel, R. P., Mohandas, N., and Steinberg, M. H., Eds.) pp 861−872, Raven Press, New York.
19. Rotter, M. A., Dragos, D., Aprelev, A., Adachi, K., and Ferrone, F. A. (2005) Molecular crowding limits the role of fetal hemoglobin in therapy for sickle cell disease, *J. Mol. Biol 347*, 1015−1023.

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