Characterization of Two Types of Fetal Hemoglobin: \( \alpha_2^6\gamma_2 \) and \( \alpha_2^3\gamma_2 \)

By Kazuhiko Adachi, Jungyop Kim, Toshio Asakura, and Elias Schwartz

The effect of differences in \( \gamma \) and \( \epsilon\gamma \) fractions of fetal hemoglobin (HbF) on the kinetics of polymerization of HbS-HbF mixtures was studied. We also examined their effect on oxygen affinity, surface hydrophobicity, mechanical stability, and solubility of HbF. Differences in \( \gamma\epsilon\gamma \) ratio did not affect the polymerization of mixtures of HbF and HbS, suggesting that the inhibitory effect of HbF on the polymerization of HbS is independent of the \( \gamma\epsilon\gamma \) ratio of HbF and is totally dependent on the fraction of HbF in the mixture. The oxygen equilibrium curve of HbF was not affected by differences in the ratio of \( \gamma \) and \( \epsilon\gamma \) in HbF. In contrast, surface hydrophobicity, mechanical stability, and solubility of HbF were affected by differences in the \( \gamma\epsilon\gamma \) ratio.

**FETAL HEMOGLOBIN (HbF)** contains two types of \( \gamma \) chain that differ in the amino acid residue at the 136th position: one type has alanine \( (\alpha) \) as its residue and the other, glycine \( (\epsilon\gamma) \). There are usually two \( \gamma \)-chain genes per chromosome, and the \( \gamma \) chain and \( \epsilon\gamma \) chains are the products of nonallelic tandem genes. HbF isolated from red blood cells of newborns contains about 70\% \( \gamma \) chain and 30\% \( \epsilon\gamma \) chain, a proportion that is constant throughout fetal development.

In contrast, the small amount of HbF found in adult red blood cells contains about 40\% \( \gamma \) chain and 60\% \( \epsilon\gamma \) chain. The transition from the fetal \( \gamma\epsilon\gamma \) ratio to the “adult” ratio takes place during the first 10 months of life.

Clinical severity in sickle cell disease is quite varied. The concept that elevation of HbF should favorably influence the kinetics of polymerization of sickle hemoglobin.

**EXPERIMENTAL PROCEDURES**

Hemoglobin S was purified from sickle cell trait hemolysates (HbA and HbS) by CM Sephadex column chromatography (Sigma, St Louis, MO). Hemoglobin F with only \( \alpha_2\gamma \) was isolated from a patient with HbS\(^a\) hereditary persistence of fetal hemoglobin (HPFH) who has a HbF level of 28\% and only \( \alpha_2\gamma \) present and hemolysate from sickle cell patients with fetal hemoglobins having various \( \gamma \) fractions, we studied the effect of the glycine and alanine residues at \( \gamma \) 136 on surface hydrophobicity, oxygen affinity, and stability of the HbF molecule.

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Under these conditions, the mixture contains HbF, HbS, and hybrid hemoglobins.

Solubility was determined by measuring the concentration of dissolved hemoglobin after completion of polymerization by filtration through a 0.45 μm membrane filter (Millipore Co, Bedford, MA). The polymer phase was collected by filtration. The fractions of HbS and HbF in solution were analyzed by ion exchange HPLC.

Oxygen equilibrium curves of HbF were determined in 0.1 mol/L phosphate buffer, pH 7.0, at 20°C with an automatic recording apparatus in the presence of an antifoaming agent (Silicon Antifoam Emulsion SAG, Union Carbide Corp, Somerset, NJ) and hemoglobin stabilizer (hexamethylphosphoramide), while removing oxygen by bubbling with nitrogen gas.

Mechanical shaking experiments were done according to a method described previously.

Two milliliters of carbonmonoxyhemoglobin (CO-Hb) solution (−50 pmol/L per heme) in 0.1 mol/L phosphate buffer, pH 8.0, were placed in a 10 x 45 mm cuvette equipped with a screw cap and were shaken with a TCS-shaker, Model 250 (Southampton, PA), at 60 Hz for various time intervals at room temperature. After shaking, the cuvette was centrifuged at 4,000 rpm for 10 minutes, and the absorption spectra of the supernatant hemoglobin solution were recorded between 500 and 700 nm. The percent of remaining CO-Hb was calculated by the change of absorbance at 569 nm.

RESULTS AND DISCUSSION

Effect of glycine and alanine at the γ136 position of HbF on HbS polymerization. The kinetics of the polymerization of FS mixture in low and high phosphate buffers showed that the delay time before polymerization of deoxy HbS increases with increases in the amount of HbF. This may be caused by the exclusion of FS hybrid hemoglobin and HbF from the polymerization,

suggesting that the initiation of polymerization of FS mixture is controlled only by amounts of HbF present in the FS mixture.

The effect of glycyl and alanyl residues of the HbF molecule on the kinetics of HbS polymerization was studied using equimolar mixtures of HbS and HbF that contained various mixtures of α2γ2 and α2γ1

When hemoglobins F and S are mixed in the oxy-state, the tetramers rapidly dissociate into dimers, which reassociate randomly to form FS hybrid hemoglobin. The amount of FS hybrid hemoglobin in the FS mixture in high phosphate buffers also follows the binomial expansion. The kinetics of the polymerization of three equimolar FS mixtures under hybrid-forming conditions was measured in 1.8 mol/L phosphate buffer, pH 7.4, at 30°C with a temperature jump method. The lines for the three FS mixtures were done in 1.8 mol/L phosphate buffer, pH 7.4, at 30°C using a temperature jump method. ▲ indicates deoxy-HbS alone; △, ○, and □ are 1:1 FS mixtures with 100% γ, 73% γ, and 45% γ, respectively. The slight difference among the lines for the three FS mixtures with different γ percentages must be attributed to slight differences in the fraction of HbS in the FS mixture, rather than to differences in the γ:α ratio. The fractions of HbS in these mixtures were 52.1% ± 1.25%, 49.1% ± 1.18%, and 48.5% ± 1.27% for mixtures with 100% γ, 73% γ, and 45% γ, respectively. The difference between 52.1% and 48.5% of HbS in the FS mixture can account for the 0.06 shift, assuming that the length of the delay time is determined by HbS concentration and that both the FS hybrid hemoglobins (α2γβ and α2γβ) are excluded from the nucleation step, as well as HbF (α2γ and α2γ). Multiple types of polymers form in high phosphate buffer. Among these are the types of polymers formed under low salt conditions. The nature of HbS polymers in high phosphate buffers (less than 1.8 mol/L) parallels those formed in gelling experiments under near physiologic conditions.
Since hybrid HbF and FS hybrid hemoglobin are excluded from all these multipolymers of HbS formed in 1.8 mol/L phosphate buffer, the exclusion of these hemoglobins from HbS polymers is even more likely under near physiologic conditions.

The solubilities of these three FS mixtures were measured at the plateau of the polymerization curve. As shown in Fig 2, although the solubility of HbS was independent of the initial hemoglobin concentration, the solubility of equimolar FS mixtures depends on the initial hemoglobin concentration. This also indicates that HbF and FS hybrid hemoglobin are excluded from polymers, and that the solubility of the FS mixture is controlled by HbS. The supernatant of FS mixture (solubility) is greater when initial hemoglobin concentration is increased because of increases in the HbF and FS hybrid hemoglobin that are excluded from polymerization. This result is similar to that in studies using AS mixtures.26 The solubility of the FS mixture in 1.8 mol/L phosphate buffer was independent of the \( \frac{\alpha_2}{\beta_2} \) ratio. It is known that the logarithmic plots of solubility of hemoglobins are linearly related to phosphate concentration.24,37 It is more likely that the solubility of FS mixture is independent of \( \frac{\alpha_2}{\beta_2} \) ratio under near physiologic conditions, although direct measurements are required to prove this.

Turbidity increased linearly with increases in initial hemoglobin concentration and was independent of the \( \frac{\alpha_2}{\beta_2} \) ratio (Fig 3). These results indicate that polymerization of FS mixture is not affected by the \( \frac{\alpha_2}{\beta_2} \) ratio in HbF, and that the inhibitory effect of HbF on the polymerization of HbS depends solely on the fraction of HbF.

Oxygen equilibrium curves of HbF with various fractions of \( \alpha_2 \gamma \). Oxygen equilibrium curves of HbF with two different fractions of \( \alpha_2 \gamma \) were determined in 0.1 mol/L phosphate buffer, pH 7.0, at 20°C by an automatic Hemox analyzer in the presence and absence of organic phosphate. As shown in Table 1, the \( P_{50} \) values for HbF containing 100% \( \alpha_2 \gamma \) and 73% \( \alpha_2 \gamma \) are the same, both in the presence and absence of organic phosphates, suggesting that glycyl and alanyl residues at the \( \gamma_{136} \) position do not affect the functional properties of HbF.

Effect of Gly and Ala at \( \gamma_{136} \) on surface hydrophobicity.

HPLC using so-called reversed phase (RP) column packing has become a powerful tool for the separation of \( \alpha_2 \gamma \) and \( \lambda \), as well as \( \alpha \) and \( \beta \) chains.127 In RP column chromatography, proteins separate according to their degree of hydrophobicity by interaction between exposed nonpolar amino acid residues on the protein and hydrophobic groups on the column matrix. In addition, in RP-HPLC, very densely distributed nonpolar groups produce strong hydrophobic interaction, necessitating the use of organic solvents for elution. Using this method, \( \alpha_2 \gamma \) can clearly separate from \( \lambda \gamma \), and the retention time for \( \alpha_2 \gamma \) is found to be shorter than that for \( \lambda \gamma \), suggesting that the alanyl residue at the \( \gamma_{136} \) position is more hydrophobic than the glycyl residue at the same position. However, this finding can not be applied directly to native tetrameric \( \alpha_2 \gamma_2 \) and \( \lambda_2 \gamma_2 \), since the solvent used for the separation causes proteins to denature.

Recently, hydrophobic interaction column chromatography that does not use organic solvents has been developed19,40 and can be used to quantify the surface hydrophobicity of proteins in the native state. We found that a high-performance size-exclusion column equilibrated with high

![Fig 2. Solubility after polymerization of the deoxy-form of HbS and 1:1 FS mixtures. Supernatant hemoglobin concentration (solubility) was determined at the plateau of polymerization. Experimental conditions and symbols are as described for Fig 1.](image)

![Fig 3. Relationship between turbidity after polymerization and hemoglobin concentration. Turbidity at the plateau of polymerization of 1:1 FS mixtures with various hemoglobin concentrations was measured spectrophotometrically at 700 nm. Experimental conditions and symbols are as described for Fig 1.](image)

<table>
<thead>
<tr>
<th>( HbF ) (% ( \alpha_2 \gamma ))</th>
<th>( P_{50} ) (mm Hg)</th>
<th>+DPG (2 mmol/L)</th>
</tr>
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<tbody>
<tr>
<td>100</td>
<td>8.63</td>
<td>11.5</td>
</tr>
<tr>
<td>73</td>
<td>6.65</td>
<td>11.7</td>
</tr>
</tbody>
</table>

Measurement of oxygen equilibrium curves of HbF (~100 μmol/L) was performed in 0.1 mol/L phosphate buffer, pH 7.0, at 20.5°C to 20.8°C. The values are the mean value of duplicate. Under the same conditions, the \( P_{50} \) values of HbA with and without DPG (2 mmol/L) were 8.30 and 13.7 mm Hg, respectively.
salt concentration behaves similarly to hydrophobic interaction chromatography, making it possible to characterize the surface hydrophobicity of native proteins with the same molecular size.32

Using this method, we studied the effect of Gly and Ala at the \( \gamma_{136} \) position on surface hydrophobicity of the HbF molecule. The elution volume of normal CO-HbF with 73% \( \alpha_2\gamma_2 \) was 28.3 mL using TSK Gel-2000 SW (7.5 x 300 mm) in 1.65 mol/L phosphate buffer, pH 7.4, at room temperature. Under these conditions, CO-HbA and CO-HbS elute much more slowly than CO-HbF.32 The elution volumes were 44 mL and 73 mL for CO-HbA and CO-HbS, respectively. The elution volume for HbF was dependent on the fraction of \( \alpha_2\gamma_2 \) and \( \alpha_2\gamma_7 \); the higher the fraction of \( \alpha_2\gamma_7 \), the larger the elution volume (Fig 4). The elution curves of the mixture of \( \alpha_2\gamma_2 \) and \( \alpha_2\gamma_7 \) broadened with higher fractions of \( \alpha_2\gamma_2 \). The elution volume for HbF with 100% \( \alpha_2\gamma_2 \) was 27.75 mL; for HbF containing 45% \( \alpha_2\gamma_7 \), 30.75 mL. However, the tetrameric \( \alpha_2\gamma_2 \) and \( \alpha_2\gamma_7 \) did not separate from each other, perhaps indicating the formation of hybrid hemoglobin between these two hemoglobins. From this result, the difference in the elution volume between HbF with \( \alpha_2\gamma_2 \) and HbF with \( \alpha_2\gamma_7 \) was calculated to be about 5 mL, under these conditions. These data indicate that the native form of \( \alpha_2\gamma_2^{136} \) has a stronger surface hydrophobicity than \( \alpha_2\gamma_2^{364} \), as suggested by the result using denatured \( \alpha_2\gamma_2 \) and \( \alpha_2\gamma_7 \) chains.1

Effect of Gly and Ala at \( \gamma_{136} \) on mechanical stability of HbF. The oxy-form of HbS denatures at a rate approximately 10 times faster than that of HbA during mechanical agitation.33 We have also found that oxy-HbF is much more stable than oxy-HbA during mechanical agitation. Our studies on the stability of hemoglobins during mechanical agitation have shown that the rate of denaturation of abnormal hemoglobins depends on the type and site of the mutation.33,41 The rate of precipitation of hemoglobin during mechanical agitation reflects the surface hydrophobicity of tetrameric and dimeric hemoglobin molecules, in addition to protein conformation and surface activity.33,41,42 In this study, the results indicate that the rate of denaturation of HbF during mechanical agitation varies with the fraction of \( \alpha_2\gamma_2 \) (Fig 5). The denaturation rates for HbF solutions containing 73% \( \alpha_2\gamma_2 \) and 35% \( \alpha_2\gamma_2 \) are 1.1 and 1.4 times faster, respectively, than that of HbF that does not contain \( \alpha_2\gamma_2 \) (100% \( \alpha_2\gamma_7 \)). From these results, it can be calculated that the denaturation rate of HbF with 100% \( \alpha_2\gamma_7 \) is 1.6 times faster than that of HbF with 100% \( \alpha_2\gamma_2 \).

The denaturation rate of hemoglobin molecules is also related to the surface hydrophobicity and the amount of dimeric and tetrameric hemoglobin present.42 Since the oxygen affinities of \( \alpha_2\gamma_2 \) and \( \alpha_2\gamma_7 \) are similar, the dissociation constants of these two fetal hemoglobins should also be similar. Our data indicate that the surface hydrophobicity of dimeric \( \alpha_2\gamma_7 \) is greater than that of \( \alpha_2\gamma_2 \).

Effect of Gly136 and Ala136 of the \( \gamma \) chain on the solubility of HbF. The solubility of HbF is significantly higher than that of HbA. This appears to be due to a weakening of one of the electrostatic contacts between neighboring molecules in HbF crystals.43 Solubility depends on solvent conditions; for instance, the higher the phosphate concentration, the lower the solubility.24,37 The solubility of HbF with various fractions of \( \alpha_2\gamma_2 \) and \( \alpha_2\gamma_7 \) was studied in 2.5 mol/L phosphate buffer and was found to be dependent on the fraction of \( \alpha_2\gamma_2 \) and \( \alpha_2\gamma_7 \) (Table 2). Solubility of HbF was higher with increased fractions of \( \alpha_2\gamma_7 \).

![Fig 4. Relationship between the elution volume on a TSK-GEL SW column and the fraction of \( \alpha_2\gamma_2 \) of HbF. CO-HbF solution (200 µL) with various ratios of \( \alpha_2\gamma_2 \) to \( \alpha_2\gamma_7 \) in 1.65 mol/L phosphate buffer, pH 7.4, was applied to a TSK-GEL-2000 SW column equilibrated with 1.65 mol/L phosphate, pH 7.4, at room temperature and was eluted with the same buffer. The elution volume of \( \alpha_2\gamma_2 \) was determined by the extrapolation of the line to 0% \( \alpha_2\gamma_2 \) (dashed line).](#)

![Fig 5. Denaturation curve of oxy-HbF with various fractions of \( \alpha_2\gamma_2 \). Oxy-HbF with differing ratios of \( \alpha_2\gamma_2 \) in 0.1 mol/L phosphate buffer (2 mL), pH 8.0, was shaken for the time intervals shown in comparison with oxy-HbA. The percentages of denatured hemoglobin were determined spectrophotometrically.](#)

<table>
<thead>
<tr>
<th>Fraction of ( \alpha_2\gamma_2 ) (mg/dL)</th>
<th>Solubility (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>28.5 ± 6.4</td>
</tr>
<tr>
<td>73</td>
<td>27.4 ± 2.8</td>
</tr>
<tr>
<td>50</td>
<td>20.5 ± 1.4</td>
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</tbody>
</table>

Table 2. Solubility of HbF With Various Fractions of \( \alpha_2\gamma_2 \) and \( \alpha_2\gamma_7 \) in 2.5 mol/L Phosphate Buffer, pH 7.4, at 22°C.
The solubility of protein depends on the protein-solvent and protein-protein interactions. The 136th position of the H helix of the HbF molecule does not appear to be a contact point when HbF aggregates to form crystals.41 The difference in the solubility of α2γ2 and α2γ2 may be related to differences in the protein-solvent interaction caused by their surface hydrophobicity. This depends on the difference in the hydrophobicity of Gly and Ala at the 136th position of the γ chain.

Our results indicate that the chain composition of fetal hemoglobin in respect to βγ and γ content does not affect two major properties of sickle hemoglobin: delay time from deoxygenation to aggregation and oxygen equilibrium. It is, therefore, very unlikely that the differences in βγ and γ content found with various β haplotype backgrounds account for the range of clinical expression of sickle cell disease. We recognize that the experiments done in this study, especially those on the kinetics of polymerization in a high phosphate buffer, were not done under physiologic conditions. Further experiments on the kinetics of the polymerization of FS mixtures under near physiologic conditions should be done to confirm our results.

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TWO TYPES OF Hb F $\alpha_2^S\gamma_2$ AND $\alpha_2^A\gamma_2$


Characterization of two types of fetal hemoglobin: alpha 2G gamma 2 and alpha 2A gamma 2

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