Probing the Importance of the Amino-Terminal Sequence of the β- and γ-Chains to the Properties of Normal Adult and Fetal Hemoglobins†

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ABSTRACT: A recombinant mutant of human fetal hemoglobin (Hb F), named rHb Oscar, has been constructed to explore the importance of the sequence of the amino-terminal region of the γ-chain to the structural and functional properties of Hb F as compared to human normal adult hemoglobin (Hb A). Substitutions in the N-terminal region of Hb A have shown this region to be important to its structural and functional properties. Recent studies of recombinant mutants of Hb A with γ-chain mutations have been used to probe the significance of the N-terminal sequence to the properties of Hb F. One of these mutants of Hb A, called rHb Felix, contains eight substitutions in the N-terminal region of the β-chain corresponding to the sequence of the γ-chain in that region [Dumoulin et al. (1998) *J. Biol. Chem. 273*, 35032−35038]. rHb Felix exhibits a 2,3-bisphosphoglycerate (2,3-BPG) response like that of Hb A, but its tetramer−dimer dissociation constant is similar to that of Hb F. In contrast, rHb Oscar contains a γ-chain with eight mutations at the N-terminal end corresponding to the sequence of the β-chain of Hb A in that region. ^{1}H NMR studies of rHb Oscar indicate a global structure like that of Hb F. rHb Oscar is not as stable against alkaline denaturation as Hb F but is more stable than Hb A, and it exhibits a stronger response to 2,3-BPG and inositol hexaphosphate as compared to Hb F. The 2,3-BPG effect in rHb Oscar also appears to be slightly enhanced compared to that in Hb A. Subzero isoelectric focusing experiments suggest that rHb Oscar does not have dissociation properties like those of Hb A. These results along with those of rHb Felix illustrate that the effects of the N-terminal region on structure and function of the Hb molecule are complicated by interactions with the rest of the molecule that are not yet well defined. However, studies of complementary mutations of Hb A and Hb F may eventually help to define such interactions and lead to a better understanding of the relationship between the amino acid sequence and the properties of the Hb molecule.

The amino acid sequence of the amino-terminal region of the β-chain is known to be important to the structural and functional properties of the hemoglobin (Hb)[1] molecule (*1*). Specifically, the β1Val and β2His residues are a part of the 2,3-bisphosphoglycerate (2,3-BPG) binding site and are located near the entrance to the central cavity in human normal adult hemoglobin (Hb A) in the deoxy form (*2*, *3*). Mutations of the β1Val residue have been shown to exhibit oxygen affinity similar to that of Hb A and to cause only localized conformational changes (*4*, *5*). One such mutation (β1Val→Gly), while causing no change in oxygen affinity, significantly lowers the tetramer−dimer dissociation constant of the Hb molecule (*6*). Individual mutations at β2His, β5Pro, β9Ser, β11Val, and β15Trp have also been shown to alter the functional properties of Hb (*7*−*11*). A detailed understanding of the influence of the N-terminal region of the β-chain on the structural and functional properties of the Hb molecule could provide valuable insights into the structure−function relationship of Hb.

In one study, a recombinant mutant of Hb A named rHb Felix was constructed with eight mutations in the N-terminal region of the β-chain corresponding to the sequence of the γ-chain in that region (*12*). There are 39 amino acid substitutions between the γ- and β-chains, which result in some distinctly different properties between human fetal hemoglobin (Hb F) and Hb A (*13*). Dumoulin et al. (*12*) reported a 70-fold decrease in the tetramer−dimer dissociation of Hb F compared to that of Hb A. Hb F is also much more stable against alkaline denaturation than is Hb A (*14*). Another difference between Hb A and Hb F is their responses to the allosteric effector, 2,3-BPG. While both Hbs have decreased oxygen affinity in the presence of 2,3-BPG, the effect is decidedly more pronounced for Hb A than for Hb F (*15*). Another important difference between Hb F and Hb

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[1] Abbreviations: Hb, hemoglobin; Hb A, human normal adult hemoglobin; rHb, recombinant hemoglobin; met-Hb, methemoglobin; Hb F, human fetal hemoglobin; 2,3-BPG, 2,3-bisphosphoglycerate; IHP, inositol hexaphosphate; NMR, nuclear magnetic resonance; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; IEF, isoelectric focusing; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CCD, charged-coupled device.

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A is in their interactions with sickle cell hemoglobin (Hb S). In mixtures with Hb S, Hb F has been shown to inhibit the polymerization of Hb (*16*), while Hb A has much less of an effect on the polymerization. It is believed that increased levels of Hb F can reduce the polymerization through the formation of asymmetric hybrids of Hb F and Hb S, i.e., $\alpha_2\beta^s\gamma$, or by a direct sparing effect on the solubility of Hb S by Hb F tetramers (*16*−*19*).

rHb Felix and several other mutants of Hb A have been used to investigate the effects of specific substitutions on the properties of Hb F (*6*). What is striking about rHb Felix and another rHb containing the single substitution β1Val→Gly is that neither of these rHbs has a significantly different response to 2,3-BPG compared to Hb A, even though the β1Val of Hb A is believed to be part of the 2,3-BPG binding site. rHb Felix does, however, have a tetramer−dimer dissociation constant like that of Hb F. On the basis of these results, it appears that the N-terminal sequence of the β-chain is very important to the tetramer−dimer dissociation of the Hb molecule. It would seem likely that the N-terminal sequence of the γ-chain is similarly important to the tetramer−dimer dissociation of Hb F. To further examine the role of the N-terminal region in the properties of Hb F, we have constructed a recombinant mutant of Hb F, named rHb Oscar, with eight mutations in the N-terminal region of the γ-chain corresponding to the amino acid sequence of the β-chain. Given the properties of rHb Felix, it was hoped that the dissociation properties of this rHb would be similar to those of Hb A, and in turn, rHb Oscar would, like Hb F, yield more hybrid tetramers in mixtures with Hb S.

This paper reports the results of functional and structural studies of rHb Oscar. The effects of anions, i.e., chloride, 2,3-BPG, and inositol hexaphosophate (IHP), on the oxygen-binding properties of rHb Oscar are presented along with proton nuclear magnetic resonance (NMR) results. Alkaline denaturation of rHb Oscar was also used to probe any significance of the N-terminal region for the denaturation properties of Hb F. The tetramer−dimer dissociation properties of rHb Oscar were investigated by subzero isoelectric focusing (IEF) of mixtures of rHb Oscar and Hb S. Similar studies of mixtures of Hb A and Hb S and of Hb F and Hb S have indicated that less hybrid tetramer is formed in the Hb F and Hb S mixtures, due to the lower dissociation rate of Hb F (*20*). Thus, the amount of hybrid tetramer formation in mixtures of rHb Oscar and Hb S would be an indication of the dissociation properties of rHb Oscar. These Hb mixtures were further examined by gelation studies to determine their effect on delaying the polymerization of Hb S.

EXPERIMENTAL PROCEDURES

Construction of rHb Oscar. An expression plasmid (pHE80) containing both α- and γ-globin genes and the *Escherichia coli* methionine aminopeptidase (MAP) gene was constructed in this laboratory (*21*). This plasmid was used as a template in a PCR-based mutagenesis method as described by Dumoulin et al. (*22*). For our rHb Oscar, eight amino acid residues were replaced at the N-terminal end of the γ-chain with the corresponding residues of the β-chain. Two synthetic oligonucleotides were used as primers to produce PCR fragments containing all of the mutations. These primers were designed to contain enough of a complementary sequence within the mutated region that the two fragments would hybridize during the second amplification. Their sequences are ACT CCC GAA GAA AAA TCT GCC GTT ACT GCT CTG TGG GGT AAA G (which contains mutations for amino acids γ5, γ7, γ9, γ10, γ11, and γ13) and GGC AGA TTT TTC TTC GGG AGT CAG GTG AAC CAT TAT TAA TAC CCT C (which contains mutations for amino acids γ10, γ9, γ7, γ5, γ3, and γ1). Each primer was paired with another synthetic oligonucleotide complementary to an area of the plasmid several hundred bases downstream of the mutation primer and containing a restriction site.

A two-step amplification procedure adapted from the overlap extension method (*23*) was employed. The first amplification was performed in two separate tubes, each containing one set of primers. The sizes of the PCR fragments were checked on an agarose gel, and the DNA was eluted and purified with Prep-A-Gene (Bio-Rad). The two DNA fragments from the first amplification were combined in the second amplification to create a longer fragment containing all of the mutations. This DNA fragment was checked for the appropriate size, eluted, purified, and subsequently digested with the appropriate restriction enzymes to allow it to be cloned back into the pHE80 plasmid. The new plasmid, pHE8001, contains eight amino acid substitutions in the γ-chain coding region: γ1Gly→Val, γ3Phe→Leu, γ5Glu→Pro, γ7Asp→Glu, γ9Ala→Ser, γ10Thr→Ala, γ11Ile→Val, and γ13Ser→Ala. DNA sequence analysis was used to confirm the entire sequence of the α- and γ-chain coding regions of the plasmid.

Preparation of rHb Oscar. The expression plasmid pHE8001 was transformed into *E. coli* JM109 cells which were grown in LB medium in a Microferm fermentor (New Brunswick Scientific, Model MF20) as described previously (*21*, *24*) until the cell density was approximately 1×10^9 cells/mL. Hemin and glucose were added during growth. The expression of the Hb and MAP genes was induced by the addition of isopropyl β-thiogalactopyranoside, and growth was subsequently 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 our laboratory (*21*, *24*, *25*).

Electrospray ionization mass spectrometric analysis of rHb Oscar was performed on a VG Quattro-BQ (Fissons Instruments, VG Biotech, Altrincham, U.K.) as described by Shen et al. (*21*). Automated cycles of Edman degradation were performed on an Applied Biosystems gas/liquid-phase sequencer (Model 470/900A) equipped with an on-line phenylthiohydantoin amino acid analyzer (Model 120A). Hb Oscar had the correct molecular weight and contained ∼12% methionine at the amino terminus.

Preparation of Other Hemoglobins. Human normal adult blood samples were obtained from a local blood bank. Hb A was isolated and purified by established methods in our laboratory (*26*). Hb S and Hb F hemosylates obtained from a blood sample of an SS donor were prepared according to the same procedure as that for Hb A. They were then equilibrated with 10 mM phosphate/0.5 mM EDTA at pH 6.8 at 25 °C and put through a FPLC Mono S column (Amersham Pharmacia cation exchanger 16/10) to separate Hb from other components. All Hb samples used in the following experi-

ments were 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 our Hb samples were obtained using a Hemox-Analyzer (TCS Medical Products, Huntington Valley, PA) at 29 °C as a function of pH in 0.1 M sodium phosphate. Oxygen-binding properties were also determined for our Hbs in 0.1 M *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, pH 7.4, and 5 mM 2,3-BPG at pH 7.4 and 37 °C and in 0.1 M HEPES plus 50 mM chloride, 2 mM IHP, or 2 mM 2,3-BPG at 29 °C. Oxygen-binding properties as a function of chloride concentration (from 0.05 to 0.15 M) have also been determined for these Hbs in 0.1 M HEPES at pH 7.2−7.4 and at 29 °C. The methemoglobin (met-Hb) reductase system of Hayashi et al. (*27*) was used in most of the Hb samples reported here in order to reduce the amount of met-Hb to less than 5%.

^{1}H NMR Spectroscopy Investigation. ^{1}H NMR spectra of our Hbs were obtained using a Bruker AVANCE DRX-300 spectrometer. All Hb samples were in 0.1 M sodium phosphate in 100% water, and the Hb concentration was about 5% (∼3 mM). The water signal was suppressed by using a jump-and-return pulse sequence (*28*). 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.

Alkaline Denaturation. Studies of the resistance of rHb Oscar to denaturation under alkaline conditions were also performed. The alkaline denaturation technique used in this laboratory was established by Singer et al. (*14*) and described previously (*20*).

Subzero Isoelectric Focusing. Isoelectric focusing was carried out at −25 °C on equimolar mixtures of rHb Oscar and Hb S. Sample preparation and running conditions were as described previously (*20*).

Studies on the Delay Time of Gelation. Delay times for various Hbs were determined using a modified version of the high phosphate method of Adachi and Asakura (*29*). Oxy-Hb samples were added to 1.8 M potassium phosphate buffer until an Hb concentration of 0.20 g/dL was reached. Samples at this concentration were subsequently combined in specific ratios of Hb S to the other Hb in a total volume of 3 mL. This mixture of Hbs was then deoxygenated under N_2 in a rotary evaporator submerged in an ice bath. The deoxygenated sample was transferred anaerobically to a cuvette containing 10 mg of nitrogen-flushed dithionite. The dithionite was dissolved by inverting the cuvette several times, after which the cuvette was kept in an ice bath for 10 min to allow the sample to settle and to cool to 1 °C. The cuvette was then placed in a thermostated cell holder at 30 °C, and the absorbance of the sample was monitored at 700 nm with a Cary 50 spectrophotometer (Varian) until the absorbance reached a maximum. The temperature of the sample was monitored with a temperature probe in the solution. The temperature of all samples rose quickly and uniformly, reaching 30 °C in less than 3 min. Delay times were determined as described by Adachi and Asakura (*29*).

RESULTS

Oxygen-Binding Studies. The oxygen-binding properties of Hb A, Hb F, and rHb Oscar in 0.1 M sodium phosphate

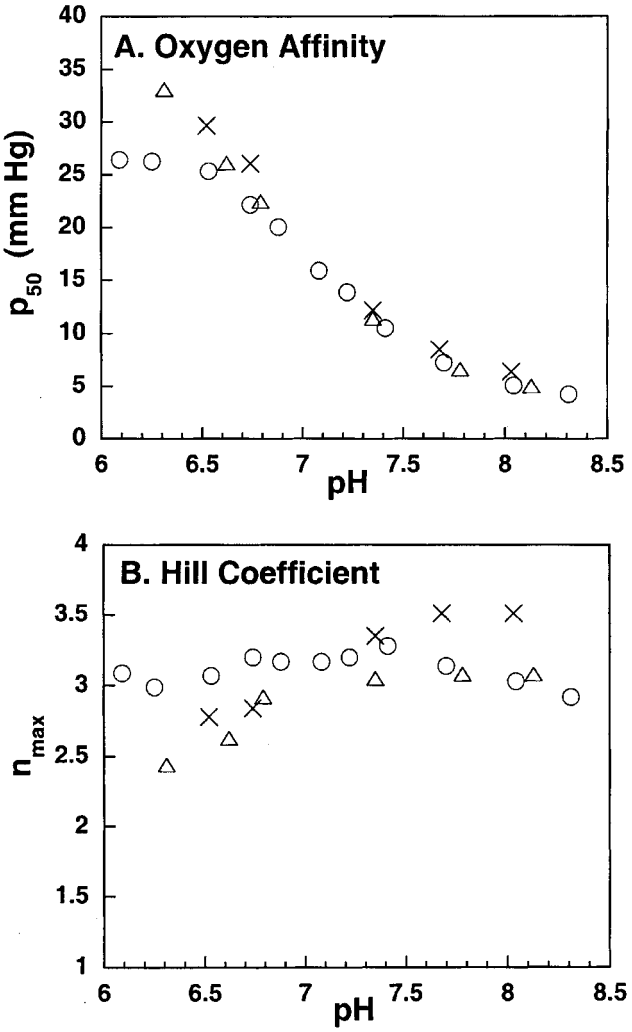

FIGURE 1: Oxygen-binding properties of Hb A (○), Hb F (×), and rHb Oscar (△) in 0.1 M phosphate as a function of pH at 29 °C.

Table 1: Effect of Chloride on Hemoglobins in 0.1 M HEPES at pH 7.3−7.4 at 29 °C[a]

NaCl concn (mM)	Hb A		Hb F		Hb Oscar	
	p_{50}	n_{max}	p_{50}	n_{max}	p_{50}	n_{max}
0	5.4	3.0	6.4	3.2	4.5	2.6
50 mM	7.3	3.0	8.2	3.1	6.7	2.9
100 mM	9.0	3.0	9.8	3.0	8.0	2.9
150 mM	10.2	2.9	10.8	3.0	10.7	2.9

[a] All data were taken in the presence of the reductase system of Hayashi et al. (*27*).

are shown in Figure 1. These data indicate no significant difference in the oxygen affinity and cooperativity of these three Hbs at pH 6.5−8.5. However, the graph of p_{50} versus pH does suggest a possible difference in these Hbs below pH 6.5. It has been known for some time that the acid Bohr effect is less pronounced for Hb F than for Hb A (*30*). The oxygen affinity data for rHb Oscar suggest that it may have an acid Bohr effect similar to Hb F.

Table 1 contains oxygen affinities and Hill coefficients for Hb A, Hb F, and rHb Oscar at several chloride concentrations. The number of chloride ions bound to Hb upon deoxygenation can be estimated by calculating from the best fit of $-\partial \log p_{50}/\partial$pH (*25*). The strength of the chloride effect is very similar for Hb F (number of Cl^- bound

Table 2: Oxygen Affinities of Hemoglobins in 0.1 M HEPES at pH 7.3−7.4[a]

	Hb A	Hb F	rHb Oscar
p_{50} (mmHg)			
−chloride (29 °C)	5.4	6.4	4.5
−chloride (37 °C)	13.5	14.3	15.7
+50 mM chloride (29 °C)	7.3	8.2	6.7
+2 mM IHP (29 °C)	60.2	34.4	56.0
+2 mM 2,3-BPG (29 °C)	14.6	13.1	15.7
+5 mM 2,3-BPG (37 °C)	33.9	28.9	49.9
log[p_{50}(Hb)/p_{50}(Hb A)]			
−chloride (29 °C)		0.074	−0.079
−chloride (37 °C)		0.025	0.066
+50 mM chloride (29 °C)		0.050	−0.040
+2 mM IHP (29 °C)		−0.240	−0.031
+2 mM 2,3-BPG (29 °C)		−0.047	0.032
+5 mM 2,3-BPG (37 °C)		−0.069	0.168
ΔΔ log p_{50} (50 mM chloride, 29 °C)		−0.024	0.041
ΔΔ log p_{50} (2 mM IHP, 29 °C)		−0.314	0.048
ΔΔ log p_{50} (2 mM 2,3-BPG, 29 °C)		−0.121	0.110
ΔΔ log p_{50} (5 mM 2,3-BPG, 37 °C)		−0.094	0.102

[a] All data were taken in the presence of the reductase system of Hayashi et al. (*27*).

= 0.25) as compared to Hb A (number of Cl^- bound = 0.3), while the chloride effect for rHb Oscar (number of Cl^- bound = 0.41) is slightly enhanced compared to Hb A.

The effect of anions on the oxygen affinity can be quantified by comparing a change in the free energy of oxygen binding, ΔG, in buffers with anions and in chloride-free HEPES buffer (*25*). The effect of anions on changes in the free energy of oxygen binding induced by mutations can, therefore, be quantified as proportional to ΔΔ log p_{50} = Δ log p_{50}(mutant) − Δ log p_{50}(Hb A), where Δ log p_{50} is the difference of free energy in buffers with anions and in chloride-free HEPES buffer. Table 2 summarizes changes in the free energy of oxygen binding in 50 mM chloride, 2 mM IHP, and 2 or 5 mM 2,3-BPG induced by Hb F and rHb Oscar compared to Hb A. The difference in response to 50 mM chloride between Hb F and Hb A and between Hb A and rHb Oscar is small, but noticeable. rHb Oscar exhibits a stronger IHP response than does Hb F, approaching the level of response of Hb A. The change in the oxygen affinity of Hb F due to IHP relative to Hb A is −0.31 unit and that of Hb Oscar is +0.05 unit. The difference in p_{50} values due to 2 mM 2,3-BPG at 29 °C among Hb F, Hb A, and rHb Oscar is minor; however, there is a significant difference in oxygen affinity when measured in the presence of 5 mM 2,3-BPG at 37 °C. There is a change of −0.10 unit in the ΔΔ log p_{50} due to 2,3-BPG binding in Hb F and a +0.10 change due to 2,3-BPG in rHb Oscar. The 2,3-BPG effect appears to be enhanced in Hb Oscar as compared to Hb A and Hb F.

^{1}H NMR Spectroscopy Investigation. The exchangeable proton resonances and ring-current-shifted proton resonances of Hb A, Hb F, and rHb Oscar in the CO form are shown in Figure 2. The ring-current-shifted resonances are an indication of the tertiary structure of the heme pocket (*31*). The resonances at ∼−1.8 and ∼−1.7 ppm have been assigned to the γ_2-CH_3 of the E11Val of the β-chain and α-chain of HbCO A, respectively (*26*, *32*). The resonance assigned to the γ_2-CH_3 of the γ-E11Val of Hb F is shifted upfield to ∼−2.0 ppm, suggesting that this group in the γ-chain of Hb F is located closer to the normal of the heme than in the

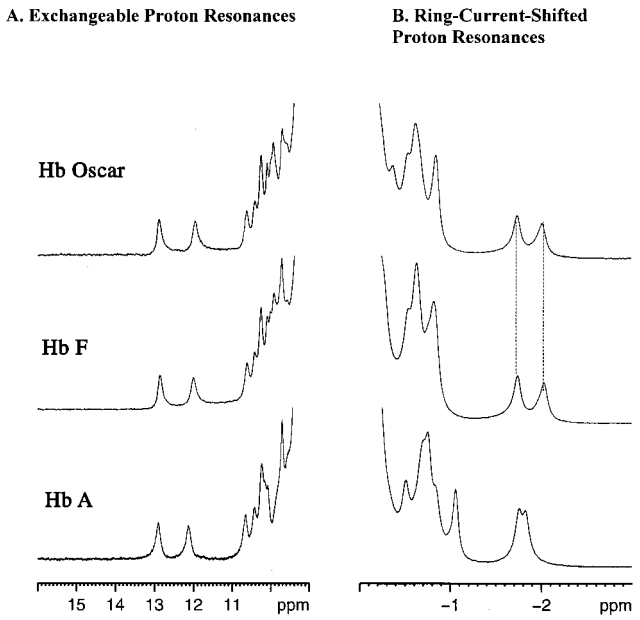

FIGURE 2: 300 MHz ^{1}H NMR spectra of 4−6% solutions of Hb A, Hb F, and rHb Oscar in the CO form in H_2O in 0.1 M sodium phosphate at pH 7.0 and 29 °C.

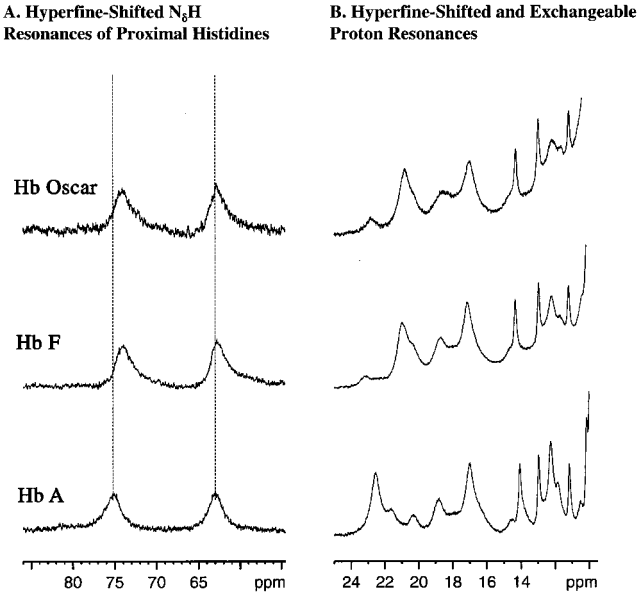

FIGURE 3: 300 MHz ^{1}H NMR spectra of 4−6% solutions of Hb A, Hb F, and rHb Oscar in the deoxy form in H_2O in 0.1 M sodium phosphate at pH 7.0 and 29 °C.

β-chain of HbCO A. These two resonances of rHb Oscar are identical to those of Hb F (Figure 2B), suggesting that the mutations in the N-terminal end of the γ-chain do not alter the tertiary structure around the heme pocket region of Hb F.

The exchangeable and hyperfine-shifted proton resonances of Hb A, Hb F, and rHb Oscar in the deoxy form are shown in Figure 3. The resonances at 63 and 76 ppm from DSS have been assigned to the hyperfine-shifted N_δH-exchangeable proton of the proximal histidine residue (α87His) of the α-chain of deoxy-Hb A and the corresponding residue of the β-chain (β92His) of deoxy-Hb A, respectively (*33*, *34*). All three Hbs in Figure 3A have a similar resonance at 63 ppm from DSS, but Hb F and rHb Oscar have resonances

Table 3: Results of Alkaline Denaturation of Hemoglobins

hemoglobin	% undenatured hemoglobin
Hb A	4.0 ± 0.5
Hb S	2.0 ± 0.2
Hb A_2	19.2 ± 0.5[a]
Hb F	80.4 ± 1.5
rHb Oscar	71.4 ± 1.5[a]

[a] These data represent an average ± the range rather than the standard deviation because there were fewer than seven data points.

that are slightly upfield shifted from that of Hb A at 76 ppm to 74 ppm from DSS. This shift is due to a difference in the conformation of the γ-chain (and the mutated γ-chain of Hb Oscar) compared to that of the β-chain. The spectral region from 10 to 25 ppm arises from the hyperfine-shifted resonances of the porphyrin ring and the amino acid residues situated in proximity to the heme pockets and the exchangeable proton resonances (Figure 3B). There are spectral changes in Hb F as compared to Hb A over the region from 16 to 20 ppm, reflecting changes in the environment of the heme pockets of the γ-chains (*35*). There are no noticeable differences in the resonances from 10 to 25 ppm between deoxy-Hb F and deoxy-rHb Oscar.

Alkaline Denaturation. The results of the alkaline denaturation of rHb Oscar are listed in Table 3 along with the results from a previous study of Hb A, Hb S, Hb A_2, and Hb F (*20*). These data indicate that rHb Oscar is slightly less stable against alkaline denaturation than Hb F but much more stable than Hb A. According to Perutz (*36*), the reason that Hb A is more susceptible to the alkaline denaturation than Hb F is because Hb A contains three internal amino acid residues, α104Cys, β1122Cys, and β130Tyr, which are ionizable at pH ~12. Thus, the ionziation of these residues can attract water molecules into the interior of the β-chain, which could change the conformation of the Hb molecule, making it more susceptible to denaturation (*36*). However, rHb (γThr112Cys), rHb (γTrp130Tyr), and rHb (γTrp130Tyr) were found to be as stable as Hb F against the alkaline denaturation, suggesting that the amino acid residues other than those mentioned above are responsible for the stability of Hb F against the alkaline denaturation as compared to Hb A (*20*). All of these results suggest that a more complex mechanism is involved in the alkaline denaturation of Hb than just a few of the amino acid residues that are in the immediate contacts in the subunit interfaces.

Subzero Isoelectric Focusing. Figure 4 shows the charge-coupled device (CCD) images of two types of gels, pH range 6−8 and pH range 7−9, run with equimolar mixtures of rHb Oscar and Hb S. The hybrid Hb band could not be well separated from the parent Hb bands in the gel with a pH range from 6 to 8 (gels 1 and 2 of Figure 4). The shapes of the Hb bands in pH 7−9 gels (gels 3−6 in Figure 4) indicate that these gels have different structures, even though they were made from the same solution.

Because the separation of the bands in these gels is poor, a Gaussian fitting program was applied to the optical density profiles of the gels. The equation

$$y = a\exp^{-(x-b)^2/c^2} + d\exp^{-(x-e)^2/f^2} + g\exp^{-(x-h)^2/i^2}$$

a sum of three Gaussians, was fit to the profile data, and the fitting parameters *a* through *i* were used to define three

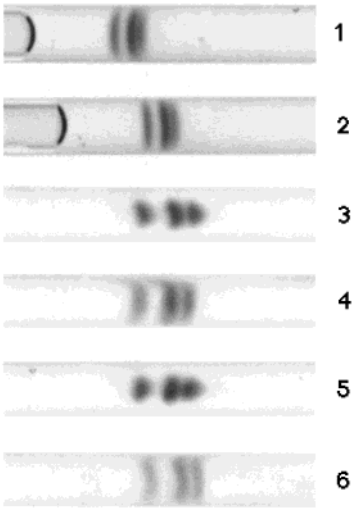

FIGURE 4: Equimolar mixtures of Hb Oscar and Hb S focused at −25 °C. The gels are oriented with the cathode to the left and the anode to the right. Gels 1 and 2 were prepared according to the standard gel recipe (pH 6−8). Gels 3−6 were prepared from a single batch of gel using pH 7−9 ampholytes. From left to right, the bands represent rHb Oscar, hybrid Hb, and Hb S. In gels 1 and 2, only two bands are clearly observed, the rHb Oscar band on the left and the hybrid Hb band overlapping with the Hb S band on the right.

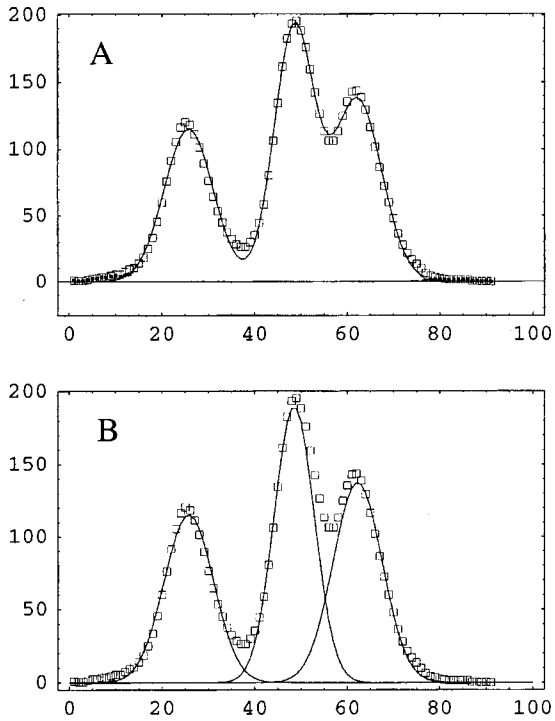

FIGURE 5: Optical density profiles of gel pattern: (A) a fit (solid line) to the optical density profile (open boxes) of gel 3 from Figure 4; (B) the three Gaussian peaks (solid lines) constructed from the fit parameters and plotted with the optical density profile (open boxes). The abscissa gives the optical density in arbitrary units, and the ordinate gives the relative position of the gel band.

separate Gaussian peaks. The area under these peaks is proportional to the amount of Hb in the band represented by the peak. The fit to the optical density profile of the third gel in Figure 4 is shown in Figure 5A. The three Gaussian peaks constructed from the fitting parameters are also shown

Table 4: Normalized Areas of the Separated Peaks for rHb Oscar, Hybrid Hb, and Hb S

	rHb Oscar	hybrid Hb	Hb S
batch 1,	0.282	0.381	0.337
5% acrylamide	0.285	0.454	0.262
	0.281	0.358	0.361
	0.288	0.446	0.266
	0.284	0.366	0.350
	0.286	0.394	0.320
batch 1 averages	0.284 ± 0.004	0.400 ± 0.048	0.316 ± 0.050
batch 2,	0.273	0.288	0.439
4% acrylamide	0.271	0.296	0.433
	0.278	0.315	0.407
	0.274	0.376	0.349
	0.274	0.346	0.380
	0.280	0.344	0.375
batch 2 averages	0.275 ± 0.005	0.328 ± 0.044	0.397 ± 0.045
averages, all data	0.280 ± 0.009	0.364 ± 0.083	0.357 ± 0.089

plotted with the original profile in Figure 5B. The normalized areas of the Gaussians constructed from the fits to 12 optical density profiles of rHb Oscar and Hb S mixtures are given in Table 4. These profiles represent two separate samples and two separate batches of gels, one using 4% acrylamide (standard recipe) and the other using 5% acrylamide. It is clear from these data that the first peak (Hb Oscar) was well separated from the others, and the data for this peak are very reproducible in each batch of gels. The values for the hybrid Hb and Hb S, however, vary considerably because the bands are too close to each other to separate them accurately. The separation problem is exacerbated by the different shapes of the bands in different gels. The large errors in the data for mixtures of rHb Oscar and Hb S make a comparison with data from other mixtures difficult. The percent of hybrid Hb found in mixtures of rHb Oscar and Hb S, 36.4 ± 8.3, may or may not be comparable to that found for mixtures of Hb F and Hb S, 44.5 ± 0.5 (*20*). On the basis of these results, it is unclear whether mixtures of rHb Oscar and Hb S contain less hybrid Hb than do mixtures of Hb F and Hb S. However, it is likely that mixtures of rHb Oscar and Hb S do not contain more hybrid Hb than do mixtures of Hb F and Hb S.

Studies on the Delay Time of Gelation. An example of these scans for mixtures of Hb S and rHb Oscar at several different ratios is given in Figure 6. The graph of delay time versus percent of Hb S for mixtures of Hb S and Hb A, Hb F, and rHb Oscar is shown in Figure 7. rHb Oscar and Hb F have a similar inhibiting effect on the polymerization of Hb S at those concentration ratios shown in Figure 7.

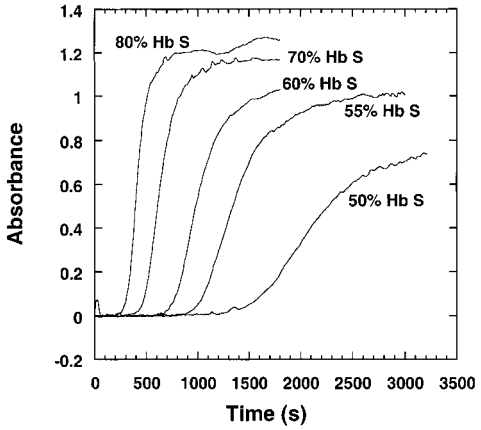

FIGURE 6: Kinetics of polymerization of mixtures of Hb S and rHb Oscar. Time courses of polymerization at various ratios of mixtures of Hb S and rHb Oscar were measured in 1.8 M phosphate buffer, pH 7.4, and at 30 °C. The final Hb concentration in the mixture was 0.2 g/dL. Delay times were determined as described by Adachi and Asakura (*29*).

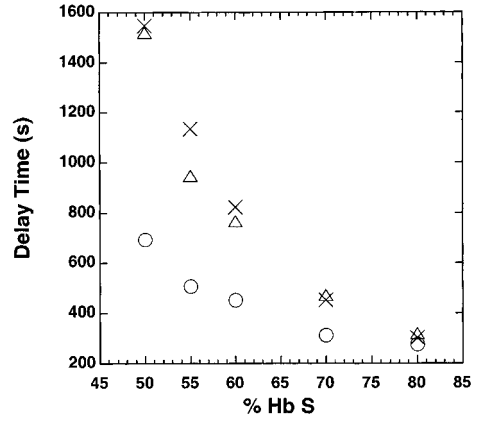

FIGURE 7: Delay time of polymerization as a function of Hb S concentration in the mixture of Hb S with Hb A (○), Hb F (×), and rHb Oscar (△). The final Hb concentration in the mixture was 0.2 g/dL. Time courses of polymerization were determined in 1.8 M phosphate buffer, pH 7.4, and at 30 °C.

DISCUSSION

rHb Oscar has eight mutations in the N-terminal region of the γ-chain corresponding to the sequence of the β-chain in that region. ^{1}H NMR studies show that rHb Oscar has tertiary structure around the heme pockets and quaternary structure in the $\alpha_1\gamma_1$ and $\alpha_1\gamma_2$ subunit interfaces similar to those of Hb F (Figures 2 and 3). Thus, the sequence of the N-terminal region of the γ-chain does not affect the overall structure of the molecule. It can be inferred from alkaline denaturation results for rHb Oscar that the substitutions in the N-terminal region do have an effect on the denaturation properties of Hb F. rHb Oscar is less resistant to alkaline denaturation than Hb F, though it is still much more resistant than Hb A (Table 3). Though the subzero IEF studies on rHb Oscar do not yield a clear value for the amount of hybrid Hb in mixtures with Hb S, they do indicate that rHb Oscar does not form more hybrid with Hb S than does Hb F (Table 4 and Figure 4). Studies of the delay time of gelation further show no improvement in the inhibition of gelation of Hb S by rHb Oscar compared to Hb F (Figure 7). These data suggest that the properties of rHb Oscar are more like those of Hb F than Hb A.

One of the most significant differences between Hb F and Hb A is that Hb F dissociates about 70 times less readily than does Hb A. In a structural comparison of Hb A and F, Frier and Perutz (*30*) found that the eight substitutions in the A helix of the γ-chains cause the A helix to move toward the E helix and away from the H helix, resulting in a tighter structure for deoxy-Hb F as compared with deoxy-Hb A. rHb Felix, which has eight mutations in the N-terminal region of the β-chain corresponding to the sequence of the γ-chain in that region, exhibits a tetramer−dimer dissociation constant similar to that of Hb F (*12*), suggesting that rHb Felix also has a tightened structure even though it has the Hb A amino acid residues at the $\alpha_1\beta_2$ and $\alpha_1\beta_1$ interfaces.

Comparison of the first 13 N-terminal amino acids of Hb A and Hb F shows that those on the γ-chain are in general bulkier than those on the β-chain. Dumoulin et al. (*12*) have suggested that the bulkier residues, γ3Phe and γ10Thr as compared to β3Leu and β10Ala, would make contact with helix H of rHb Felix and, in turn, push the A helix away from the H helix, leading to a tighter structure for rHb Felix. rHb Oscar does not form more hybrids with Hb S than does Hb F, indicating that rHb Oscar and Hb F have similar tetramer−dimer dissociation constants (*20*). Thus, amino acid substitutions in the N-terminal end of the A helix of Hb F alone are not sufficient to loosen the tetramer−dimer strength or stability of Hb F.

When considering the data of the delay time of deoxy-Hb S polymerization, one has to keep in mind that this kinetic assay as modified by Adachi and Asakura (*29*) was done at high salt buffer, generally in 1.8 M potassium phosphate buffer. It has been shown that the solubility of deoxy-Hb S or Hb mixtures in high ionic strength media does not consistently reflect their properties in more physiological conditions (*37*, *38*). This is, in part, attributed to the shielding effect of intermolecular electrostatic interactions that appear to be involved in deoxy-Hb S polymerization (*39*, *40*). The recent study of the pH dependence of the tetramer−dimer equilibrium shows that the 70-fold difference in the association equilibrium constant between Hb F and Hb A decreases progressively from pH 7.5 to pH 9.0 (*41*). This difference was attributed to the difference in the pK_a of 8.1 of γ1Gly compared to the pK_a of 7.1 of β1Val. The dextran-C_{sat} method (*42*) was, hence, applied to get more reliable data for rHb Oscar on inhibition of polymerization of Hb S in a near physiological condition, i.e., at 50 mM potassium phosphate and at pH 7.5. With 12 g/dL dextran, the solubilities in the mixture of 50% Hb S/50% Hb F and of 50% Hb S/50% rHb Oscar are 10.89 ± 1.04 g/dL [compared to 8.8 g/dL (*42*)] and 11.59 ± 1.07 g/dL, respectively (C.-H. Tsai and C. Ho, unpublished results). This suggests that the effect of rHb Oscar on inhibiting polymerization of Hb S is similar to that of Hb F.

Since the mutations in rHb Oscar do not lead to the formation of more hybrid with Hb S, other sites in the Hb F molecule will have to be considered. Intrinsic differences at the $\alpha_1\beta_2/\alpha_1\gamma_2$ and/or $\alpha_1\beta_1/\alpha_1\gamma_1$ interfaces are the likely sources for the increased stability of Hb F. Several investigators (*43*, *44*) have attributed the greater stability of Hb F compared with Hb A to increased electrostatic interactions as well as to increased hydrophobicity upon conversion of the deoxy to the oxy forms. There are only four substitutions between the β- and γ-chains at the $\alpha_1\beta_1$ interface (βP51A, βC112T, βH116I, βP125E) and one at the $\alpha_1\beta_2$ interface (βE43D). A recent study of rHb (βH116I) (*6*) showed that amino acid substitution with a more hydrophobic residue in the $\alpha_1\beta_1$ interface has a marginal effect on tetramer−dimer dissociation. rHb (βE43D), however, was shown to increase the tetramer−dimer dissociation of Hb A by 20 times (*6*), suggesting that increased electrostatic interaction by increasing net negative charges at the $\alpha_1\beta_2$ interface can enhance the tetramer strength of Hb A. Hence, it is reasonable to expect that replacement of γ43Asp with a less negatively charged residue, i.e., Glu, or a nonpolar residue, i.e., Leu, or a positively charged residue, i.e., Lys, may loosen the tetramer strength of Hb F and produce more hybrid with Hb S than does Hb F.

One of the significant differences between Hb F and Hb A is their response to the allosteric effector, 2,3-BPG (Table 2). According to the crystal structure and ^{31}P NMR studies, β143His is one of the residues involved in the binding of 2,3-BPG to Hb A (*45*). The lowered 2,3-BPG response in Hb F and the lack of a His residue in position 143 of the γ-chain have suggested an important role for β143His in the binding of 2,3-BPG to Hb A. However, recent ^{1}H NMR and oxygen-binding studies of rHb (βH143S) (*46*) have indicated that β143His is not essential for the binding of 2,3-BPG in the neutral pH range because of its very low pK_a values (pK = 4.7 in the deoxy form and pK = 5.7 in the CO form). Our present oxygen-binding studies on rHb Oscar, which has the same sequences in the 2,3-BPG binding sites as does Hb F, but responds to 2,3-BPG more than does Hb F (Table 2), are consistent with this conclusion.

Despite 39 amino acid residues of the β- and γ-chains being different, a comparison of the X-ray structures of deoxy-Hb A and deoxy-Hb F has shown that the only detectable differences in the tertiary structure between the β- and γ-chains appear in the two N-terminal segments (*30*). Because of the shift of the N-terminus due to the substitution of β3Phe for γ3Leu, an anion bound between β1Val and β82Lys in deoxy-Hb A is absent from deoxy-Hb F, and the distance between the two phosphate groups of 2,3-BPG and β2His is increased in deoxy-Hb F. Frier and Perutz (*30*), hence, suggested that such a shift of the N-terminus might contribute to the lower affinity of deoxy-Hb F for 2,3-BPG. The stronger 2,3-BPG effect in rHb Oscar and the lack of the bulkier residues in the N-terminus of rHb Oscar would, therefore, suggest a shorter distance between the two phosphate groups of 2,3-BPG and γ2His in rHb Oscar than in Hb F. In addition, the distance between γ1Gly→Val and γ82Lys is likely to be less in rHb Oscar than in Hb F, leading to the restoration of the anion effect as seen in the enhanced chloride effect of rHb Oscar as compared to Hb F (Table 2).

Both β1Val/γ1Gly and β2His/γ2His are located in the central cavity of the Hb molecule. The excessive positive charge in the central cavity has been suggested to play an important role in the oxygen-binding properties of the Hb molecule (*47*−*49*). It is known that increasing the positive charge distribution by amino acid substitution(s) in the central cavity can enhance the anion effect in Hb A (*25*, *48*). β1Val of Hb A has a pK_a value of 6.6, while γ1Gly of Hb F has a pK_a value of 8.1 in both the oxy and deoxy forms (*50*). Hence, rHb Oscar should have a more negatively charged N-terminal end than that of Hb F in the neutral pH range. Decreasing positive charges within the central cavity has been shown to reduce anion effects on oxygen-binding properties of Hb molecules (*25*). Thus, rHb Oscar would likely have a reduced anion effect rather than an increased one. The fact that rHb Oscar exhibits an increased anion effect (Tables 1 and 2) suggests that other factors, such as shortening the relative distances among the positively charged residues in the central cavity as described above, may compensate for this difference in the anion effect.

When considering the oxygen-binding data, it is important to note that the mass spectrometry of rHb Oscar indicated that about 12% of the samples still contained methionine

(Met) at the N-terminus of the mutated γ-chains. This extra Met residue is a consequence of expressing the Hb in *E. coli*, but it is usually removed efficiently by methionine aminopeptidase (*21*, *24*). The efficiency of removal of the extra Met residues in the α-, β-, and γ-chains of Hb by MAP is known to be sequence specific for the N-terminal amino acid residues (for a discussion, see ref *24*). Most rHbs expressed in our laboratory contain less than 5% of Hb with an extra Met, and this amount of extra Met does not affect the results of functional studies. A mutant of Hb A, rHb (β1Val + Met), which has an additional Met at the N-terminus, exhibits reduced response to 2,3-BPG and IHP in the CO-binding kinetic properties (*4*). As a corollary, the effect of 12% of Met at the N-terminus of rHb Oscar would likely be to decrease its response to 2,3-BPG and IHP. Thus, our values for the response of rHb Oscar to 2,3-BPG or IHP would likely be a lower limit for the actual value, not an upper limit. In conclusion, the mutations at the N-terminal region of the γ-chain are important for the 2,3-BPG and IHP response of Hb F.

rHb Felix, which has eight mutations in the N-terminal region of the β-chain corresponding to the sequence of the γ-chain in that region, exhibits a tetramer−dimer dissociation constant similar to that of Hb F, suggesting that the N-terminal sequence of the β-chain plays a role in tetramer−dimer dissociation of the Hb molecule. We hoped that rHb Oscar, which has eight mutations in the N-terminal region of the γ-chain corresponding to the sequence of the β-chain in that region, would exhibit dissociation properties similar to those of Hb A and, in turn, yield more hybrid in mixtures with Hb S than Hb F. The IEF and gelation studies of rHb Oscar have shown no more hybrid formed in mixtures with Hb S than Hb F, suggesting that the properties of rHb Oscar are not reciprocal to those of Hb Felix. Thus, the A-helix of the β-chain behaves differently in the environment of Hb F than in the environment of Hb A. The substitutions in the A-helix may still be important to the dissociation properties and oxygen-binding properties of Hb F, but they do not completely account for the differences observed in the properties of Hb F and Hb A when considered from the perspective of Hb F. It is clear that the relationship of the N-terminal region to the properties observed in Hb F is a complicated one. Future recombinant mutants of Hb F may shed more light on this problem, but a more thorough understanding is likely to come from studies of recombinant mutants of Hb F studied in conjunction with mutants of Hb A containing reciprocal mutations.

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