Enhanced Inhibition of Polymerization of Sickle Cell Hemoglobin in the Presence of Recombinant Mutants of Human Fetal Hemoglobin with Substitutions at Position 43 in the γ-Chain†

Ming F. Tam,‡ Jun Chen,§ Tsuey-Chyi S. Tam,§ Ching-Hsuan Tsai,§ Tong-Jian Shen,# Virgil Simplaceanu,§ Timothy N. Feinstein,§ Doug Barrick,# and Chien Ho*,§

Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan, Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213, and Department of Biophysics, Johns Hopkins University, Baltimore, Maryland 21218

Received February 18, 2005; Revised Manuscript Received July 6, 2005

ABSTRACT: Four recombinant mutants of human fetal hemoglobin [Hb F ($\alpha_2\gamma_2$)] with amino acid substitutions at the position 43 of the γ-chain, rHb (γD43L), rHb (γD43E), rHb (γD43W), and rHb (γD43R), have been expressed in our *Escherichia coli* expression system and used to investigate their inhibitory effect on the polymerization of deoxygenated sickle cell hemoglobin (Hb S). Oxygen-binding studies show that rHb (γD43E), rHb (γD43W), and rHb (γD43R) exhibit higher oxygen affinity than human normal adult hemoglobin (Hb A), Hb F, or rHb (γD43L), and all four rHbs are cooperative in binding O_2. Proton nuclear magnetic resonance (NMR) studies of these four rHbs indicate that the quaternary and tertiary structures around the heme pockets are similar to those of Hb F in both deoxy (T) and liganded (R) states. Solution light-scattering experiments indicate that these mutants remain mostly tetrameric in the liganded (R) state. In equimolar mixtures of Hb S and each of the four rHb mutants (γD43L, γD43E, γD43R, and γD43W), the solubility (Csat) of each of the pairs of Hbs is higher than that of a similar mixture of Hb S and Hb A, as measured by dextran-Csat experiments. Furthermore, the Csat values for Hb S/rHb (γD43L), Hb S/rHb (γD43E), and Hb S/rHb (γD43R) mixtures are substantially higher than that for Hb S/Hb F. The results suggest that these three mutants of Hb F are more effective than Hb F in inhibiting the polymerization of deoxy-Hb S in equimolar mixtures.

Sickle cell anemia patients who have fetal hemoglobin [Hb F ($\alpha_2\gamma_2$)][1] levels exceeding 20% tend to experience milder clinical symptoms than other sickle cell patients with a normal level of Hb F (*1*, *2*). It is believed that increased levels of Hb F can reduce the polymerization of deoxy-Hb S through the formation of asymmetric hybrids of Hb F with Hb S ($\alpha_2\beta^S{}_2$), that is, $\alpha_2\beta^S\gamma$ (*3*). In Hb S, the β6 position is valine replacing a glutamic acid in a normal β-chain of human normal adult hemoglobin [Hb A ($\alpha_2\beta_2$)]. Hb A has been shown to dissociate into dimers more readily than Hb F (*4*, *5*), which could lead to the formation of more hybrid tetramers in mixtures of Hb A and Hb S compared to mixtures of Hb F and Hb S. Hence, a comparison of the structures of Hb A and Hb F could suggest possible sites for mutation that may increase the tetramer−dimer dissociation constant of Hb F and, in turn, yield more hybrid tetramers in mixtures with Hb S. Our working hypothesis is that if a mutant of Hb F, when mixed with Hb S, can produce greater amounts of these hybrids, then this mutant Hb F can exhibit greater inhibition of the polymerization of Hb S. Such a mutant Hb F could form the basis for possible gene therapy for sickle cell patients.

The Hb A tetramer dissociates at the $\alpha_1\beta_2$ interface (*6*). Thus, dimers are of the form $\alpha_1\beta_1$ and $\alpha_2\beta_2$. Hb A and Hb F differ by 39 amino acid residues between the β- and γ-chains (*7*). Between the β- and γ-chains, there are only four amino acid substitutions that occur at the $\alpha_1\beta_1$ interface (β51Pro → γAla, β112Cys → γThr, β116His → γIle, and β125Pro → γGlu), while a single transposition is observed at the $\alpha_1\beta_2$ interface (β43Glu → γAsp). These interface residues are the most likely places where a substitution could affect the dissociation properties of the Hb molecule. A recombinant Hb A (rHb A), including all five of these substitutions in the γ-chain, was constructed and is referred to as Hb A/F by Dumoulin et al. (*4*). Its tetramer−dimer dissociation constant ($K_d = 0.14\ \mu M$) is between that of Hb A ($K_d = 0.68\ \mu M$) and Hb F ($K_d = 0.01\ \mu M$) in the ligated form. The K_d value of a recombinant hemoglobin (rHb) with

† This work is supported by a research grant from the National Institutes of Health (R01HL-24525).

* Address all correspondence to Dr. Chien Ho, Department of Biological Sciences, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, PA, 15213. Phone, 412-268-3395; fax, 412-268-7083; e-mail, chienho@andrew.cmu.edu.

‡ Academia Sinica. This work was carried out during a sabbatical leave at Carnegie Mellon University.

§ Carnegie Mellon University.

Johns Hopkins University.

[1] Abbreviations: Hb F, human fetal hemoglobin; Hb S, sickle cell hemoglobin; Hb A, human normal adult hemoglobin; Csat, solubility; rHb, recombinant hemoglobin; HbO_2, oxyhemoglobin; HbCO, carbonmonoxyhemoglobin; deoxy-Hb, deoxyhemoglobin; met-Hb, methemoglobin; P_{50}, partial O_2 pressure at 50% saturation; n_{max}, Hill coefficient; IPTG, isopropyl β-thiogalactopyranoside; 2,3-BPG, 2,3-bisphosphoglycerate; NMR, nuclear magnetic resonance; DSS, 2-2-dimethyl-2-silapentane-5-sulfonate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethane-sulfonic acid.

10.1021/bi050300z CCC: $30.25
Published on Web 08/18/2005

a β43Glu → γAsp mutation is 0.21 μM, which accounts for most of the tetramer−dimer strength of the penta-substituted Hb A mutant (*8*).

In this study, we have used our *Escherichia coli* expression system (*9*) to construct four rHb F with mutations at γ43, namely rHb (γD43L), rHb (γD43E), rHb (γD43W), and rHb (γD43R), in the hope that these Hb F mutants could exhibit stronger inhibition of the polymerization when mixed with Hb S. Oxygen-binding and proton nuclear magnetic resonance (NMR) studies were then conducted to characterize the functional and structural changes brought about by these substitutions in Hb F. The solubility (Csat) as measured by a modified dextran-Csat method (*10*) was used to investigate the inhibitory effect of these mutations on the polymerization of deoxy-Hb S.

MATERIALS AND METHODS

Construction of Plasmids. An expression plasmid (pHE8) containing both α- and γ-globin genes in addition to the *E. coli* methionine aminopeptidase (MAP) gene was constructed in our laboratory (*9*). The 1.0-kb SmaI−NsiI fragment of pHE8, which contains the α- and γ-globin genes, was inserted into plasmid pTZ18U (Bio-Rad). The resultant plasmid (pTH8) was used in the mutagenesis experiments. Two synthetic oligonucleotides, 5′-ACTCAGCGTTTCTTCTTAAGTTTCGGCAACCTGTC-3′ and 5′-CAGCGTTTCTTTGAAAGCTTCGGCAACCTGTC-3′, were used as mutation primers to generate Asp → Leu and Asp → Glu substitutions, respectively, at residue 43 of the γ-globin gene on plasmid pTH8. The wild-type γ-globin gene of pHE8 was then replaced by the mutated γ-globin genes in pTH8. The QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used to make the γ43Asp → Trp substitution, using the primers 5′-CCCGTGGACTCAGCGTTTCTTTTGGTCCTTCGGCAACCTGTCTTC-3′ and 5′-GAAGACAGGTTGCCGAAGGACCAAAAGAAACGCTGAGTCCACGGG-3′ in the PCR experiment. The γ43Asp → Arg mutant was generated similarly using the primers 5′-CCCGTGGACTCAGCGTTTCTTTCGATCGTTCGGCAACCTGTCTTC-3′ and 5′-GAAGACAGGTTGCCGAACGATCGAAAGAAACGCTGAGTCCACGGG-3′. The mutations on the γ-globin gene were confirmed by DNA sequencing.

Production and Purification of Hemoglobins. The expression plasmids were transformed into *E. coli* JM109 cells and grown in TB medium (*11*) in a Microferm fermentor (New Brunswick Scientific, model MF20) or a Biostat fermentor (B. Braun Biotech, Inc., model Biostat-C-20) as described previously (*9, 12*). Cells were grown to a density of approximately 1×10^9 cells/mL. Isopropyl β-thiogalactopyranoside (IPTG) was added to induce the expression of the Hb and MAP genes. Hemin and glucose were added during the induction period. Cell growth was continued for another 4 h after IPTG induction, then harvested by centrifugation and stored at −80 °C. Isolation and purification of the Hbs were performed according to the established protocol developed in our laboratory (*9, 12*).

Edman degradation and electrospray ionization mass spectrometric analyses of the four mutant rHbs were carried out to assess the N-terminal processing of the proteins and confirm the mutations on the recombinant Hbs (*9, 12*). The four mutant rHbs had the correct molecular weight and contained ∼1% methionine at the amino terminus.

Preparation of Other Hemoglobins. Human normal adult blood samples were obtained from the local blood bank, and Hb A that was stripped of 2,3-bisphosphoglycerate (2,3-BPG) was isolated and purified by established methods in our laboratory (*13*). Hemolysates obtained from a blood sample of an SS donor were prepared according to the same procedure as that for Hb A. They were equilibrated with 10 mM phosphate/0.5 mM EDTA at pH 6.8 and 25 °C. The samples were then passed through a Mono S column (Amersham Pharmacia cation exchanger 16/10) to separate Hb S and Hb F from other components. Hb S samples were free of 2,3-BPG. The Hb samples were then frozen in liquid nitrogen and stored in the CO-liganded form at −80 °C until they were used.

Oxygen-Binding Studies. Oxygen dissociation curves for the Hb samples were obtained using a Hemox-Analyzer (TCS Medical Products, Huntington Valley, PA) at 29 °C in 0.1 M sodium phosphate buffer and pH range 5.6−8.2. A methemoglobin (met-Hb) reductase system was utilized (*14*) to reduce the amount of met-Hb to less than 5% in all samples tested. Partial pressure at 50% oxygenation (P_{50}) and the Hill coefficient (n_{max}) were determined from each curve. The accuracy of P_{50} measurements (in mmHg) is ±8%, while that of n_{max} is ±10%.

^{1}H NMR Spectroscopy Investigation. ^{1}H NMR spectra of the four rHb F mutants as well as Hb A and Hb F were obtained at 29 °C on a Bruker AVANCE DRX-300 spectrometer operating at 300-MHz. All Hb samples were in 0.1 M sodium phosphate buffer at pH 7.0 and 100% water. The Hb concentration was about 4% (2.5 mM in terms of heme). The water signal was suppressed by using a jump-and-return pulse sequence (*15*). Proton chemical shifts are referenced to the methyl proton resonance of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) indirectly by using the water signal, which occurs at 4.76 ppm downfield from that of DSS at 29 °C, as the internal reference.

Dextran-Csat Assay. This assay was performed as reported (*10*) with modifications for micro-sample handling. The dextran-Csat developed by Bookchin et al. (*10*) is a convenient procedure to investigate effects of Hb S modifications or mixtures with non-S Hbs on polymerization under conditions that avoid problems associated with high ionic strength buffers during Csat measurements (*16*). Our protocol was developed using a total working volume of 100 μL, which is 5−7 times smaller than that reported in the original procedure, reducing the total Hb needed per measurement to about 8−14 mg per tube. Hb samples used were converted from the CO-liganded to the O_2-liganded form using a procedure developed in our laboratory (*13*). They were then concentrated to >200 mg/mL and changed into 50 mM potassium phosphate (pH 7.5) using a Centricon concentrator (Amicon). A stock solution of dextran (average mass of 66 700 Da, Sigma), 320 mg/mL, was prepared in 50 mM potassium phosphate at pH 7.5 using a volumetric flask. To a 200-μL PCR tube, 37.5 μL of the stock dextran solution was added with a M50 Microman Pipet (Gilson, Inc., Middleton, WI). All other solutions were delivered with gastight glass syringes. Hb S alone or 50% Hb S plus 50% non-Hb S sample was deposited into the tube to give the desired protein concentration. Buffer (50 mM potassium phosphate, pH 7.5) was then added to yield a volume of

95 μL. The sample was mixed thoroughly then covered with mineral oil. Finally, 5 μL of a 1 M stock solution of sodium dithionite (oxygen free) dissolved in 50 mM potassium phosphate (oxygen free) was delivered to the bottom of the PCR tube and stirred with a gastight syringe. The presence of sodium dithionite ensured anaerobic condition for the hemoglobins and brought the total assay volume to 100 μL. After incubation for 30 min at 37 °C in a water bath, the resulting gel under the oil layer was carefully disrupted with a metal plunger, and the tubes were spun at room temperature in a tabletop microfuge (Eppendorf Model 5415) at 14 000 rpm for 20 min. The gel disruption was repeated twice more, followed by a final 30-min spin, and the supernatant without the mineral oil was collected. There is a sufficient volume of the soluble phase after centrifugation to permit triplicate measurements of the Hb concentrations over a range of initial Hb concentrations from 80 to 140 mg/mL. Samples before and after dextran-Csat assay were converted to cyanomet-Hb using Drabkin's solution, and the absorbance was measured at 540 and 700 nm (*17*). In handling these highly concentrated and viscous protein solutions for spectrophotometric measurements, samples were prepared by weight dilution in an analytical balance. Each data-point reported in this paper represents the mean of at least two parallel experiments and three replicate measurements each.

Light Scattering Measurements. To determine average molecular weights, Hb samples were run on a size-exclusion chromatography (SEC) column, and multi-angle static light-scattering measurements of the samples were performed. Hb samples were first dialyzed extensively against 50 mM sodium phosphate buffer (pH 7.5) at concentrations of 5 mg/mL. Samples (50-μL) were injected onto a TosoHaas G3000PWXL SEC column equilibrated in 50 mM sodium phosphate at a flow rate of 0.5 mL/min. Following chromatographic separation, multi-angle light-scattering measurements were made on an in-line MiniDAWN multi-angle light-scattering detector (Wyatt Technologies, Santa Barbara CA). Concentrations were monitored using an in-line Optilab DSP differential refractive index detector (Wyatt Technologies). In addition, dynamic light scattering was measured by taking one of the 90° signals from the Minidawn and computing the autocorrelation function in a QELS detector (Wyatt Technologies). Weight-averaged molecular weights (M_w) and hydrated radii (determined from the translational diffusion constant using the Stokes−Einstein equation) were calculated using the ASTRA 4.0 software package (Wyatt Technologies).

RESULTS AND DISCUSSION

Oxygen-Binding Studies. We have constructed four recombinant mutants of Hb F with amino acid substitutions at the 43 position of the γ-chain, which is located at the $\alpha_1\gamma_2$ subunit interface, namely rHb (γD43L), rHb (γD43E), rHb (γD43W), and rHb (γD43R). Figure 1 shows the O_2-binding properties of Hb A, Hb F, and the four mutants of Hb F in 0.1 M sodium phosphate at 29 °C. Plots of P_{50} versus pH (where P_{50} is the oxygen pressure at 50% saturation) indicate no significant difference in the oxygen-binding affinity among Hb A, Hb F, and rHb (D43L) from pH 6.8 to 8.2 (Figure 1A). However, these plots suggest a possible difference in the properties of these three Hbs below pH 6.5. rHb (γD43E), rHb (γD43W), and rHb (γD43R) exhibit higher oxygen affinity than Hb A, Hb F, and rHb (γD43L) at all pH values below 8.0. All four rHbs are cooperative in binding O_2 with Hill coefficients (n_{max}) ranging from 2.1 to 3.2 (Figure 1B).

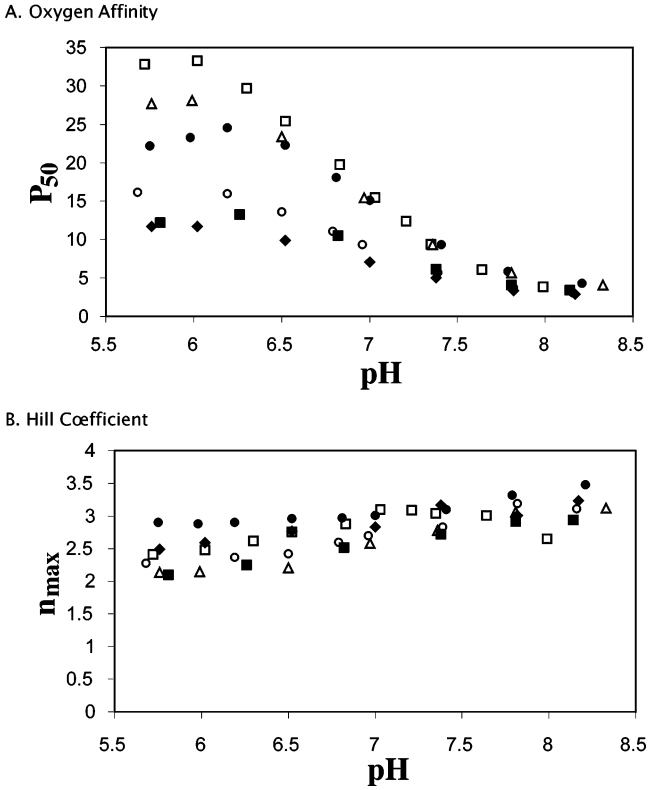

FIGURE 1: Oxygen-binding properties of Hb A (●), Hb F (□), rHb (γD43L) (△), rHb (γD43E) (○), rHb (γD43W) (◆), and rHb (γD43R) (■) in 0.1 M sodium phosphate buffer at 29 °C as a function of pH: (A) oxygen affinity; and (B) Hill coefficient.

Table 1: Bohr Effects of Hb A, Hb F, and Four Hb F Mutants in 0.1 M Sodium Phosphate Buffer (pH 6.5−8.3) at 29 °C[a]

hemoglobin	$-\Delta\log P_{50}/\Delta pH$[b]	% reduction[c]
Hb A	0.42 (pH 6.52−8.21)	-
Hb F	0.56 (pH 6.52−7.99)	-
rHb (γD43L)	0.41 (pH 6.50−8.33)	26.8
rHb (γD43E)	0.39 (pH 6.50−8.16)	30.4
rHb (γD43W)	0.36 (pH 6.52−8.17)	35.7
rHb (γD43R)	0.37 (pH 6.82−8.14)	33.9

[a] All rHb data are taken in the presence of a methemoglobin reductase system (*14*). Met-Hb formation did not exceed 4.3% in any sample, either during sample preparation or measurement. [b] The Bohr effect is measured by $-\Delta\log P_{50}/\Delta pH$, which gives the number of H^+ ions released upon ligand binding over the pH range specified. [c] The reduction of Bohr effects of the four Hb F mutants is relative to that of Hb F.

The Bohr effect, an important functional property, can be expressed as the number of hydrogen ions released upon oxygenation and measured as $\Delta H^+ = -\Delta\log P_{50}/\Delta pH$ (*18*). Table 1 summarizes the number of H^+ ions released per heme over the pH range from 6.5 to 8.2 for Hb A, Hb F, and the four Hb F mutants. All four Hb F mutants have a noticeable decrease in the number of H^+ ions released compared to Hb F over the specified pH range, suggesting that they possess a slightly lower alkaline Bohr effect than Hb F. rHb (γD43L) and rHb (γD43E) exhibit a Bohr effect only slightly less than that of Hb A. In spite of these differences in the oxygen-binding properties among these mutants and wild-type Hb

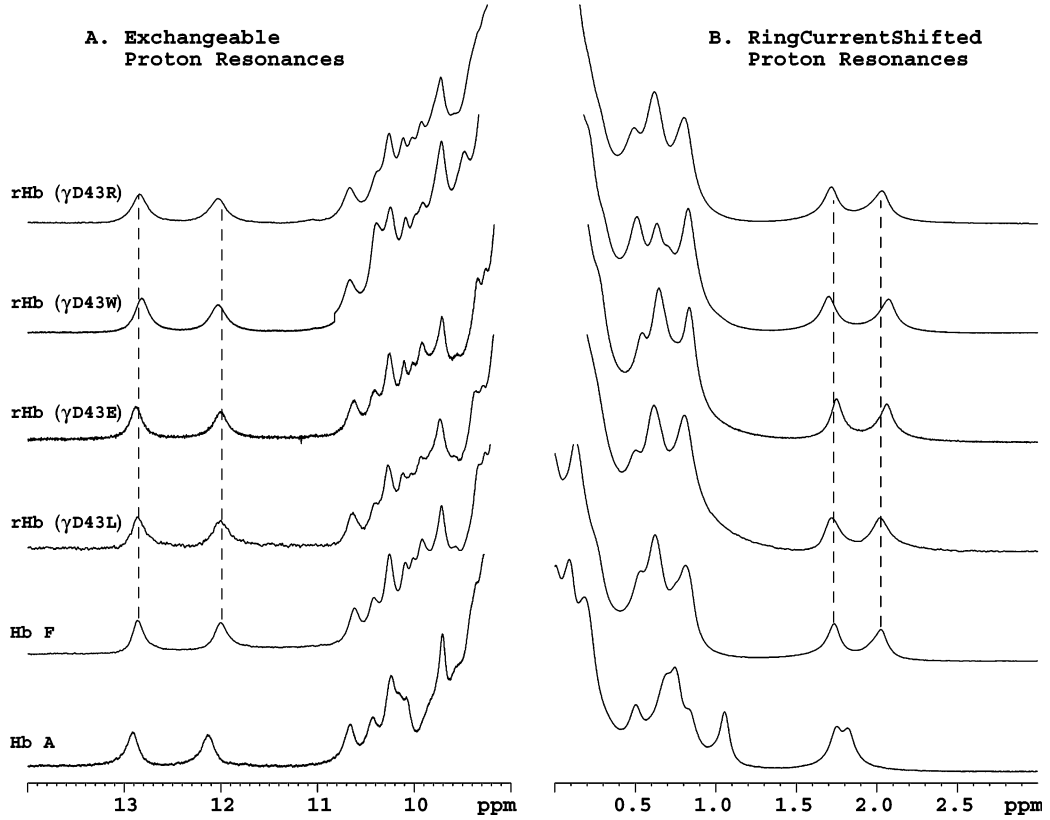

FIGURE 2: The 300-MHz ^{1}H NMR spectra of 4−6% solutions of Hb A, Hb F, rHb (γD43L), rHb (γD43E), rHb (γD43W), and rHb (γD43R) in the CO form in 0.1 M sodium phosphate buffer at pH 7.0 and 29 °C: (A) exchangeable proton resonances; and (B) ring-current-shifted proton resonances.

F, our results show clearly that these mutants can function as oxygen carriers.

It is of interest to note that the mutant rHb (γD43L) has oxygen affinity close to that of Hb F. In contrast, rHb (γD43E), rHb (γD43W), and rHb (γD43R) all possess higher oxygen affinity than that of Hb F. Moreover, the oxygen affinity of Hb F and of these four mutants of Hb F is increased in accordance with the size of the substituted amino acid residue: Asp < Leu < Glu < Arg < Trp. In Hb F and rHb (γD43L), the presence of γ43Asp or γ43Leu at the $\alpha_1\gamma_2$ subunit interface permits maximal oxygen release, but the replacement of Asp by γ43Glu, γ43Trp, and γ43Arg may introduce some conformational rearrangements around the heme pocket as suggested from the ring-current-shifted proton resonances (see below) resulting in perturbation of O_2 binding.

^{1}H NMR Studies of rHb F Samples. The exchangeable proton resonances and ring-current-shifted proton resonances of Hb A, Hb F, and the four mutants of rHb F in the CO form are shown in Figure 2. The ring-current-shifted proton resonances are an excellent indication of the tertiary structure of the heme pocket (*19*). The resonances at ∼−1.75 and ∼−1.85 ppm have been assigned to the γ_2-CH_3 of the E11Val of the α-chain and β-chain of HbCO A, respectively (*20*, *21*). Since the α-chain of Hb F is the same as that of Hb A, we can assume that the resonance at ∼−2.0 ppm is due to the γ_2-CH_3 of the E11Val of the γ-chain of HbCO F. These two resonances of rHbCO (γD43L) are essentially identical to those of HbCO F. The ∼−1.8-ppm resonance of rHbCO (γD43E) is shifted slightly upfield, and that at −2.0-ppm peak is shifted about 0.1 ppm upfield compared to those of HbCO F. For rHbCO (γD43W), the resonance at ∼−1.8 ppm is shifted about 0.1 ppm downfield and that at −2.0 ppm is shifted upfield by about 0.1 ppm compared to those of HbCO F. The −1.8-ppm-resonance of rHbCO (γD43R) is shifted slightly downfield, and that at −2.0-ppm signal is shifted slightly upfield relative to those of HbCO F. These results indicate that, upon mutating γ43Asp, the heme pocket of the γ-chain is more perturbed than that of the α-chain, as expected, but the changes are minimal.

The resonances in the downfield portion of the ^{1}H NMR spectrum of HbCO arise from the exchangeable protons in the subunit interfaces (*19*). The resonances at ∼12.8 ppm and ∼12.0 ppm have been assigned to the H-bonds between α122His and β35Tyr and α103His and β131Gln in the $\alpha_1\beta_1$ subunit interface (*22*, *23*), respectively. On the basis of the similarity of the crystal structures of Hb A and Hb F (*24*), we can assume that the H-bond pattern in the $\alpha_1\beta_1$ and $\alpha_1\gamma_1$ interfaces should be very similar as shown in Figure 2A. These two resonances of rHbCO (γD43L) are essentially identical to those of Hb F, indicating no significant perturbations around the $\alpha_1\gamma_1$ interface of this mutant. The resonance for the H-bond between α122His and γ35Tyr of rHbCO (γD43E) is shifted downfield by about 0.1 ppm relative to that of HbCO F, but the resonance at 12.0 ppm of rHbCO (γD43E) remains the same as that of HbCO F. For rHbCO (γD43W), the resonance at 12.8 ppm is shifted about 0.1 ppm upfield and that at 12.0 ppm is shifted downfield by about 0.1 ppm compared to those of HbCO F. The 12.8-ppm resonance of rHbCO (γD43R) is shifted slightly upfield, and that at the 12.0-ppm signal is shifted downfield by about 0.1 ppm relative to those of HbCO F.

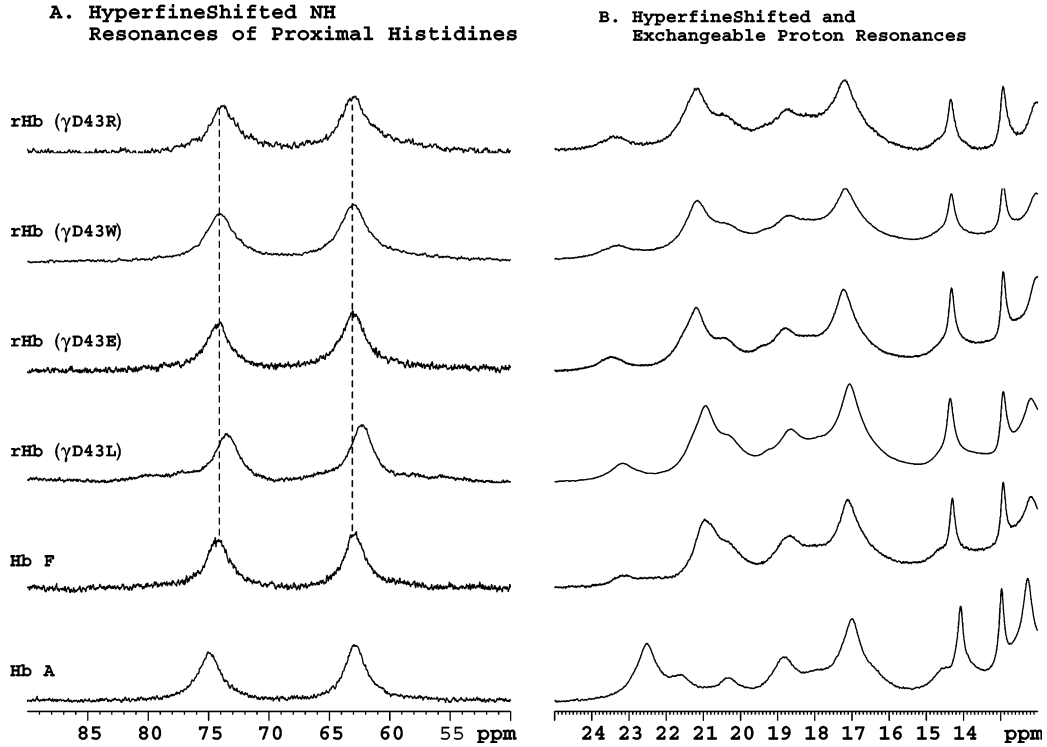

FIGURE 3: The 300-MHz ^{1}H NMR spectra of 4−6% solutions of Hb A, Hb F, rHb (γD43L), rHb (γD43E), rHb (γD43W), and rHb (γD43R) in the deoxy form in 0.1 M sodium phosphate buffer at pH 7.0 and 29 °C: (A) hyperfine-shifted $N_\delta H$ resonances of proximal histidines; and (B) hyperfine-shifted and exchangeable proton resonances.

Figure 3 shows the hyperfine-shifted resonances and exchangeable proton resonances of the four mutants in the deoxy form. The resonance at 63 ppm has been assigned to the hyperfine-shifted NH-exchangeable proton of the proximal histidine residue α87His of the α-chain of deoxy-Hb A, and the peak at 74 ppm has been assigned to the corresponding β92His of the β-chain of deoxy-Hb A (*25*, *26*). As shown in Figure 3A, the resonance at 63 ppm is the same in both deoxy-Hb A and deoxy-Hb F as expected. The hyperfine-shifted NH-exchangeable proton resonance of the proximal histidine of the γ-chain of deoxy-Hb F is shifted upfield by 1 ppm compared to that of the β-chain of deoxy-Hb A, reflecting a slight difference in the heme environment between the β- and γ-chains (*24*, *27*). These two resonances of rHb (γD43E), rHb (γD43W), and rHb (γD43R) are indistinguishable from those of Hb F, suggesting that no perturbation has been introduced into the heme pocket at the proximal histidyl residues by the mutation. However, these two resonances of rHb (γD43L) are shifted upfield by 1 ppm relative to those of Hb F, indicating a slight difference in the conformation between the proximal histidines and heme group of the mutant compared to that of Hb F. The resonances in the spectral region from 13 to 24 ppm arise from the hyperfine-shifted resonances of the porphyrin ring and the amino acid residues located in proximity to the heme pockets as well as the exchangeable proton resonances (Figure 3B) (*19*). There are no noticeable differences in the resonances from 13 to 24 ppm between deoxy-Hb F and the four mutants in the deoxy form.

Taking all these data together, we can conclude that, whether the negatively charged γ43Asp of Hb F is exchanged for a neutral, negative, or positive residue, the overall structures of these mutants are similar to those of Hb F in both deoxy (T) and liganded (R) states.

Dextran-Csat Measurements. We have shown previously with delay-time gelation and Csat measurements that Hb S with amino acid substitutions at α114 and β87 can inhibit the formation of the Hb S polymer (*28*). In this study, we used a modified version of the method of Bookchin et al. (*10*) to measure the solubilities of Hb S and of equimolar mixtures of Hb S and either Hb A, Hb F, or each of the four mutants of Hb F. This method provides a convenient way to screen the effects of amino acid substitutions on the polymerization of Hb S under conditions that avoid problems associated with high ionic strength buffers (*10*). With our modified protocol, a Csat value can be obtained with less than 50 mg of proteins for each trial. However, it should be noted that there are limitations in using the dextran-Csat method; that is, it is not under physiological conditions.

The dextran-Csat results are shown in Figure 4 and listed in Table 2. A greater increase in dextran-Csat occurs upon mixing equimolar quantities of Hb S with Hb F than with Hb A. Our data for Hb S alone, 50%Hb S/50%Hb A, and 50%Hb S/50%Hb F are similar to the published results (*10*), indicating that our procedures and data are reliable. However, within the range of 80−140 mg/mL, we have observed a gradual increase in Csat values for all samples tested. The dextran-Csat value of an equimolar mixture of Hb S with rHb (γD43W) is similar to that of Hb S/Hb F. The Csat values obtained for Hb S/rHb (γD43L), Hb S/rHb (γD43E), and Hb S/rHb (γD43R) are similar at either 80 or 100 mg/mL initial Hb concentration and significantly higher than that for Hb S/Hb F (Table 2). At higher initial protein concentrations (120 and 140 mg/mL), Hb S/rHb (γD43E) and Hb S/rHb (γD43R) have higher Csat values than that of Hb S/rHb F (γD43L). Even though γD43 of Hb F is located at the $\alpha_1\gamma_2$ interface, it is also solvent-accessible. Whether the charged guanidino or glutamyl side chain has better

Table 2: Dextran-Csat Values of Equimolar Mixtures of Hb S and Non-S Hbs in 50 mM Potassium Phosphate Buffer at pH 7.5 and 37 °C[a]

initial [Hb] (mg/mL)	dextran-Csat (mg/mL)						
	Hb S	Hb S/Hb A	Hb S/Hb F	Hb S/Hb (γD43L)	Hb S/Hb (γD43E)	Hb S/Hb (γD43W)	Hb S/Hb (γD43R)
80	23.7 ± 0.9	55.5 ± 0.3	69.9 ± 1.8	77.6 ± 0.7	77.0 ± 1.7	75.6 ± 1.9	77.0 ± 2.2
100	21.0 ± 0.6	58.3 ± 0.3	78.6 ± 1.9	84.0 ± 1.2	86.7 ± 0.9	81.1 ± 1.6	85.5 ± 1.3
120	22.4 ± 1.8	63.0 ± 1.0	87.3 ± 2.8	92.7 ± 3.0	95.8 ± 0.3	86.0 ± 2.0	95.1 ± 0.1
140	29.5 ± 1.0	66.9 ± 0.2	93.0 ± 3.0	97.0 ± 1.3	104.3 ± 0.1	90.7 ± 1.8	102.2 ± 0.3

[a] Each value represents the mean of triplicate measurements from at least two different sample preparations.

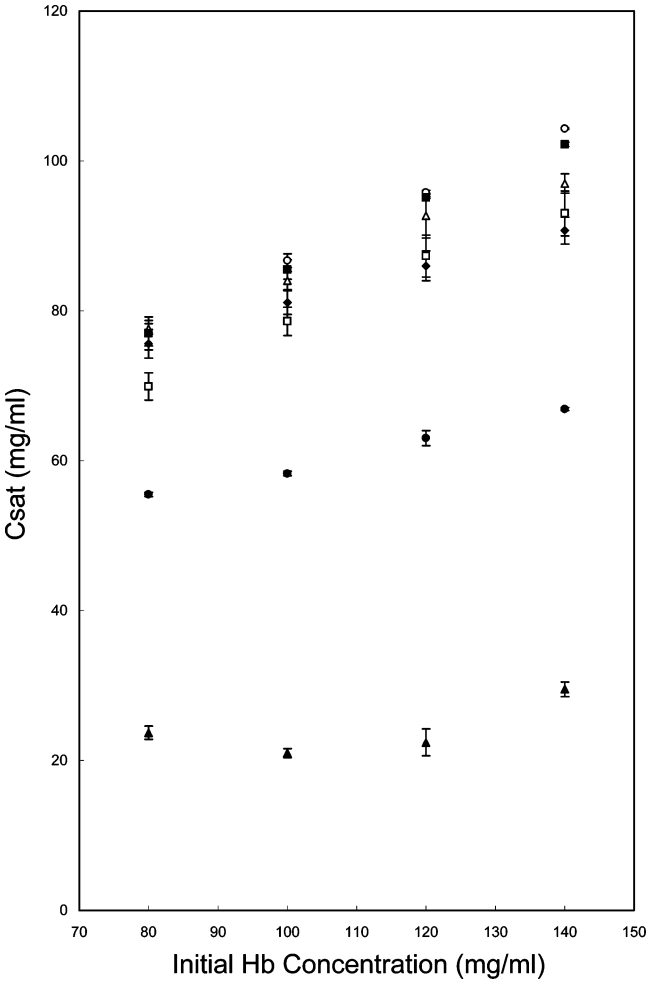

FIGURE 4: Effect of varying initial total Hb concentrations on the dextran-Csat values of deoxy Hb S alone (▲) and of equimolar mixtures of deoxygenated Hb S and Hb A (●), Hb S and Hb F (□), Hb S and rHb (γD43L) (△), Hb S and rHb (γD43E) (○), Hb S and rHb (γD43W) (◆), and Hb S and rHb (γD43R) (■) at 37 °C.

interaction with solvents and facilitates protein solubilization remains to be investigated. The Csat values that we have obtained for the rHb F mutants show a dependence on the initial protein concentration (Figure 4). The initial rHb concentrations used in the Csat experiments are significantly lower than the physiological concentration of Hb inside a red blood cell. The reason for the observed dependence of Csat on the total Hb concentration is not clear, but may be due to the "crowding" effect of dextran on the solution mixture.

Light-Scattering Studies. The quaternary structures of the wild-type hemoglobins and the Hb F mutants were assessed with light scattering. The results are summarized in Table 3 and show clearly that Hb A, Hb S, Hb F, and the four Hb F

Table 3: Molecular Weight and Radius of Gyration of Hemoglobins in the CO Form in 50 mM Sodium Phosphate Buffer Determined by Light Scattering[a]

hemoglobin	$M_w \times 10^4$ (g/mol)	R_w (nm)
Hb A	6.8 ± 0.3	2.9 ± 0.1
Hb S	6.3 ± 0.2	2.9 ± 0.2
Hb F	6.4 ± 0.3	3.0 ± 0.1
rHb (γD43L)	6.3 ± 0.2	3.1 ± 0.1
rHb (γD43E)	6.4 ± 0.2	3.0 ± 0.1
rHb (γD43W)	6.3 ± 0.3	2.9 ± 0.1
rHb (γD43R)	6.8 ± 0.5	3.0 ± 0.1

[a] Each value represents an average of multiple independent runs.

γ43 mutants have approximately the same radius of gyration (~3.0 nm). In accordance with the calculated molecular weights, these macromolecules exist in tetrameric form. Dumoulin et al. (*4*, *5*) have suggested that Hb A dissociates more readily into dimers than Hb F. It remains possible that the mutations studied here increase tetramer dissociation, but at the concentrations used for light-scattering measurements (~150 μM total dimer at loading), mass action strongly favors the formation of the tetramers. Hence, we picked up only the tetrameric form of the macromolecules in the light-scattering measurements and cannot resolve the modest changes in equilibrium dissociation between Hb A and the Hb F mutants.

The results presented in this paper as well as those reported in the literature have raised a number of interesting questions regarding the factors that affect the hybrid formation between Hb S and non-S Hbs and the sparing effect of Hb F on the inhibition of polymerization of Hb S. Early work indicated that a distribution very close to binominal was observed for Hb S mixed with Hb F in the oxy form as reported by Bunn and McDonough (*29*). Eaton and Hofrichter (*30*) carried out a thermodynamic analysis of the polymerization of Hb S with Hb F based on a binominal distribution with an assumption that the K_d value for Hb S and Hb F was the same. As mentioned earlier, Dumoulin et al. (*4*) reported that the tetramer−dimer dissociation constant for Hb F is about 70 times less than that of Hb A in the ligated form. Using a subzero isoelectric focusing technique, we found that the percentages of the hybrid form in the CO form between Hb A and Hb S, Hb S and Hb A_2, and Hb F and Hb S are 47.8, 49.4, and 44.5, respectively (*31*). These results indicate that the hybrid formation between Hb S and Hb F in the ligated form does not follow a binominal distribution, consistent with the smaller K_d value for Hb F compared to that for Hb A. The oxygen affinity of red cells from the fetus and the newborn has been known to be higher than that of adult red blood cells (*7*). However, the O_2 affinity for Hb F in the absence of 2,3-BPG is very comparable to that of Hb A (*7*, *31*−*33*). It appears that the sparing effect of Hb F on the

polymerization of Hb S inside red blood cells could be affected by a number of factors, for example, the extent and nature of hybridization of Hb F with Hb S, 2,3-BPG, degrees of oxygenation of Hb A, Hb S, and Hb F, and so forth. Hence, additional studies are clearly needed to elucidate the factors that affect the hybrid formation and the sparing effect of Hb F on the polymerization of Hb S.

In conclusion, it is known that when two hemoglobins (such as Hb S−Hb A or Hb S−Hb F) are mixed in the oxy state, the tetramers are in fairly rapid dissociation equilibrium with dimers, which reassociate randomly to form hybrid tetramers (Hb AS or Hb FS) (*7*). Kinetic and thermodynamic studies of the polymerization of cross-linked Hb AS hybrid hemoglobin suggested that Hb AS polymerized similarly or identically to Hb S (*34*). Thus, Hb A does not inhibit the polymerization of Hb S through the formation of Hb AS but can prevent the aggregation of Hb AS by acting as an inert component which simply dilutes the Hb S molecules. In contrast, kinetic and thermodynamic studies of cross-linked Hb FS hybrid hemoglobin have shown that Hb FS does not polymerize (*35*). The effect of cross-linked Hb FS on the aggregation of Hb S has shown that Hb FS could inhibit the polymerization of deoxy-Hb S, but to a lesser extent than does Hb F. These results thus explain the stronger inhibitory effect of Hb F on the polymerization of deoxy-Hb S than that shown by Hb A. We report here three Hb F mutants that have an even stronger inhibitory effect on the polymerization of deoxy-Hb S than that of the wild-type Hb F. The mechanism of this inhibition needs to be further investigated.

REFERENCES

1. Perrine, R. P., Pembrey, M. E., John, P., Perrine, S., and Shoup, F. (1978) Natural history of sickle cell anemia in Saudi Arabs. A study of 270 subjects, *Ann. Intern. Med. 88*, 1−6.
2. Powars, D. R., Weiss, J. N., Chan, L. S., and Schroeder, W. A. (1984) Is there a threshold level of fetal hemoglobin that ameliorates morbidity in sickle cell anemia?, *Blood 63*, 921−926.
3. Bookchin, R. M., Nagel, R. L., and Balazs, T. (1975) Role of hybrid tetramer formation in gelation of haemoglobin S, *Nature 256*, 667−668.
4. Dumoulin, A., Manning, L. R., Jenkins, W. T., Winslow, R. M., and Manning, J. M. (1997) Exchange of subunit interfaces between recombinant adult and fetal hemoglobins. Evidence for a functional inter-relationship among regions of the tetramer, *J. Biol. Chem. 272*, 31326−31332.
5. Dumoulin, A., Padovan, J. C., Manning, L. R., Popowicz, A., Winslow, R. M., Chait, B. T., and Manning, J. M. (1998) The N-terminal sequence affects distant helix interactions in hemoglobin. Implications for mutant proteins from studies on recombinant hemoglobin felix, *J. Biol. Chem. 273*, 35032−35038.
6. Park, C. M. (1973) Isoelectric focusing and the study of interacting protein systems: ligand binding, phosphate binding, and subunit exchange in hemoglobin, *Ann. N.Y. Acad. Sci. 209*, 237−257.
7. Bunn, H. F., and Forget, B. G. (1986) *Hemoglobin: Molecular, Genetic and Clinical Aspects*, W. B. Saunders Co., Philadelphia, PA.
8. Chen, W., Dumoulin, A., Li, X., Padovan, J. C., Chait, B. T., Buonopane, R., Platt, O. S., Manning, L. R., and Manning, J. M. (2000) Transposing sequences between fetal and adult hemoglobins indicates which subunits and regulatory molecule interfaces are functionally related, *Biochemistry 39*, 3774−3781.
9. Shen, T.-J., Ho, N. T., Zou, M., Sun, D. P., Cottam, P. F., Simplaceanu, V., Tam, M. F., Bell, D. A., Jr., and Ho, C. (1997) Production of human normal adult and fetal hemoglobins in *Escherichia coli*, *Protein Eng. 10*, 1085−1097.
10. Bookchin, R. M., Balazs, T., Wang, Z. P., Josephs, R., and Lew, V. L. (1999) Polymer structure and solubility of deoxyhemoglobin S in the presence of high concentrations of volume-excluding 70-kDa dextran. Effects of non-S hemoglobins and inhibitors, *J. Biol. Chem. 274*, 6689−6697.
11. Lech, K., and Brent, R. (1987) *Escherichia coli*, Plasmids and Bacteriophages, in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, O. D., Seidman, J. G., Smith, J. A., and Struhl, K., Eds.), Vol. I, p 1.0.1−1.2.2, Wiley, New York.
12. Shen, T.-J., 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.
13. Lindstrom, T. R., and Ho, C. (1972) Functional nonequivalence of α and β hemes in human adult hemoglobin, *Proc. Natl. Acad. Sci. U.S.A. 69*, 1707−1710.
14. Hayashi, A., Suzuki, T., and Shin, M. (1973) An enzymic reduction system for metmyoglobin and methemoglobin, and its application to functional studies of oxygen carriers, *Biochim. Biophys. Acta 310*, 309−316.
15. Plateau, P., and Guéron, M. (1982) Exchangeable proton NMR without base-line distorsion, using new strong-pulse sequences, *J. Am. Chem. Soc. 104*, 7310−7311.
16. Magdoff-Fairchild, B., Poillon, W. N., and Bertles, J. F. (1976) Thermodynamic studies of polymerization of deoxygenated sickle cell hemoglobin, *Proc. Natl. Acad. Sci. U.S.A. 73*, 990−994.
17. Drabkin, D. L. (1946) Spectrophotometric studies XIV. The crystallographic and optical properties of the hemoglobin of man in comparison with those of other species, *J. Biol. Chem. 164*, 703−723.
18. Wyman, J. (1964) Linked functions and reciprocal effects in hemoglobin: a second look, *Adv. Protein Chem. 19*, 223−286.
19. Ho, C. (1992) Proton nuclear magnetic resonance studies on hemoglobin: cooperative interactions and partially ligated intermediates, *Adv. Protein Chem. 43*, 153−312.
20. Lindstrom, T. R., Norén, I. B. Charache, S., Lehmann, H., and Ho, C. (1972) Nuclear magnetic resonance studies of hemoglobins. VII. Tertiary structure around ligand binding site in carbonmonoxyhemoglobin, *Biochemistry 11*, 1677−1681.
21. Dalvit, C., and Ho, C. (1985) Proton nuclear Overhauser effect investigation of the heme pockets in ligated hemoglobin: conformational differences between oxy and carbonmonoxy forms, *Biochemistry 24*, 3398−3407.
22. Simplaceanu, V., Lukin, J. A., Fang, T. Y., Zou, M., Ho, N. T., and Ho, C. (2000) Chain-selective isotopic labeling for NMR studies of large multimeric proteins: application to hemoglobin, *Biophys. J. 79*, 1146−1154.
23. Chang, C. K., Simplaceanu, V., and Ho. C. (2002) Effects of amino acid substitutions at beta 131 on the structure and properties of hemoglobin: evidence for communication between alpha 1 beta 1- and alpha 1 beta 2-subunit interfaces, *Biochemistry 41*, 5644−5655.
24. Frier, J. A., and Perutz, M. F. (1977) Structure of human foetal deoxyhaemoglobin, *J. Mol. Biol. 112*, 97−112.
25. Takahashi, S., Lin, A.-K. C., and Ho, C. (1980) Proton nuclear magnetic resonance studies of hemoglobins M Boston (α58E7 His → Tyr) and M Milwaukee (β67E11 Val → Glu): spectral assignments of hyperfine-shifted proton resonances and of proximal histidine (E7) NH resonances to the α and β chains of normal human adult hemoglobin, *Biochemistry 19*, 5196−5202.
26. La Mar, G. N., Nagai, K., Jue, T., Budd, D. L., Gersonde, K., Sick, H., Kagimoto, T., Hayashi, A., and Taketa, F. (1980) Assignment of proximal histidyl imidazole exchangeable proton NMR resonances to individual subunits in hemoglobins A, Boston, Iwate and Milwaukee, *Biochem. Biophys. Res. Commun. 96*, 1172−1177.
27. Davis, D. G., Charache, S., and Ho, C. (1969) Nuclear magnetic resonance studies of hemoglobins. III. Evidence for the nonequivalence of α- and β-chains in azide derivatives of methemoglobins, *Proc. Natl. Acad. Sci. U.S.A. 63*, 1403−1409.
28. Ho, C., Willis, B. F., Shen, T.-J., Ho, N. T., Philip Sun, D.-Z., Tam, M. F., Suzuka, S. M., Fabry, M. E., and Nagel, R. L. (1996) Roles of alpha 114 and beta 87 amino acid residues in the polymerization of hemoglobin S: implications for gene therapy, *J. Mol. Biol 263*, 475−485.
29. Bunn, H. F., and McDonough, M. (1974) Asymmetric hemoglobin hybrids. An approach to the study of subunit interactions, *Biochemistry 13*, 988−993.
30. Eaton, W. A., and Hofrichter, J. (1990) Sickle cell hemoglobin polymerization, *Adv. Protein Chem. 40*, 63−279.

31. Larson, S. C., Fisher, G. W., Ho, N. T., Shen, T.-J., and Ho, C. (1999) A biochemical and biophysical characterization of recombinant mutants of fetal hemoglobin and their interaction with sickle cell hemoglobin, *Biochemistry 38*, 9549−9555.
32. Allen, D. W., Wyman, J., and Smith, C. A. (1953) The oxygen equilibrium of foetal and adult hemoglobin, *J. Biol. Chem. 203*, 81−97.
33. Fang, T.-Y., Zou, M., Simplaceanu, V., Ho, N. T., and Ho, C. (1999) Assessment of roles of surface histidyl residues in the molecular basis of the Bohr effect and of β143 histidine in the binding of 2,3-bisphosphoglycerate in human normal adult hemoglobin, *Biochemistry 38*, 13423−13432.
34. Adachi, K., and Asakura, T. (1984) Polymerization of AS hybrid hemoglobin. Potent inhibitory effect of hemoglobin A on the polymerization of AS hybrid hemoglobin, *J. Biol. Chem. 259*, 2108−2112.
35. Nibu, K., and Adachi, K. (1985) Effect of FS ($\alpha_2\gamma\beta^S$) hybrid hemoglobin on Hb S nucleation and aggregation, *Biochim. Biophys. Acta 829*, 97−102.

BI050300Z