Sickle Hemoglobin Polymer Stability Probed by Triple and Quadruple Mutant Hybrids*

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As part of an effort to understand the interactions in HbS polymerization, we have produced and studied a recombinant triple mutant, D6A(α)/D75Y(α)/E121R(β), and a quadruple mutant comprising the preceding mutation plus the natural genetic mutation of sickle hemoglobin, E6V(β). These recombinant hemoglobins expressed in yeast were extensively characterized, and their structure and oxygen binding cooperativity were found to be normal. Their tetramer-dimer dissociation constants were within a factor of 2 of HbA and HbS. Polymerization of these mutants mixed with HbS was investigated by a micromethod based on volume exclusion by dextran. The elevated solubility of mixtures of HbS with HbA and HbF in dextran could be accurately predicted without any variable parameters. Relative to HbS, the copolymerization probability of the quadruple mutant/HbS hybrid was found to be 6.2, and the copolymerization probability for the triple mutant/HbS hybrid was 0.52. The pure quadruple mutant had a solubility slightly above that of its hybrid with HbS. One way to explain these results is to require significant cis-trans differences in the polymer and that HbA assemble above 42.5 g/dL. A second way to explain these data is by the modification of motional freedom, thereby changing vibrational entropy in the polymer.

Sickle cell disease is due to a single DNA base change encoding for the amino acid at the 6th position of the two β-globin chains of the hemoglobin tetramer. This surface mutation of glutamic acid to valine does not affect the oxygen carrying chains of the hemoglobin tetramer. This surface mutation of the 14-stranded polymers (4, 5).

Interactions of molecules within the 14-stranded structure are complex in many ways. First of all, the existence of two β and two α subunits means that there are two possible regions of interaction for each α or β amino acid when polymers form. Such differences are known as cis-trans differences and were first proposed to explain the unusual behavior of HbC-Harlem (6). Second, because there are so many different interactions possible within a 14-stranded polymer it is also possible that the interactions are different for the same amino acid in different strands. A proposal utilizing both of the above features has recently been put forward as a way to explain quantitatively the polymerization of HbS/HbA mixtures by having four molecules use the second β in a favorable contact (7). Third, even when an interaction is identified, the effect of a given amino acid on polymer stability may depend not only on its identity but also on the local environment. For example, it has recently been proposed that the mutation of β88 Leu → Ala increases solubility because of the tightness of fit of the mutation rather than the intrinsic hydrophobicity of the group (8). Finally, interactions at separate sites may not provide additive stability, even when the sites are physically well separated, as has been seen in a triple mutant involving the β68 sickle mutation plus β88 Leu → Ala and β95 Lys → Ile (9). In short, the interactions within the fiber are sufficiently subtle that it is of paramount importance to learn how different sites are able to contribute to polymer stability to have any hope of a rational approach to designing drugs for the treatment of this disease.

In disentangling such issues many strategies are possible, and no single approach is assured of success. In addition to pursuing the known contact sites within the double strand, another fruitful avenue is to expand upon previously known mutants to develop further the detailed knowledge of regions in which some understanding already exists.

Recently the systematic study of a series of recombinant sickle hemoglobin tetramers with substitution at one to three sites in addition to β6 has been reported (10). These three mutations (E121R/β, D75Y/α, and D6A/α) were all chosen because they are known to enhance polymerization (10–12), and yet the locations of the mutations were well separated to attempt to avoid concerted interaction among the sites.

Given the enhanced polymer stability brought about by the three mutation sites (E121R/β/D75Y/α/D6A/α), the immediate question was whether the enhancement could overcome the resistance to assembly posed by the presence of Glu at β6. The study of these mutants is all the more interesting because the structural basis of their enhancement is somewhat obscure, using presently accepted models for the HbS polymer (13, 14), despite the fact that some of the mutants have been previously studied.
To probe gelation strength in these experiments, solubility was measured in a newly developed assay that replaces an amount of scarce mutant Hb with dextran, thereby crowding the solution and promoting assembly using only small quantities of the hemoglobin mutant (15). With 12 g/dl dextran, HbS has a solubility of 35.3 mg/ml, the mutant HbS/E121R(β) gels at 24.3 mg/ml, the mutant HbS/E121R(β)/D75Y(α) gels at 11.8 mg/ml, and the quadruple mutant HbS/E121R(β)/D75Y(α)/D6A(α) gels at a mere 7.0 mg/ml. This enhancement of polymerization is consonant with the behavior of these sites in the SAD mouse construct in which the combination of HbS, Hb-Antilles (E23I(β)), and HbD Punjab (E121Q(β)) are expressed to produce an important research tool, i.e., a sickling mouse model (16). In previous studies it was found that the solubility of the above quadruple mutant was quite close to the solubility of HbSAD, which is 6.1 mg/ml.

A fruitful approach in understanding the behavior of natural mutants has been to study their polymerization when mixed with HbS. This permits their hybridization by the spontaneous dissociation of the hemoglobin tetramer into its constituent dimers (4). This approach is applicable here since, as we show, the tetramer-dimer dissociation constants of the recombinant mutants are close to that of HbS. Particularly when the hybrids can be compared with the simultaneous presence of all the sites, the important cis-trans issues can be explored. In this work we report the polymerization of a triple mutant (α6, α75, and β121) in hybrids with HbS. We contrast this with the polymerization of a quadruple mutant (the triple mutant plus the β6 mutation of HbS) mixed with HbS. By determining solubilities at a variety of fractional mixtures, the adequacy of the description of the mixtures can be tested, and the relative probability of copolymerization can be determined. The latter is directly related to the free energy difference of the hybrid relative to HbS.

**EXPERIMENTAL PROCEDURES**

**Reagents and Plasmids**—The restriction endonucleases, alkaline phosphatase, and DNA ligase were from Roche Molecular Biochemicals or New England Biolabs. The DNA sequencing kit and the PCR reagent kit were obtained from United States Biochemical Corp. The GeneClean kit was from BIO 101, Inc. The plasmid kit was from Qiagen. The α5S-labeled dATP was from Amersham Biosciences. Dextran and DPG1 were purchased from Sigma. The nucleotides used to make the mutations were synthesized by the protein sequencing facility at Rockefeller University. The construction of pGS189 and pGS389 plasmids has been described elsewhere (30, 31). All other reagents were of the highest purity available.

**Construction of the Triple Mutant D6A(α)/D75Y(α)/E121R(β)—** From the recombinant plasmid of the quadruple mutant D6A(α)/D75Y(α’)/E56V(β’)/E121R(β’), pAD190 (17), the formation of the triple mutant was excised and used to replace the XhoI site of pGS389 to give the recombinant plasmid pGS389(α6α75/β121β6).

**Yeast Expression System and Purification of Hemoglobin Mutants—** Recombinant pGS389(α6α75/β121β6) was transformed into Saccharomyces cerevisiae GS112 citr strain using the lithium acetate method described previously (17). The transformants were selected using a complete medium first without uracil and then without uracil and leucine. To express the D6A(α)/D75Y(α)/E121R(β) triple mutant, the yeast strain was grown in 12 liters of yeast extract plus peptone medium in a New Brunswick Fermentor Bioflo IV for 3 days using ethanol as a carbon source. The promoter controlling the transcription of the globin genes was induced for 20 h by adding galactose (Pfanstiehl or United States Biochemical Corp., containing <0.1% glucose) to a final concentration of 3%. The collection and breakup of the cells after bubbling with CO gas and purification have been described previously (17).

**Analytical Methods—** SDS-PAGE of the recombinant mutants was performed on the Phast System from Amersham Biosciences. The protein bands were stained with Coomassie Brilliant Blue R-250. Isoelectric focusing was performed on the pH 7–10 Hb-Resolve system from Isolab. The α- and β-globin chains from recombinant hemoglobins were separated by reverse phase HPLC on a Vydac C18 column with a gradient of 20–60% acetonitrile containing 0.1% trifluoroacetic acid. Amine acid analysis of globin chains isolated by this procedure was performed on a Beckman 6300 instrument with a System Gold data handling system. The spectrum of each mutant was recorded on a Shimadzu 1601 UV-visible spectrophotometer.

**Mass Spectrometry Analysis—** Electrospray mass spectrometric analysis of the purified recombinant mutant Hb tetramers was performed with a Finnigan-MAT TSQ-700 triple quadrupole mass spectrometer as described previously (10).

**Functional Studies—** The oxygen binding curves of the hemoglobins were determined at 37 °C on a modified Hem-O-Scan instrument (Aminco). Before the measurements, the purified Hb sample was dialyzed in 50 mm bis-Tris, pH 7.5, and converted to the oxy form. These samples were concentrated using Centricon, Centiprep, MicroCon ultrafiltration devices (10,000 molecular weight cut-off, Amicon) to a final concentration of 0.6 m M. To measure the effect of anions on the oxygen affinity of these hemoglobin mutants, an aliquot of a solution of 5.6 mm DPG or 2.5 m NaCl in 50 mm bis-Tris, pH 7.5, was added to the Hb sample to achieve the desired final concentration.

**Measurement of Gelation Concentration (Cm)—** The gelation concentration of the hemoglobin mutants or hemoglobin mixtures was determined by the "Dextran-Csat" micromethod as described previously (15). The concentrated hemoglobin sample in the oxy form in 50 mm potassium phosphate buffer, pH 7.5, was mixed with dextran at a final concentration of 120 mg/ml. Mineral oil was layered on top, and fresh sodium dithionite solution (50 mm final concentration) was added anaerobically below the Hb/dextran mixture using a gas-tight syringe. After stirring and incubation for 30 min in a 37 °C water bath, the resulting gel under the oil layer was disrupted with a narrow wire loop, and the tubes were centrifuged in a microcentrifuge for 30 min. The clear supernatant was carefully separated from the aggregated Hb, and the hemoglobin concentration was measured spectrophotometrically and verified by amino acid analysis after acid hydrolysis of an aliquot.

**Copolymerization Theory—** The analysis uses a modified form of the extensively used copolymerization theory (5, 18). The solubility observed, cS, is related to the solubility of pure HbS, denoted cS. Because of reproporation among the subunits, if the additive species is a fraction X of total molecules, then, (1 – X) is pure HbS molecules, 2X(1 – X) are hybrids, and X2 are pure tetramers of the additive species. These fractions are denoted X1, X2, and X3, respectively. Each of these species copolymerizes with a probability denoted εi, which is related to the free energy difference between having HbS polymerized and the ith species polymerized. By this definition, copolymerization probability εi is identically 1 for the case of hypothetical “hybrids” of HbS with HbS, i.e., HbS mixed with itself. Then using Equations 22–25 of Eaton and Hofrichter (5) it is straightforward to show that if εi is the initial concentration, cS is the concentration of the pure polymer, εi is the specific volume of the monomer, cS is the concentration of the dextran, and vi is the specific volume of the dextran, then

\[ \frac{X_{S}}{X_{S} - c_{S}} = \frac{X_{i}}{X_{i} - c_{i}} \]

\[ \frac{\chi}{\chi_{S}} = \frac{1 + \epsilon_{i}}{1 + \epsilon_{i} - \epsilon_{i} - c_{i}} \]

where \( \chi \) is the activity coefficient of the monomers at solubility, and \( \chi_{S} \) is the activity coefficient of monomers at cS. The expression for \( \Gamma \) has been modified from that used in the footnote to Table III.2 of Eaton and Hofrichter (5) to include the volume exclusion due to the dextran in which the solubility is measured. The volume of the dextran molecules has been taken here as 0.664 m M as measured directly by Bookchin et al. (17). This is almost equal to the Hb molar specific volume v.1

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1 The abbreviations used are: DPG, 2,3-diphosphoglycerate; bis-Tris, 2-[bis(2-hydroxyethyl)aminol]-2-hydroxyethyl)propane-1,3-diol; HPLC, high pressure liquid chromatography; FPLC, fast protein liquid chromatography.
Activity coefficients are calculated based on scaled particle theory, described by Minton (19), using the volumes described above and assuming spherical particles. Because the first equation cannot be simply inverted to give an expression of $c_+$, it is solved numerically.

**Circular Dichroism Measurements**—The CD spectra of HbA and recombinant mutants were measured on a Jasco J-715 spectropolarimeter at 25 °C as described previously (10).

**Measurement of Tetramer-Dimer Dissociations**—This measurement was performed on the CO-ligated recombinant hemoglobins on a Superose-12 HR 10/30 column using an Amersham Biosciences HPLC system (20). The absorbance of the eluent was measured at 405 nm with the Amersham Biosciences on-line mercury lamp detection system with a 5-mm flow cell. For the double mutant D6V/β/E121R(β), $K_D$ was calculated as described previously using the parameters ($V_0$, $V_t$) that were determined from the standard dimeric Hb Rothschild and tetrameric cross-linked Hb (20). For recombinant mutants D75Y(α)/E6V(β)/E121R(β), D6A(α)/D75Y(α)/E6V(β)/E121R(β), and D6A(α)/D75Y(α)/E121R(β), the elution volume ($V_t$) at low or high concentrations was not within the standards presumably due to the interaction of their strong positive charges with Superose-12 column. Thus, their $K_D$ values were calculated by curve fitting the experimental volume data allowing $V_0/V_t$ to vary (10).

**RESULTS**

**Purification and Characterization of D6A(α)/D75Y(α)/E121R(β)**—The recombinant mutant D6A(α)/D75Y(α)/E121R(β) lacking the sickle mutation was expressed in yeast and purified by CM-cellulose 52 chromatography (9, 10, 17, 21). Its purity was verified by chromatography on an Amersham Biosciences FPLC Mono S column. It showed one band upon isoelectric focusing in the pH 6–8 Hb-Resolve system from Isolab, and its migration was consistent with the loss of four negative charges per αβ subunit compared with HbA (Asp → Ala and Asp → Tyr) and a substitution equivalent to the loss of their strong positive charges with Superose-12 column. Thus, their $K_D$ values were calculated by curve fitting the experimental volume data allowing $V_0/V_t$ to vary (10).

**HPLC Analysis and Amino Acid Analysis**—The purified recombinant HbS mutants were analyzed by reverse phase HPLC using a denaturing solvent to separate the globin chains (Fig. 2). The β-chain containing a substitution of Arg for Glu at β121 eluted at 30.5 min ahead of the normal β-chain from HbA (35.0 min). The elution position of the α-chain from D6A(α)/D75Y(α)/E121R(β) coincided with the α-chain from natural HbA (43.2 and 42.7 min, respectively), indicating that the replacement of the hydrophilic residue Asp at the α-chain by nonpolar amino acids Ala and Tyr does not alter their chromatographic behavior significantly on the reverse phase C_4 column. Amino acid analysis of each isolated subunit gave the expected composition (Table 1). For the mutated β-chain containing Glu-121(β) → Arg and mutated α-chain containing Asp-6(α) → Ala and Asp-75(α) → Tyr, the values for Arg, Glu, Asp, and Ala were in reasonable accord with theoretical values. The values for the other amino acids were also in good agreement with the known composition of this mutant.

**Mass Spectrometry**—Mass spectrometric analysis of the mutant D6A(α)/D75Y(α)/E121R(β) by the electrospray ionization mass spectrometry method gave the expected mass for both α and β subunits. The molecular mass of the β-chain containing
Glu-121(\beta) \to \text{Arg} agreed with the expected mass. The mass difference from the sickle \(\beta\)-chain was within experimental error of the expected difference of 27 Da between a Glu (129 mass units) and an Arg (156 mass units). The measured molecular mass for the \(\alpha\)-chain of D6A(\alpha)/D75Y(\alpha)/E121R(\beta) was also consistent with the calculated value within experimental error.

Circular Dichroism—The circular dichroism spectra of the recombinant hemoglobins including natural HbA, E6V(\beta)/E121R(\beta), D6A(\alpha)/D75Y(\alpha)/E6V(\beta)/E121R(\beta), and D6A(\alpha)/D75Y(\alpha)/E121R(\beta) in liganded forms were analyzed and com-

**Fig. 3. CD spectra of HbA and recombinant hemoglobins.** Top, far UV CD spectra. Measurements were made by using a 1-mm quartz cell, and the samples were in 50 mM phosphate buffer, pH 7.0, at 25°C. The protein concentration was 1 \muM. Bottom, CD spectra in the Soret region. Measurements were made using a 10-mm quartz cell with conditions of the sample otherwise as given for the top panel. deg, degrees.
pared with that of HbA (Fig. 3). In the far ultraviolet region from 200 to 250 nm, comparison of all five spectra indicated that their CD profiles were practically identical showing that no change occurred in helical content and showing that there were no adverse effects of the mutations on their secondary structures. In the Soret region, the maximum ellipticity at 414 nm was identical to that of HbA. The minor differences at 412 nm observed between the individual Hbs were no greater than the differences between the natural HbA and HbS. Thus, the overall features of these CD curves are identical to those of native HbA, indicating no adverse effects on the heme pocket by the amino acid replacements.

Tetramer-Dimer Dissociations—Since these recombinant hemoglobins have quite different surface charges than HbA and HbS, their tetramer-dimer dissociation was investigated. The two other previously reported recombinant HbS hemoglobins, E6V/E121R and D75Y/E6V/E121R, were also included for comparison. For the double mutant E6V/E121R, $K_d$ was calculated using the GraFit program as described previously (20) using the parameters ($V_d$ and $V_t$) that were determined from the standard dimeric Hb Rothschild and tetrameric cross-linked Hb (Fig. 4A). The $K_d$ values of the double and triple mutants were calculated by curve fitting the experimental volume data, allowing $V_d/V_t$ to vary (Fig. 4, B–D) (20).

As shown in Table II, the tetramer-dimer dissociation constants for the liganded recombinants were in the range of 0.3–1.0 μm, which are the same within experimental error for native HbS ($K_d = 0.4$ μM) (Table II). These results show that the amino acid replacements in all these recombinants have no significant effect on the dissociation behaviors at the $\alpha_1\beta_2$ interfaces in the liganded hemoglobins.

Functional Studies—The oxygen binding properties of the mutant D6A/D75Y/E6V/E121R were determined at a hemoglobin concentration of 0.6 mM (Table III). Its $P_{50}$ is 6 mm Hg with a Hill's coefficient ($n$) value of 2.8 compared with a value of 5 mm Hg and $n = 2.8$ for D6A/D75Y/E6V/E121R under the same conditions with a 2-fold higher value of $P_{50}$. The D6A/D75Y/E6V/E121R shows a somewhat decreased response to chloride similar to that of D6A/D75Y/E6V/E121R. These results suggest that the replacement of the amino acid from Glu to Val at position 6 of D6A/D75Y/E6V/E121R does not affect the oxygen affinity.

Analysis of Polymerization Data—The mixtures of HbS with HbF and HbA provide valuable control experiments for analysis using the dextran assay as employed here. HbF does not polymerize of its own (i.e., $e_2 = 0$) nor does it copolymerize (i.e., $e_2 = 0$). This allows the solubility data for the HbS/HbF mixtures to be compared with a prediction from the theory since there are no unknown parameters (5). Fig. 5 shows the comparison of the theory with the solubility data. The HbS/HbF
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**Table II**

| Hemoglobin                  | $K_d$ |
|-----------------------------|-------|
| HbA                         |       |
| E6V($\beta$) (HbS)          | 0.68 ± 0.05$^a$ |
| E6V($\beta$/E121R($\beta$) | 0.42 ± 0.03$^b$ |
| D75Y($\alpha$/E6V($\beta$/E121R($\beta$) | 1.04 ± 0.11$^c$ |
| D6A($\alpha$/D75Y($\alpha$/E6V($\beta$/E121R($\beta$) | 0.31 ± 0.07$^d$ |
| D6A($\alpha$/D75Y($\alpha$/E121R($\beta$) | 0.35 ± 0.04$^e$ |
| D6A($\alpha$/D75Y($\alpha$/E121R($\beta$) | 0.69 ± 0.24$^f$ |

$^a$ Values from Ref. 28.  
$^b$ Values from Ref. 20.  
$^c$ Calculated using the parameters ($V_v$ and $V_t$) that were determined from the standard dimeric Hb Rothschild and tetrameric cross-linked Hb.  
$^d$ Calculated by curve fitting the experimental volume data with $V_v/V_t$.  
$^e$ Values from Ref. 20.  
$^f$ Calculated by curve fitting the experimental volume data with $V_v/V_t$, allowed to vary.

**Table III**

Functional properties of natural HbS and recombinant hemoglobin mutants

The procedures used to calculate the $P_{o,a}$ and $n$ values are described in the text, and the Hb concentration for oxygen dissociation curves was 0.6 mM in 50 mM bis-Tris, pH 7.5.

| Hb                | Additive | $P_{o,a}$ | $n$ |
|-------------------|----------|-----------|-----|
| D6A($\alpha$/D75Y($\alpha$/E6V($\beta$/E121R($\beta$) | 0        | 5   | 2.8 |
| 1.2 mM DPG        | 9        | 2.8 |
| 0.5 mM NaCl       | 7        | 2.8 |
| D6A($\alpha$/D75Y($\alpha$/E121R($\beta$) | 0        | 6   | 2.8 |
| 1.2 mM DPG        | 11       | 2.7 |
| 0.5 mM NaCl       | 8        | 2.7 |

$^a$ Values from Ref. 10.

**DISCUSSION**

Functional Character of the Mutants—Since these recombinant hemoglobins have increased positive charge at the protein surface, circular dichroism and tetramer-dimer dissociation were measured to ensure that the overall conformation and tetramer stability of these recombinant hemoglobins was not compromised. The circular dichroism results indicate that overall structures of these recombinant hemoglobins expressed in yeast were practically the same as those of natural Hb. The tetramer-dimer dissociation studies suggested that the amino acid replacements in all these recombinants have no significant effect on the strength of the interaction at the $\alpha_2\beta_2$ interfaces. Therefore, we conclude the effect of the amino acid substitutions in the recombinant hemoglobin on the polymerization process arises from tetrameric interactions.

**Cis-Trans Effects on Polymerization**—It is completely unexpected that the hybrid quadruple mutant polymerizes with almost the same probability as the full mutant. One would have expected, given the polymerization of the hybrid, that the addition of sites of favorable interaction in the full quadruple mutant would significantly increase the net strength of interaction. Clearly if the mutant sites on both halves of the molecule interact with their neighbors within the polymer the hybrid should have only half the interaction strength. It might seem that the present result could be achieved if only one-half of the tetramer sites were engaged or if the其它 sites within the polymer interact much like the $\beta 6$ site itself. However, since the hybrids would have two ways to be positioned within the polymer, the probability would be reduced (just as seen for HbA/HbS hybrids).

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**Note:** The text refers to various scientific studies and experiments involving hemoglobin mutants and their interactions, including polymerization and dissociation constants. The tables and figures provide specific data points and methodologies used in these studies. The discussion section highlights the functional characteristics of the mutants and the implications of their interactions at various interfaces, emphasizing the complexity and variability in hemoglobin behavior.
short, having only half the mutation sites potent does not solve the dilemma.

The preceding example in which one-half of the sites are ineffectual is a special case of the situation where the site interactions are asymmetric. Such an idea was first proposed to describe the polymerization of HbC-Harlem (6). It is possible to treat the asymmetric (cis-trans) case in general. Suppose that in the full quadruple mutant, although both sets of mutation sites interact with the polymer, they do so with different strength, labeled $\Delta G_+$ and $\Delta G_-$. In the full mutant since both sites are involved in the net stability, the net change in free energy is $\Delta G_+ + \Delta G_-$. In the hybrid, either one configuration ($\Delta G_+$) or the other ($\Delta G_-$) is active; the net probability is the average of the two possible configurations. In this explanation, it is a coincidence that the net probability for copolymerizing the hybrid, $(\exp(\Delta G_+/RT) + \exp(\Delta G_-/RT))/2$, is about the same as that for the copolymerization of full mutant, $\exp(\Delta G_+ + \Delta G_-)/RT$. Following this approach, a consistent set of values can be found with probabilities of 11.8 and 0.51 implying a favorable interaction energy of 1.47 kcal/mol and an unfavorable energy (penalty) of $-0.40$ kcal/mol (The details are described under Appendix.

The structural implication of this view is that one set of contacts stabilizes the polymer, while the same mutation sites on the other side of the molecule make a second set of contacts that weakly destabilize the polymer.

These conclusions then have implications for analysis of the triple mutant. The diminished likelihood for polymerization of the triple mutant hybrid is the result of competition between the repulsive effects of burial of $\beta 6$ Glu and the attractive strength of the three other mutation sites. Since the strength of the attraction is known from the quadruple mutant analysis above, the strength of the repulsion can be determined (as discussed in detail under “Appendix.”). This analysis directly gives the probability of positioning Hb so that $\beta 6$ Glu is in the receptor pocket as 0.045. Although this probability is small, it translates into an effective solubility of 42.5 g/dl for HbA, which is an accessible concentration and one found in some erythrocytes. Very few experiments have directly probed the possibility that HbA can polymerize in low phosphate buffers. In one study, using the Bencs oxygen equilibrium method, no effects on $P_{50}$ were seen on HbA to concentrations around 50 g/dl (21). However, it might be argued that if the kinetics are sufficiently slow, such a scanning method might miss the effect of polymerization. On the other hand, there are reports of the assembly of HbA in high phosphate buffers (22). In this case it should also be noted that these aggregates do not show polymers in differential interference contrast microscopy. In short, the evidence is inconclusive as to whether polymers form in HbA at such concentrations as required by the cis-trans analysis.

Vibrational Entropy—The nonadditivity of the interactions can be explained in another way that does not invoke asymmetry. In the assembly of HbS to form fibers, a significant entropic penalty (35.5 kcal/mol) is incurred by the removal of hemoglobin monomers from solution in which they could freely rotate and translate (23). The stabilization from the various intermolecular contacts is nowhere near this great, and assembly would be thermodynamically forbidden without the declaration of lost entropy by vibrations of the molecules about their equilibrium positions in the polymer structure. Such redeclared entropy is thought to account for about 26.5 kcal/mol (24). Polymer stability thus arises from the additive contributions of contact energy and the free energy due to recovered vibrational motion. These can be competitive with a stronger bond resulting in greater localization of the molecule within the aggregate, leading to lessened vibrations. Such an effect is believed to be operative in HbC-Harlem (29). Thus the similarity of the copolymerization probability for the quadruple mutant and its hybrids with HbS may be the consequence of such an interplay. The strengthened contacts in the quadruple mutant in this explanation have been offset by a somewhat diminished vibrational entropy because of the multiplicity of contact points. In the case of the mutant hybrids, the molecules are somewhat freer, albeit less strongly held. If this explanation is correct, it should make a noticeable effect on the kinetics of polymerization of the quadruple mutant. In fact there is preliminary evidence for such a kinetic effect.3

Nonadditivity has been seen previously in the triple mutant E6V($\beta$)/L88A($\beta$)/K95I($\beta$) relative to the constituents E6V($\beta$)/L88A($\beta$) and E6V($\beta$)/K95I($\beta$) (9). In that case, both double mutants significantly increased the solubility of HbS, but the triple mutant had the same elevated solubility as E6V($\beta$)/K95I($\beta$). An analysis of the mutant E6V($\beta$)/L88A($\beta$) revealed that the enhancement of the solubility was due to vibrational restriction since the contact energy was the same as that of pure HbS as determined by kinetics and by modeling of the structure (8). On the other hand, the mutation of K95I replaces a charged group, and the result is expected to involve changed contact energy as well as vibrational entropy. Given that, it is plausible that the restriction of molecular motion that occurs in the K95I mutation involves the same restriction as that of the L88A mutation. Hence there would be no added effect of the L88A site since, in effect, its mode of action has already been preempted by the K95I mutant.

Structural Interpretation—Finally we turn to the question of how to understand the enhancements observed in terms of fiber structure. The $\alpha 6$ mutation known as HbSarawa ($\alpha 6$ Asp $\rightarrow$ Ala) is found to create a lower solubility tetramer (12.0 versus 14.7 g/dl) when combined with $\beta 6$ subunits (11) in addition to its enhancement as a part of the mutants discussed here (10). Nevertheless, the $\alpha 6$ site has no nearby polymer contacts within 5Å on adjacent tetramers. This mutation may engender some small changes internal to the tetramer (25). This is borne out by the observation that this mutation raises oxygen affinity ($P_{50}$ decreases by about 40%), although its cooperativity, as measured by the Hill $n$ value, is essentially unchanged (10, 11, 26).

The $\alpha 75$ mutation ($\alpha 75$ Asp $\rightarrow$ Tyr), known as Hb Winnipeg, decreases solubility even more than the $\alpha 6$ mutation (again when combined with $\beta 6$ subunits) in addition to the effects seen here. For this site the solubility drops to 7.8 versus 14.7 g/dl (11). $\alpha 75$ has an extensive set of contacts in the polymer. The naturally occurring Asp contacts attractive positive charged groups (Lys at $\beta 66$, $\beta 144$, and $\alpha 60$) as well as repulsive groups (Asp at $\beta 21$, $\beta 73$, and $\alpha 64$). Hence it is plausible that placement of a Tyr at $\alpha 75$ would not sacrifice much, if any, charge-charge stability, whereas it would gain stability from contact with several hydrophobic groups ($\beta 70$ Ala, $\beta 88$ Leu, $\beta 91$ Leu, and $\beta 145$ Tyr) as well as its contact with $\beta 19$ Asn and $\beta 87$ Thr.

Mutations at $\beta 121$ are the most well known of the three sites studied. Hybrids of HbS with HbD Punjub (HbD Los Angeles) ($\beta 121$ Glu $\rightarrow$ Gln) or HbO Arab ($\beta 121$ Glu $\rightarrow$ Lys) enhance polymerization (with an effect trans to the $\beta 6$ in the contact site). The double mutant HbS plus $\beta 121$ Glu $\rightarrow$ Arg also favors polymerization (10). At first sight the effects of the mutation on polymerization might seem plausible since $\beta 121$ is involved in several contacts along the double strand axis (3). However, the details prove to be problematic. The native, negatively charged

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2 K. Adachi, personal communication.

3 Z. Hu and F. A. Ferrone, unpublished results.
Glu is close to the positive charge of Lys-17 in both cis and trans position. The replacement Arg or Lys β121 thus makes an unfavorable contact with these Lys amino acids. β121 is also close to β116 and 117 in the trans position from which repulsion would be expected between His (116 and 117) and Lys or Arg. The remaining residues within 5 Å of the Arg at β121 are β16 Gly, β13 Ala, and β114 Pro. Thus it is difficult to understand in simple structural terms the origin of the enhanced polymerization brought about by the substitution at β121. In passing it should be noted that β23 is also thought to have interactions with β116 and 117 (27). The possibility that a mutant containing both β23 and β121 would exhibit mutual interactions through the intermediary of β116-117 made it less desirable for the present study to combine both β23 and β121 in the same mutant.) In sum, while there is no question that these mutations enhance polymerization, only changes at α75 are readily reconciled with the established polymer structures.

Vibrational entropy might provide a way to reconcile structural data and the unambiguous stabilization of the polymer. The ionic interactions that appear to be present in the native forms at α75 and β121 in HbS could serve more to limit flexibility (by specificity of contacts) rather than to add much stab-

bility by the strength of the contacts. The latter is especially pertinent if internal water can substitute for the ion pair lost in the mutation. On the other hand, in the mutant, the repulsive interactions add flexibility in positioning the molecules within the polymer and thus paradoxically make an entropic contribution to stability. The most interesting case would be that of α6 since the native Asp is thought to form hydrogen bonds with two Ser residues, α1 and α7. The loss of these hydrogen bonds is thus thought to loosen the Hb structure (25).

Summary—It is of course possible that part of the stability gain is vibrational and part arises from, e.g., the successful burial of Tyr α75. If vibrational entropy is indeed a cause of the nonadditivity seen, it points to the necessity of probing the details of the thermodynamics of assembly by other means than solubility alone since a single measurement is incapable of separating the contact energy terms from the vibrational terms. Kinetic measurements are one way of doing this since equilibrium nucleation theory is successfully used to describe the nucleation process, and the nuclei have a different balance of energetic terms than does the infinite polymer. It may also be possible to discern such entropic effects by the direct observation of polymer motion; such studies are presently underway. Regardless of whether an unexpected assembly of HbA or the vibrational motion of Hb polymers proves to be the correct explanation, the value of performing such hybrid experiments is clear in exposing the intricacies of the assembly process in HbS.

APPENDIX

We denote by subscript λ each unique configuration of the mutant relative to receptor. Then for the mutant there is an energy difference ΔG i relative to having HbS in the same configuration. Hence for each λ the microscopic copolymerization probability $\tilde{e}_\lambda$ may be defined as

$$\tilde{e}_\lambda = \exp(\Delta G_i / RT).$$  \hspace{1cm} (Eq. A1)

If $n_\lambda$ is the relative frequency of each of these unique energy states then

$$e_i = \frac{\sum_\lambda n_\lambda \tilde{e}_\lambda}{\sum_\lambda n_\lambda} \hspace{1cm} i = 1, 2, 3$$  \hspace{1cm} (Eq. A2)

The copolymerization probability $e_i$ is the quantity directly inferred from the observation of copolymerization. This differs from the microscopic probability $\tilde{e}_\lambda$ that was encountered by the molecular species. Typically $\Sigma n_\lambda$ is the total number of sites (14 strands) times the number of alternatives, 2, for a total denominator of 28. To illustrate, consider the copolymerization of HbA first in a fiber in which all sites are equivalent. There are two possible orientations of an HbA/HbS hybrid. In one orientation the β6 Val contacts its receptor site, and the β6 Glu remains solvated. Labeling that case as 1, $\tilde{e}_1 = 1$ so that the probability of that microstate is the same as in HbS. The reverse case, in which the Glu is placed into the hydrophobic pocket, will have a large energetic penalty. Labeling that configuration as 2, with a large penalty it follows that $\tilde{e}_2 \rightarrow 0$, and the probability of that microstate is very small. Therefore the net observed copolymerization probability for the hybrid is $e = (14 \times 1 + 14 \times 0)/(14 + 14) = 0.5$. Recently it has been proposed that four outer strands involve both β6 sites, which causes a different weighting (7). For that case, the energetic penalties give microprobabilities of 1 or 0 again (since if one had contact makes the probability almost 0, two such contacts will be indistinguishable). Of the 28 β6 sites, 18 have effectively 0 probability,
while now only 10 states are unpenalized. For this model, \( e_2 = (10 \times 1 + 18 \times 0)/28 = 0.357 \).

We turn to the analysis of the data at hand. For the hybrids of the quadruple mutant and HbS, the added mutations can either be distal (trans) or proximal (cis) to the active (i.e., buried) \( \beta \) site so that both possibilities must be considered in the sum. Let the two possibilities be denoted \( \tilde{e}_+ \) and \( \tilde{e}_- \). Then the hybrid has a measured copolymerization probability of \( e_2 = (\tilde{e}_+ + \tilde{e}_-)/2 \). The probability for the full, unhybridized mutant is \( e = \tilde{e}_- \tilde{e}_+ \). (The probabilities multiply since the free energies are additive.) From these equations, it is possible to solve uniquely for \( \tilde{e}_+ \) and \( \tilde{e}_- \). We take \( \tilde{e}_- \) as the smaller value.

To describe the triple mutant, we modify the quadruple mutant description by the addition of a \( \beta \) Glu site, which includes a penalty for polymerization. If the copolymerization penalty when \( \beta \) Glu is buried is denoted as \( e_X \), then there are two possibilities as shown in Fig. 6. The penalty may accrue for \( \tilde{e}_+ \) or \( \tilde{e}_- \), i.e., we may have either \( \tilde{e}_+ \tilde{e}_- \) or \( \tilde{e}_- \tilde{e}_X \). Now we can have either \( e_2 = (\tilde{e}_+ \tilde{e}_X + \tilde{e}_- \tilde{e}_X)/2 \) or \( e_2 = (\tilde{e}_+ + \tilde{e}_- \tilde{e}_X)/2 \). In addition, it is known from HbA/HbS polymerization experiments that \( \tilde{e}_X \) must be small. Because of this requirement, it is apparent that the second option above becomes \( e_2 = (\tilde{e}_+ + \tilde{e}_- \tilde{e}_X)/2 \sim \tilde{e}_x/2 \), which the data excludes. Hence the triple mutant must be analyzed by solving \( e_2 = (\tilde{e}_+ \tilde{e}_X + \tilde{e}_- \tilde{e}_x)/2 \).

Note that each time a probability product is generated the entropy arguments circumscribe.

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