A Biochemical and Biophysical Characterization of Recombinant Mutants of Fetal Hemoglobin and Their Interaction with Sickle Cell Hemoglobin[†,‡]

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Received March 30, 1999; Revised Manuscript Received June 7, 1999

ABSTRACT: Three recombinant mutants of human fetal hemoglobin (Hb F) have been constructed to determine what effects specific amino acid residues in the γ chain have on the biophysical and biochemical properties of the native protein molecule. Target residues in these recombinant fetal hemoglobins were replaced with the corresponding amino acids in the β chain of human normal adult hemoglobin (Hb A). The recombinant mutants of Hb F included rHb F (γ112Thr → Cys), rHb F (γ130Trp → Tyr), and rHb F (γ112Thr → Cys/γ130Trp → Tyr). Specifically, the importance of γ112Thr and γ130Trp to the stability of Hb F against alkaline denaturation and in the interaction with sickle cell hemoglobin (Hb S) was investigated. Contrary to expectations, these rHbs were found to be as stable against alkaline denaturation as Hb F, suggesting that the amino acid residues mentioned above are not responsible for the stability of Hb F against the alkaline denaturation as compared to that of Hb A. Sub-zero isoelectric focusing (IEF) was employed to investigate the extent of hybrid formation in equilibrium mixtures of Hb S with these hemoglobins and with several other hemoglobins in the carbon monoxy form. Equimolar mixtures of Hb A and Hb S and of Hb A_2 and Hb S indicate that 48−49% of the Hb exists as the hybrid tetramer, which is in agreement with the expected binomial distribution. Similar mixtures of Hb F and Hb S contain only 44% hybrid tetramer. The results for two of our recombinant mutants of Hb F were identical to the results for mixtures of Hb F and Hb S, while the other mutant, rHb F (γ130Trp → Tyr), produced 42% hybrid tetramer. The sub-zero IEF technique discussed here is more convenient than room-temperature IEF techniques, which require Hb mixtures in the deoxy state. These recombinant mutants of Hb F were further characterized by equilibrium oxygen binding studies, which indicated no significant differences from Hb F. While these mutants of Hb F did not have tetramer−dimer dissociation properties significantly altered from those of Hb F, future mutants of Hb F may yet prove useful to the development of a gene therapy for the treatment of patients with sickle cell anemia.

Sickle cell anemia patients who have fetal hemoglobin [Hb F[1] ($\alpha_2\gamma_2$)] levels exceeding 20% tend to experience milder clinical symptoms than other sickle cell patients (*1*, *2*). It is believed that increased levels of Hb F reduce sickling through the formation of asymmetric hybrids of Hb F and sickle cell hemoglobin [Hb S ($\alpha_2\beta^S{}_2$)], namely, $\alpha_2\beta^S\gamma$ (*3*). Apparently, these hybrids are not incorporated in the Hb S polymer (*4*). If a mutant Hb F could be created which would form a hybrid with Hb S more readily than natural Hb F, then this mutant Hb could be the basis for a possible gene therapy for sickle cell patients. Finding appropriate sites for mutation of Hb F which could achieve this objective is a challenging task. However, studies of the tetramer−dimer dissociation and the denaturation properties of several Hbs may suggest which amino acid residues are most important to the hybrid formation. Studying the formation of hybrid tetramers directly can also be a significant problem due to a rapid equilibrium between liganded Hb tetramers and dimers in solution.

Tetramer−Dimer Dissociation of Hb

It is now well established that the tetramer dissociates at the $\alpha_1\beta_2$ interface (*5*). Thus, dimers are of the form $\alpha_1\beta_1$ and not $\alpha_1\beta_2$. Hb A has been shown to dissociate into dimers more readily than Hb F (*6*), which could lead to the formation of more hybrid tetramers in mixtures of Hb A and Hb S compared to that in mixtures of Hb F and Hb S. Hence, a comparison of these Hbs could suggest possible sites for mutation which might increase the dissociation rate of Hb F and, in turn, yield more hybrid tetramers in mixtures with Hb S. Hb A and Hb F differ by 39 amino acid residues within

[†] This work was supported by research grants from the National Institutes of Health (HL-24525 to C.H.) and the National Science Foundation Science and Technology Center (MCB-8920118 to G.W.F.).

[‡] Preliminary results were presented at the International Conference on Oxygen Binding and Oxygen Activating/Sensing Heme Proteins, September 27 to October 2, 1998, Asilomar Convention Center, Pacific Grove, CA.

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[1] Abbreviations: Hb, hemoglobin; Hb A, human normal adult hemoglobin; Hb S, sickle cell hemoglobin; Hb F, human fetal hemoglobin; rHb, recombinant hemoglobin; IEF, isoelectric focusing; MAP, methionine aminopeptidase; K_d, tetramer−dimer dissociation constant for Hb.

10.1021/bi990740o CCC: $18.00
Published on Web 06/26/1999

the β (or γ) chain (*7*). These amino acid substitutions result in a difference of two net charges between the β and γ chains. Most of these amino acid residues reside on the surface of the Hb molecule and, therefore, are not likely to contribute significantly to the functional properties of Hb F (*7*). Many of the internal substitutions involve replacement of one nonpolar residue with another. There are only four substitutions between the β and γ chains which occur at the $\alpha_1\beta_1$ interface (β51Pro → Ala, β112Cys → Thr, β116His → Ile, and β125Pro → Glu) and one substitution which occurs at the $\alpha_1\beta_2$ interface (β43Glu → Asp). These interfaces are the most likely places where a substitution would affect the Hb's dissociation and/or denaturation properties. If it is assumed the tetramer−dimer interaction is important in the formation of hybrid tetramers, then it would seem that the substitution at the $\alpha_1\gamma_2$ interface should be the most important substitution to the dissociation of the Hb F tetramer. However, if the monomer−dimer interaction is also important to hybrid tetramer formation, then the four substitutions at the $\alpha_1\gamma_1$ interface should be important to the dissociation of the Hb F tetramer. Recently, a recombinant Hb including all five of these substitutions in the β chain was constructed by Dumoulin et al. (*6*), but the tetramer−dimer dissociation properties of this mutant did not account for the differences observed between Hb A and Hb F. This result suggests a more complex mechanism may be involved in the dissociation of Hb than just the immediate contacts at the subunit interfaces. It has been suggested that electrostatic interactions play a role in the assembly of Hb tetramers and that such interactions might also have an influence on hybrid tetramer formation (*8*, *9*).[2]

Alkaline Denaturation of Hb

Other possible sites for mutation in Hb F which might affect hybrid formation can be inferred from alkaline denaturation studies of several Hbs. According to Perutz (*10*), the reason that Hb A is more susceptible to alkaline denaturation than Hb F and several other hemoglobins is because it contains three internal amino acid residues that are ionizable at pH ~12.0. Two of these residues (β112Cys and α104Cys) are at the $\alpha_1\beta_1$ interface and would prevent the association of the subunits when ionized. Ionization of the third residue (β130Tyr) would attract water to the interior of the β chain, which could change the conformation of the molecule making it more susceptible to dissociation (*10*). Two of these three important residues are substituted in Hb F; β112Cys corresponds to γ112Thr, and β130Tyr corresponds to γ130Trp. Because of these substitutions, it is likely that the interaction between subunits is stronger for the $\alpha\gamma$ dimer than for the $\alpha\beta$ dimer. If the interaction between the α chain and a mutant γ chain were not as strong as it is in Hb F, then perhaps the mutant of Hb F would be more likely to form a hybrid tetramer with Hb S. Three mutants have been created to test this possibility: rHb F (γ112Thr → Cys), rHb F (γ130Trp → Tyr), and rHb F (γ112Thr → Cys/γ130Trp → Tyr). First, the alkaline denaturation results for these recombinant fetal Hbs are presented, and then the amount of hybrid tetramer formation with Hb S and the oxygen dissociation curves are given.

Hybrid Tetramer Formation

If a recombinant mutant of Hb F is to be considered for a gene therapy for sickle cell anemia, then its hybrid tetramer formation with Hb S should be characterized. To isolate the hybrid tetramer for quantitation, dissociation of the tetramers must be prevented or slowed dramatically. In conventional isoelectric focusing (IEF), a hybrid tetramer band of liganded Hb is visible only transiently because the rate of the electrophoretic separation process is much slower than the rate at which tetramer equilibrium is achieved (*5*). Deoxy-Hb, however, dissociates much less so than liganded forms (*11*−*13*). Therefore, it is possible to separate the hybrid from the parent Hbs if the entire separation experiment is performed in an anaerobic atmosphere. Such separations have been achieved by several groups (*14*−*16*), but these methods are cumbersome. The Hbs must be mixed in the oxy form and then deoxygenated. Great care must be taken to remove oxygen from the running buffers and from the gels. Even trace amounts of oxygen in the system undermine the experiment (*5*).

Another way to reduce the the level of dissociation of Hb without removing the ligands is by lowering the temperature. Perrella and Rossi-Bernardi (*17*) showed that lowering the temperature to −25 °C sufficiently reduces the dissociation so that the hybrid can be separated from the parent Hbs. This method has been well established by Perrella and others (*17*−*20*), making it easier or more convenient to achieve than the anaerobic method. In the current work, quantitative sub-zero IEF was used to determine the amount of hybrid tetramer in equilibrium mixtures of Hb S and Hb A, Hb F, Hb A_2, and the three mutants of Hb F described above.

EXPERIMENTAL PROCEDURES

Construction of Plasmids. An expression plasmid (pHE9) that contains both α- and γ-globin genes and the *Escherichia coli* methionine aminopeptidase (MAP) gene was recently constructed in this laboratory (*21*). The 1.0 kb *Sma*I−*Nsi*I fragment of pHE9 which contains the α- and γ-globin genes was inserted into plasmid pTZ18U (Bio-Rad), and the resultant plasmid pTH9 was used in the mutagenesis experiments. Two synthetic oligonucleotides 5′-GATTGCCAAAACGCACACCAGCACATT-3′ and 5′-CACCATCTTCTGATAGCTAGCCTGCACCTC-3′ were used as primers to introduce mutations γ112Thr → Cys and γ130Trp → Tyr, respectively, into the γ-globin gene of plasmid pTH9. The site-directed mutagenesis was performed as described previously (*22*, *23*). The mutated γ-globin genes in pTH9 then replaced the wild-type γ-globin gene of pHE9. The plasmids pHE901 and pHE902 thus formed contain mutations γ112Thr → Cys and γ130Trp → Tyr, respectively. The third plasmid pHE903 which contains mutations γ112Thr → Cys and γ130Trp → Tyr was constructed by inserting the 0.92 kb *Sma*I−*Bsu*36I fragment of pHE901 into the 5.92 kb *Sma*I−*Bsu*36I fragment of pHE902.

[2] Bunn and co-workers (*8*, *9*) investigated the effect of surface charges on the rate of assembly of Hb dimers and found the importance of electrostatic interactions in subunit assembly. They provided a plausible explanation for the distribution of normal and mutant Hbs in various hematologic disorders. Since their model only considered the surface charge on Hb subunits and not that in the subunit interfaces, their conclusion is not directly relevant to this investigation of the amino acid substitutions in the $\alpha_1\beta_1$ or $\alpha_1\gamma_1$ interface (γ112Thr → Cys) and in the interior (near the $\alpha_1\gamma_1$ interface) of the Hb molecule (γ130Trp → Tyr). Furthermore, these amino acid replacements do not involve a change in the net charge under the physiological conditions.

Production and Purification of Hemoglobins. Expression plasmids were transformed into *E. coli* cells and grown in LB medium in a Microferm fermentor (New Brunswick Scientific, model MF20) as described previously (*21*, *23*) until the cell density was approximately 1×10^9 cells/mL. Isopropyl β-thiogalactopyranoside (IPTG) was added to induce the expression of the Hb and MAP genes, and hemin and glucose were also added during growth. After the addition of IPTG, growth was continued for another 4 h. Cells were harvested by centrifugation and stored at −80 °C. Isolation and purification were performed according to the standard methods developed in this laboratory (*21*, *23*, *24*). Purified Hbs were stored in the CO-liganded form at −80 °C. Before Hbs were quenched for the IEF experiments, they were exchanged into a 20 mM sodium phosphate buffer at pH 7.5.

Preparation of Hemoglobins. Human normal adult blood samples were obtained from a local blood bank, and Hb A was isolated and purified by established methods in our laboratory (*25*). Hb F was isolated from cord blood samples, and Hb S was isolated from blood samples of SS or AS donors. Hb F and Hb S were prepared as described previously (*26*). All Hb samples used in these experiments were frozen in liquid nitrogen and stored in the CO-liganded form at −80 °C until they were used.

Alkaline Denaturation. The methods for alkaline denaturation used in this work were established by Singer et al. (*27*). The denaturing solution (1.6 mL of $^1/_{12}$ N KOH at pH 12.7) was kept at 20 °C in a water bath. A 10% Hb solution was prepared, and 0.1 mL of this solution was added to the denaturing solution and allowed to react. After exactly 60 s, 3.4 mL of the precipitating solution [800 mL of 2 M $(NH_4)_2SO_4$ and 2 mL of 10 N HCl] was added and mixed. This solution was immediately filtered, the absorbance of the filtrate measured at 540 nm, and the concentration determined. Readings from at least three different samples of a given Hb were then averaged to determine the percentage of undenatured Hb in the sample.

Preparation of the Gels. The polyacrylamide gels used in these experiments were very similar to those described by Perrella and Rossi-Bernardi (*28*), with some minor changes. Gels (10 cm long and 3.4 mm in diameter) were made six at a time with the following composition: 1.08 mL of acrylamide/bisacrylamide solution (4.1 g/dL acrylamide and 0.17 g/dL methylene-bisacrylamide), 0.34 mL of Bio-Lyte ampholyte (40% solution from Bio-Rad), 1.28 mL of ethylene glycol (EGOH), 2.65 mL of deionized water, 0.105 mL of ethyl acrylate (from ACROS Organics), 0.90 mL of methanol, 5 μL of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), and 9 μL of 10% ammonium persulfate. The gel solution was kept on ice throughout the preparation. The first four ingredients were deoxygenated before the ethyl acrylate was added under N_2. Before methanol and TEMED were added under N_2, the solution was swirled for 10 min to dissolve the ethyl acrylate. Ammonium persulfate was added immediately before pouring the gels in a cold room at 4−6 °C. A layering solution of 20% EGOH and 15% methanol was quickly placed on top of the gels, and the gels were polymerized in an ice bath for 1 h. Gels could be stored for up to 2 weeks at −25 °C.

Quenching the Hemoglobins. Hemoglobins were mixed in equimolar amounts and incubated at room temperature for at least 1 h prior to quenching. The mixtures were quenched by adding the Hb sample to a solution of EGOH and 20 mM sodium phosphate buffer kept at −25 °C or at lower temperatures (below −30 °C). The final composition of the quenched solution was 50% EGOH and 50% aqueous buffer. Quenching was performed by adding 0.24 mL of the sample (25 μL at a time) to 0.72 mL of quench buffer and rapidly stirring with magnetic stir bars or with a motorized stirring wand. Both methods of stirring yielded similar results. The temperature of the quenched solution was allowed to equilibrate for 10 min between additions.

Isoelectric Focusing. Isoelectric focusing was carried out at −25 °C in an electrophoresis cell similar to that described by Perrella and co-workers (*17*, *18*). The cathodic solution contained 30% EGOH, 10% methanol, 2% pH 7−9 Bio-Lyte ampholyte, and 2% pH 8−10 Bio-Lyte ampholyte. The anodic solution contained 30% EGOH, 10% methanol, and 0.6% pH 6−8 Bio-Lyte ampholyte. Gels were prefocused at a constant voltage of 800 V for 1 h. Hb samples were loaded with Hamilton syringes which were cooled to −25 °C before loading. Gels were focused at a constant voltage of 800 V for at least 14 h.

Densitometry. Focused gels were imaged using a 12-bit, thermoelectrically cooled charge-coupled device (CCD) camera (Photometrics Ltd., Tuscon, AZ) equipped with a 50 mm Nikkor lens (Nikon, Inc., Melville, NY) and an emission filter with maximum transmittance at 530 nm (530 DF 25, Chroma Technologies, Brattleboro, VT) placed between the lens and the camera faceplate. IEF gels were imaged within 1 h of electrophoresis to ensure that significant diffusion of the hemoglobins had not occurred. The tubes containing gels were placed on top of a light box previously checked to ensure uniform illumination over the region concerned. Camera bias was subtracted from all images. To further ensure constant illumination, a background image was acquired over the same region of the lightbox and used afterward for flat-fielding images (*29*). Example gel images from several sub-zero IEF experiments following bias subtraction and flat fielding are shown in Figures 1 and 2. Brightness values were converted to optical density (OD) by comparison to neutral density filters (Oriel Corp., Stratford, CT) imaged in exactly the same manner and likewise bias subtracted and the images flat-fielded. Generation of standard optical density curves and gel quantitation were performed using the gel analysis package provided in NIH Image 1.55 (developed at the National Institutes of Health and available over the Internet at http://rsb.info.nih.gov/nih-image/) using the full 12-bit pixel depth. Pixel values across the tube diameter corresponding to Hbs usually ranged between 0 and 0.5 OD. Subsequent processing is shown in Figure 3. The area under each peak is directly proportional to the amount of Hb present in the respective band. When peaks slightly overlapped, droplines were drawn by hand at the lowest point of the curve, separating them.

We also modified the NIH Image program to test the effect of flat fielding on image intensity values and gel quantitation. In this case, flat fielding was not performed, and instead, a separate standard curve was generated for each pixel location in the gel to compensate for any possible small differences in source illumination. Since this procedure gave nearly identical results, but took much longer to perform, we preferred the flat-fielding approach and applying one optical

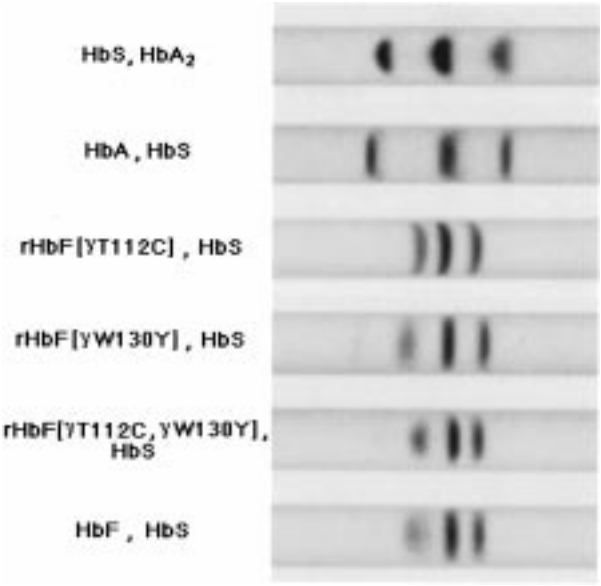

FIGURE 1: Equimolar mixtures of Hb S and six other hemoglobins focused at −25 °C. The gels are oriented with the anode to the left and the cathode to the right. Note that these gels came from separate experiments, and neighboring gels are not necessarily aligned by isoelectric point. All hemoglobins except for Hb A_2 traveled farther into the gel than Hb S (thus, the bands for Hb A, Hb F, and the mutant Hbs are to the left).

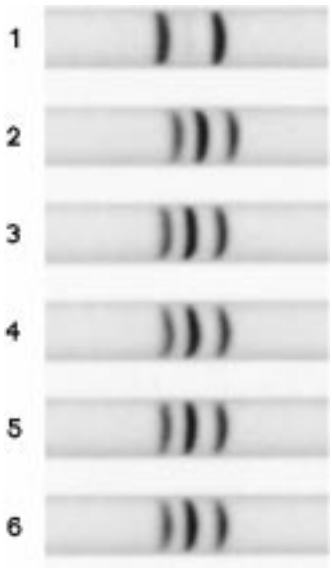

FIGURE 2: Equimolar mixtures of rHb F (γ112Thr → Cys) and Hb S focused at −27 °C. The gels are oriented with the anode to the left and the cathode to the right. Gel 1 contains a sample that was mixed during quenching. Gels 2−6 contain samples mixed before quenching. From left to right, the bands are rHb F (γ112Thr → Cys), hybrid Hb, and Hb S, respectively.

density standard to all pixel locations. All of the results reported here were based on flat fielding of both gel and neutral density filter images.

Oxygen Binding Studies. Oxygen dissociation curves for our Hb samples were obtained using a Hemox-Analyzer (TCS Medical Products, Huntington Valley, PA) at 29 °C in 0.1 M sodium phosphate buffer at various pHs between 6.0 and 8.5. After the measurements were taken, the samples were checked for the presence of methemoglobin (met-Hb). Data from those samples with levels of met-Hb exceeding 5% were discarded. Oxygen equilibrium parameters were derived by fitting the Adair equations to each equilibrium oxygen binding curve by a nonlinear least-squares procedure. P_{50}, a measure of oxygen affinity, was obtained at 50% saturation of the oxygen binding curve. The Hill coefficient (n_{max}), a measure of the cooperativity of the oxygenation process, was determined from the maximum slope of the Hill plot by linear regression. The accuracy of these P_{50} measurements (millimeters of Hg) was ±5%, and that of n_{max} was ±7%.

Table 1: Results of Alkaline Denaturation Experiments[a]

hemoglobin	% undenatured Hb
Hb A	4.0 ± 0.5
Hb S	2.0 ± 0.2
Hb A_2	19.2 ± 0.5[c]
Hb F[b]	80.4 ± 1.5,[b] 82.7 ± 0.4,[b,c] 93.4 ± 2.1[b]
rHb F (γ112T → C)	91.3 ± 0.7
rHb F (γ130W → Y)	87.4 ± 0.2[c]
rHb F (γ112T → C/γ130W → Y)	83.2 ± 0.4[c]

[a] Hbs were denatured in 1.6 mL of $^1/_{12}$ N KOH at pH 12.7 and precipitated after 60 s with 3.4 mL of a solution made up of 800 mL of 2 M $(NH_4)_2SO_4$ and 2 mL of 10 N HCl. The solution was then filtered, and the absorbance of the filtrate was measured at 540 nm. [b] These data represent three different Hb F samples purified from different sources. [c] These data represent an average ± the range rather than the standard deviation because there were fewer than seven data points.

RESULTS

Alkaline Denaturation. The resistance of our recombinant mutant fetal Hbs to alkaline denaturation was determined and compared to that of Hb F, Hb A, Hb S, and Hb A_2. The results of these experiments are presented in Table 1. Hb A and Hb S are not very resistant to alkaline denaturation, which is indicated by values for undenatured Hb of <5%. The results for Hb F, Hb A, and Hb S are consistent with previously reported values (*27*). Each of our three recombinant mutant fetal Hbs has a high resistance to alkaline denaturation similar to that of Hb F. Hb A_2 is more resistant to denaturation than Hb A, but it clearly does not have the marked stability of Hb F.

Sub-Zero Isoelectric Focusing. Sub-zero IEF experiments were performed with Hb A, Hb F, Hb A_2, and three recombinant mutants of Hb F in mixtures with Hb S. The percentage of hybrid tetramer in each of these mixtures is listed in Table 2. Results for mixtures of Hb A and Hb S and of Hb A_2 and Hb S yield values for hybrid percentage that are consistent with the expected binomial distribution. Mixtures of Hb F and Hb S, however, contain less hybrid than would be indicated by a binomial distribution.

Initially, reactions of Hb mixtures were quenched at −25 °C before the IEF experiments. A measure of the accuracy of this quenching method can be determined by quenching each Hb at −25 °C before it is mixed with another Hb at the same temperature. If the association−dissociation interactions of the Hbs were completely quenched, then there would be no hybrid tetramer in the gels run with this sample. When Hb A and Hb S were quenched separately at −25 °C, there was 1−2% hybrid measured in the gels, indicating that a small amount of interaction between the Hbs did occur despite quenching, but was not likely to dramatically affect the results. Similar results were found for mixtures of Hb

Table 2: Results from Sub-Zero Isoelectric Focusing Experiments

parent Hbs	quenching temperature (°C)	no. of experiments	no. of gels	% hybrid[a]
Hb A and Hb S	−25	8	40	47.8 ± 0.6
Hb S and Hb A_2	−25	4	22	49.4 ± 0.7
Hb F and Hb S	−25	5	30	44.5 ± 0.4
rHb F (γT112 → C) and Hb S	−25	2	10	43.1 ± 0.7
rHb F (γW130 → Y) and Hb S	−25	2	9	40.6 ± 0.8
rHb F (γT112 → C/γW130 → Y) and Hb S	−25	1	5	43.1 ± 0.4
Hb A and Hb S	−30	2	10	47.3 ± 0.4
Hb S and Hb A_2	−30	2	10	49.3 ± 0.3
Hb F and Hb S	−30	3	15	44.5 ± 0.5
rHb F (γT112 → C) and Hb S	−30	3	15	44.6 ± 0.5
rHb F (γW130 → Y) and Hb S	−30	3	13	42.4 ± 0.3
rHb F (γT112 → C/γW130 → Y) and Hb S	−30	3	15	44.1 ± 0.5

[a] Data represent the average from all gels ± the standard deviation.

A_2 and Hb S and of Hb F and Hb S when they were quenched separately. Therefore, a quench temperature of −25 °C was deemed sufficient for these Hb mixtures.

Interestingly, when the mutants of Hb F were quenched separately with Hb S at −25 °C, 5−6% hybrid tetramer was found on IEF, indicating that these Hbs were not as efficiently quenched at this temperature as other Hbs studied previously. Why this should be so is not known at this time. It is important to our equilibrium measurements, however, that there be as little interaction between the Hbs as possible during and after the quenching process. Hence, these mutant Hbs were quenched separately with Hb S at temperatures ranging from −30 to −35 °C to reduce such interaction. Subsequently, IEF experiments were performed at −25 to −28 °C, and the results indicated about 2% hybrid tetramer. Thus, these mutant Hbs are as efficiently quenched at temperatures below −30 °C as are the naturally occurring Hbs at the higher temperature. Moreover, when Hb A and Hb F were quenched with Hb S at temperatures below −30 °C, the results were identical to those realized at −25 °C.

Once this difference in quenching temperature was established, all subsequent experiments contained a control gel for which the Hbs were quenched separately. If the control gel contained more than 2% hybrid, the experiment was discarded. This is illustrated in Figure 2. In the first gel, the two Hbs were quenched separately below −30 °C. For the remaining five gels, the two Hbs were incubated at room temperature and then quenched. Thus, the sample run in the first gel confirmed that the temperature of the quench solutions was sufficiently low. The OD profiles of the first two gels in Figure 2 are presented in Figure 3. Generally, three to six gels in each IEF experiment contain the same mixture of two Hbs. So far, six combinations of Hbs have been studied. The results of several IEF experiments performed on different days were averaged together. These results are presented in Table 2 along with the number of IEF experiments and the total number of gels that were averaged in each case.

Oxygen Binding Studies. The oxygen dissociation curves for Hb A, Hb F, Hb A_2, rHb F (γ112Thr → Cys), rHb F (γ130Trp → Tyr), and rHb F (γ112Thr → Cys/γ130Trp → Tyr) are shown in Figure 4. For comparison, some of the data from Figure 4 are listed in Table 3. It is clear from

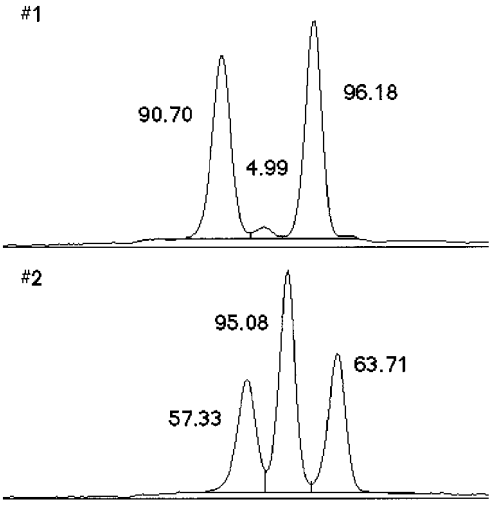

FIGURE 3: Optical density profiles of gels 1 and 2 from Figure 2. The areas under the peaks are directly proportional to the amount of Hb in the band. The peaks from left to right represent rHb F (γ112Thr → Cys), hybrid Hb, and Hb S, respectively.

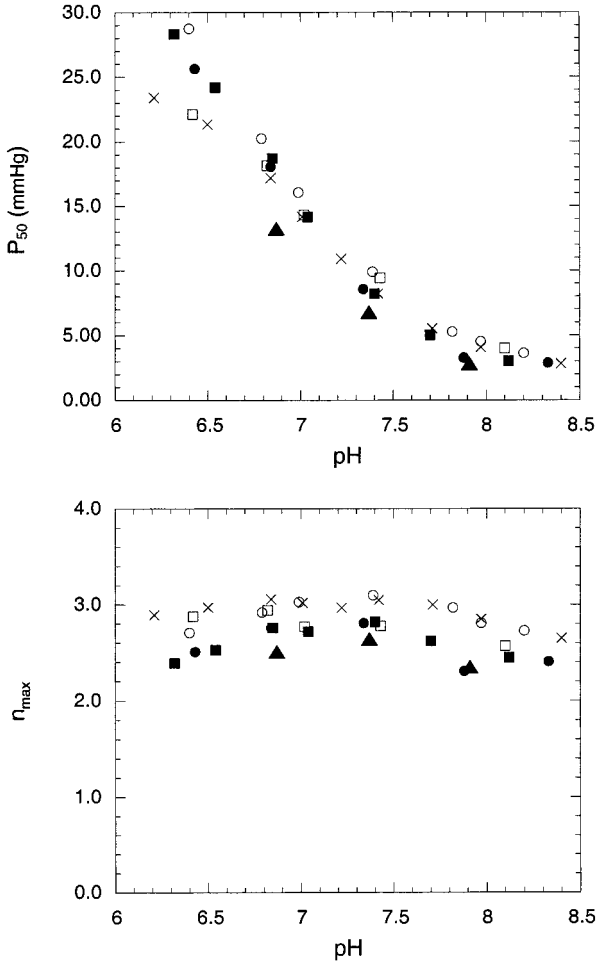

FIGURE 4: Oxygen binding properties of Hb A (×), Hb A_2 (□), Hb F (■), rHb F (γ112Thr → Cys) (●), rHb F (γ130Trp → Tyr) (○), and rHb F (γ112Thr → Cys/γ130Trp → Tyr) (▲) in 0.1 M phosphate as a function of pH at 29 °C.

Figure 4 and Table 3 that rHb F (γ112Thr → Cys) has an oxygen affinity and a cooperativity very similar to those of Hb F. The mutant rHb F (γ130Trp → Tyr) also exhibits an oxygen affinity similar to that of Hb F, while its cooperativity may be slightly higher, more like that of Hb A. Results for

Table 3: Oxygen Binding Data for Hb A, Hb A_2, Hb F, and Mutants of Hb F in 0.1 M Phosphate at 29 °C

sample	pH	P_{50} (mmHg)	n_{max}
Hb A	7.4	8.2	3.0
Hb A_2	7.4	9.5	2.8
Hb F	7.4	8.2	2.8
rHb F (γ112T → C)	7.3	8.6	2.8
rHb F (γ130W → Y)	7.4	9.9	3.1
rHb F (γ112T → C/γ130W → Y)	7.4	6.8	2.6

the double mutant, however, seem to suggest that it has a slightly higher oxygen affinity and slightly lower cooperativity than Hb F.

DISCUSSION

It remains to be determined whether Hbs which reduce the amount of polymerization in mixtures with Hb S will prove to be useful to the development of a gene therapy for sickle cell anemia. Current drug therapies designed to increase the levels of Hb F in red blood cells of sickle cell anemia patients have met with considerable success (*30*). However, these drug therapies are not equally effective for all sickle cell patients, and therapies which use cytotoxic drugs (like hydroxyurea) are not recommended for use in children due to the possibility of long-term damage in differentiating cells. It is highly likely that gene therapy will be important in the treatment of a variety of hemoglobinopathies, including sickle cell anemia. However, so far the realization of such therapies has been hampered by inefficient gene transfer and low levels of expression of the transferred gene. Most strategies require high levels of expression of the transferred gene to be effective. Perhaps, a reasonable approach may be to find a mutation which would make the transferred gene more effective at lower levels of expression. Toward this end, recombinant mutants of Hb F which form hybrid molecules with Hb S more readily than their natural counterparts would be more effective at preventing Hb S polymerization, thus requiring less of the mutant Hb to produce the desired benefit.

It is clear from the results presented in this work that the three recombinant mutants of Hb F described above may not be suitable candidates for such a gene therapy. The data from the sub-zero IEF experiments indicate that mixtures of rHb F (γ112Thr → Cys) or rHb F (γ112Thr → Cys/γ130Trp → Tyr) with Hb S contain the same amount of hybrid as mixtures of Hb F and Hb S. These data suggest that the substitution at the $\alpha_1\gamma_1$ interface at position γ112 has no effect on hybrid formation in these mixtures. Experiments with mixtures of rHb F (γ130Trp → Tyr) and Hb S indicate that the substitution at position γ130 may have only a minor effect on hybrid formation.

More striking than the results of the sub-zero IEF are those of the alkaline denaturation of these recombinant mutants of Hb F. All three mutant Hbs are equally resistant to denaturation as Hb F, whereas Hb A has a much lower resistance to denaturation than Hb F. If the substitutions at positions γ112 and γ130 above were important to the stabilization of Hb F against alkaline denaturation, then the mutations in our recombinant fetal Hbs would have made those Hbs less resistant to denaturation. Thus, it is clear from the alkaline denaturation studies that the amino acid substitutions at positions γ112 and γ130 do not play a significant role in the stabilization of Hb F on the basis of this criterion.

Oxygen binding data for the recombinant mutants of Hb F do not indicate any significant changes in the functional behavior of these mutants. The two mutations at positions γ112 and γ130 do not, individually, have an impact on the oxygen affinity of the molecules, but in conjunction, they may effect a slight increase in the oxygen affinity (Table 3 and Figure 4). The mutant rHb F (γ130Trp → Tyr) exhibits a cooperativity much like Hb A, while rHb F (γ112Thr → Cys) exhibits an only slightly lower cooperativity, similar to that of Hb F. The cooperativity of the double mutant appears to be somewhat lower than that of Hb F.

Since these first three mutant Hbs do not possess the desired dissociation properties for gene therapy, other sites in the Hb F molecule will have to be considered which may result in an increased level of hybrid formation with Hb S. The most immediately obvious target residues are the remaining substitutions at the $\alpha_1\gamma_1$ and $\alpha_1\gamma_2$ interfaces. One or more of these sites could be considered for mutation. However, recent results of Dumoulin et al. (*6*) suggest that the amino acid substitutions at the $\alpha_1\gamma_1$ and $\alpha_1\gamma_2$ interfaces have no significant effect (at least collectively) on the tetramer−dimer dissociation properties of the Hb molecule. These researchers constructed a rHb in which all five of these interface sites were substituted in the β chain. The tetramer−dimer dissociation constant (K_d) of this rHb was determined to be intermediate between that of Hb A and that of Hb F. Thus, the tetramer−dimer dissociation properties of Hb F cannot be explained completely by the substitutions at the $\alpha_1\gamma_1$ and $\alpha_1\gamma_2$ interfaces. Another recent publication by Dumoulin et al. (*31*) suggests that the tetramer−dimer dissociation properties of Hb F may be due in large part to substitutions in its amino-terminal sequence. In this study, a new rHb (Hb Felix) was constructed which contains a β chain with the N-terminal 18-amino acid sequence of Hb F. The K_d of Hb Felix is very similar to that of Hb F. Thus, the eight substitutions in this N-terminal region may be more important to the hybrid tetramer formation than those at the interfaces and could prove to be better targets for mutation in the design of mutant fetal Hbs. On the basis of these results, the next step in this research will be to construct a mutant Hb F in which the N-terminal region of the γ chain is replaced by the corresponding region of the β chain. Given the properties of Hb Felix, it is reasonable to expect that the dissociation properties of this new recombinant mutant of Hb F may be similar to those of Hb A. Hence, this mutant Hb would be expected to produce more hybrid with Hb S than does Hb F.

Another Hb considered in this paper is Hb A_2 ($\alpha_2\delta_2$). This Hb seems to be equally as effective as Hb F at inhibiting polymerization of Hb S (*32*, *33*). Hb A and Hb A_2 differ by 10 amino acid residues between the β and δ chains, and there is no difference in the net charge between these two chains (*7*). It may be possible to construct a mutant of Hb A_2 which would be more likely to form hybrid tetramers with Hb S than ordinary Hb A_2. Sub-zero IEF results for the mixtures of Hb S with Hb A_2 and with Hb A indicates that the association and dissociation properties of Hb A_2 are similar to those of Hb A, which is consistent with the results of Manning and co-workers (*34*) that indicate similar tetramer−dimer dissociation constants for Hb A_2 and Hb A. Since Hb

A_2 forms nearly 50% hybrid tetramer in mixtures with Hb S, increasing the amount of hybrid tetramer would require a mutation which would destabilize the Hb A_2 tetramer, but not the hybrid tetramer.

ACKNOWLEDGMENT

We extend our appreciation to Dr. Michele Perrella for many helpful suggestions with regard to improving our sub-zero isoelectric focusing techniques.

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BI990740O