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Effects of β6 amino acid hydrophobicity on stability and solubility of hemoglobin tetramers

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The relationship between different amino acids at the β6 position of hemoglobin and tetramer stability was addressed by a site-directed mutagenesis approach. Precipitation rates during mechanical agitation of oxyhemoglobins with Gln, Ala, Val, Leu and Trp at the β6 position increased 2, 5, 13, 21 and 53 times, respectively, compared with that for Hb A. There was a linear relationship between the log of the precipitation rate constant and amino acid hydrophobicity at the β6 position, suggesting that enhanced precipitation of oxy Hb S during mechanical agitation results in part from increased hydrophobicity of β6 Val. Deoxyhemoglobin solubility increased in the order of β6 Ile, Leu, Val, Trp, Gln, Ala and Glu suggesting that hydrophobic interactions between β6 Val and the acceptor site of another hemoglobin molecule during deoxy-Hb S polymerization not only depend on hydrophobicity but also on stereospecificity of the amino acid side chain at the β6 position. Furthermore, our results indicate that hydrophobic amino acids at the β6 position which promote tetramer instability in the oxy form do not necessarily promote polymerization in the deoxy form.

Hemoglobin S; Stability; Solubility; Polymerisation; Hydrophobicity; Mutagenesis

1. INTRODUCTION

Sickle cell disease results from a glutamic acid to valine change at the 6th position in the β-globin chain of hemoglobin, which causes low solubility and rapid polymerization of hemoglobin tetramers upon deoxygenation. The structural and functional properties of Hb S and Hb A in dilute solution are very similar [1,2]. Furthermore, since the kinetics of ligand binding and dissociation for Hb A and Hb S are also identical [3,4], it is implied that the β6 substitution in Hb S has little or no effect on protein conformation in dilute solution.

There is, however, one property of Hb S that differs significantly from Hb A in dilute solution. The oxygen and carbon monoxide complexes of Hb S precipitate much more rapidly than the corresponding complexes of Hb A upon mechanical agitation [5]. Extensive studies have also shown that the rate of mechanical denaturation of abnormal hemoglobins depends on the type and site of the mutation [6–13]. Precipitation of oxy Hb S after mechanical agitation can be attributed to the Glu→Val change at the β6 position; however, the structural basis of precipitation and the relationship between instability of Hb S and accumulation of the membrane-associated hemoglobins in red cells of patients with sickle cell disease are not clear.

Recombinant DNA approaches promise to help define the relationship between protein structure and function and to increase our knowledge of basic molecular mechanisms involved in polymerization and stability of hemoglobin [14–18]. Previous studies show that deoxyhemoglobin solubility is lower with Ile and Leu at the β6 position than with Val, and that both hydrophobic substitutions accelerate polymerization [16,18]. More recent studies show that tryptophan at the β6 position does not promote tetramer polymerization, suggesting that there may be steric hindrance at the acceptor site of a lateral contact on polymers [17]. These results indicate that low solubility of deoxyhemoglobin is not unique for β6 Val, and that acceleration of deoxyhemoglobin polymer formation depends on the type of amino acid at the β6 position. The precise relationship between hydrophobicity, β6 amino acid side chains, tetramer stability in the oxy form, as well as solubility and polymerization of deoxyhemoglobins is not at all clear. In this report we have utilized a site-directed mutagenesis approach to evaluate the effects of β6 amino acid hydrophobicity on hemoglobin stability and solubility.

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2. MATERIALS AND METHODS

The pLcIIFXβ-globin expression vector was essentially used as described by Nagai et al. [14]. Fusion protein is isolated from *E. coli* and cleaved with Factor Xa, resulting in liberation of authentic β globin which is then assembled into hemoglobin tetramers [15].

For mutagenesis, the expression vector pLcIIFXβ was digested with *Sac*I and *Hin*dIII, and the approximately 1 kb insert was subcloned into M13mp18 as described previously [14,15]. The constructs were verified by DNA sequence analysis of single-stranded mutagenized

cDNA employing the dideoxy chain-termination technique [20]. Following sequence verification, the *SacI/HindIII* fragment was subcloned back into the expression vector.

Cultures of *E. coli* (M5219) containing each expression vector were grown at 30°C overnight, and fusion protein was isolated. Cells from overnight growths at 30°C were lysed and treated with DNase I, and soluble proteins were removed by centrifugation [18]. After 3–5 washes with 0.5% (v/v) Triton X-100, the resultant pellet was dissolved in 8 M urea/50 mM Tris buffer and the fusion protein was purified by ion-exchange chromatography (CM-Sepharose). The partially-purified fusion protein was dialyzed against distilled water, lyophilized, solubilized in 1% (v/v) formic acid, and further purified on a Sephacryl S-200 column (3 × 100 cm) equilibrated in 1% (v/v) formic acid/1 mM DTT. Cleavage of fusion protein with activated Factor Xa (PIERCE, Rockford, IL) results in liberation of authentic β globin. Confirmation of the N-terminal protein sequence for recombinant β6 Val, Ala, Leu and Trp globins was accomplished by Edman degradation employing an automatic protein microsequencer (Applied Biosystems 477A pulsed-liquid protein sequencer).

Tetrameric hemoglobins were directly reconstituted in cleavage buffer [17] following the addition of cyanoheme and native α-globin chains in the CO form to the recombinant β-globin chains. Reconstituted hemoglobin was reduced with sodium dithionite under an atmosphere of CO gas, and then purified by CM-52 ion-exchange chromatography [15]. The recombinant hemoglobin tetramers were further purified on a Mono S column (Pharmacia, Piscataway, NJ).

Hemoglobin concentration was determined spectrophotometrically using millimolar extinction coefficients of 12.5 at 555 nm and 13.5 at 541 nm for monomeric deoxy- and oxyhemoglobin, respectively [20]. Precipitation rates during mechanical agitation of oxyhemoglobins and solubility of deoxyhemoglobins were calculated as described previously [21].

3. RESULTS AND DISCUSSION

3.1. *Mechanical stability of hemoglobins modified at the β6 position*

The oxy form of Hb S denatures approximately 10 times faster than oxy-Hb A during mechanical agitation in 0.1 M phosphate buffer, pH 8.0 at room temperature [5]. Under the same conditions, precipitation rates after mechanical agitation of recombinant and native Hb S were very similar (data not shown). Effects of amino acid hydrophobicity on precipitation after mechanical agitation were studied by engineering hemoglobins with Ala, Leu and Trp at the β6 position. The rate of precipitation of hemoglobin mutants with β6 Ala, Val, Leu and Trp increased 5, 13, 21 and 53 times, respectively, compared to β6 Glu (Hb A) (Fig. 1). These results and our previous results on Hb Machida (a naturally-occurring variant containing Gln at the β6 position) [21] were evaluated with respect to the role of amino acid hydrophobicity of the β6 position in facilitating tetramer instability in the oxy form.

A variety of approaches has been used for determining hydrophobicity values for amino acids including measurements of relative solubilities in water/organic solvents, empirical calculations, measurements of chromatogram retention times and partitioning between different solvents [22–28]. Relative hydrophobicity values for tryptophan vary widely depending on the method; however, when hydrophobicity values derived from the water/octanol partion coefficients of *N*-acetyl amino

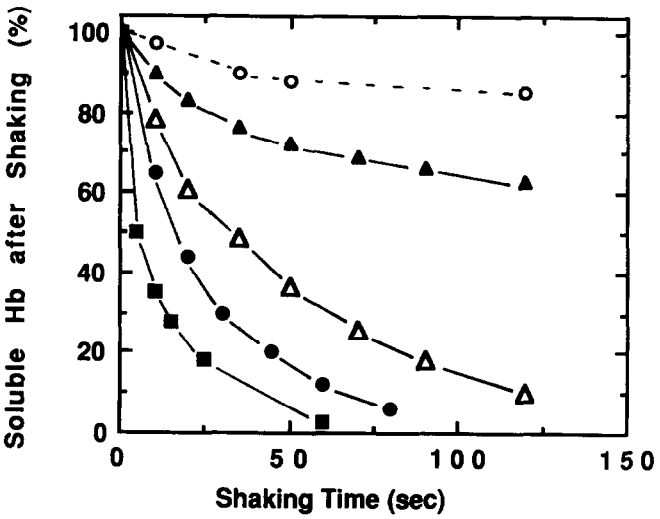

Fig. 1. Mechanical stability of the oxy form of native and recombinant hemoglobins. The oxy forms of various β6-substituted Hbs (~30 μM) were mechanically agitated, insoluble denatured hemoglobins were removed by centrifugation, and the concentration of soluble hemoglobin was determined. Values on the y axis represent % of hemoglobin remaining in solution after mechanical agitation. Hemoglobins included β6 Glu (○), Ala (▲), Val (△), Leu (●) and Trp (■).

acid amide derivatives were used [23], there was a linear relationship between the log of the precipitation rate constant and amino acid hydrophobicity at the β6 position (Fig. 2). These results suggest that enhanced precipitation of oxy Hb S after mechanical agitation results, at least in part, from increased hydrophobicity of valine at the β6 position. Fluorescence studies suggest that tryptophan at the β6 position is exposed on the surface of hemoglobin [16], and our results indicate that the presence of hydrophobic amino acid side chains on the surface may facilitate instability of hemoglobin.

Understanding the structural basis of Hb S instability is important, since instability of oxy-Hb S is believed to be the primary cause of oxidant damage of the red cell membrane in sickle cell disease [29]. The denaturation rate of oxy Hb S depends on experimental conditions, such as speed of shaking, angle of tube inclination, pH, temperature and hemoglobin concentration [5–12]. A possible explanation for the mechanical instability of oxy-Hb S has been presented [30–32]. Mechanical agitation generally increases the rate of unfolding of proteins at the air/water interface. Agitation may also help to remove denatured proteins from the interface and allow for newly undenatured material to reach the surface [31]. In addition, upon coating with foam, a dramatic increase of interphase occurs as air bubbles are introduced into the bulk of the solution, which may accelerate denaturation [31,32].

At hemoglobin concentrations low enough to induce dissociation into dimers, instability increases exponentially with decreasing hemoglobin concentrations [9]. These findings suggest that instability may depend on dissociation of tetramers to dimers. It then follows that

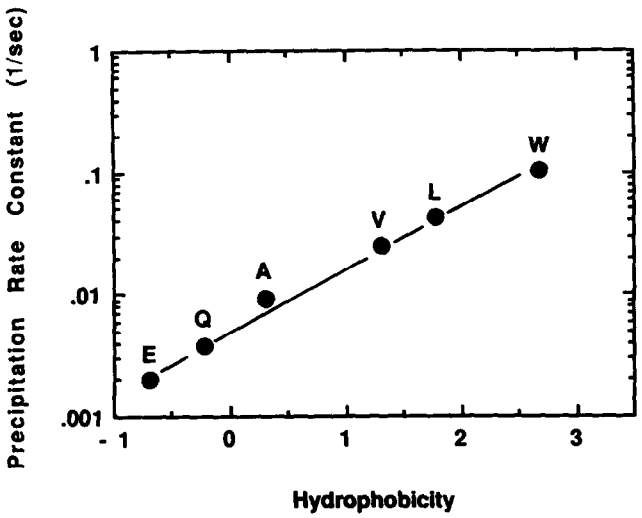

Fig. 2. Relationship between amino acid hydrophobicity at the β6 position and precipitation rate constants. Precipitation rate constants (y axis) for various recombinant and naturally-occurring Hbs were calculated from results of Fig. 2, and plotted vs. hydrophobicity (x axis) of the amino acid at the β6 position. Hydrophobicity values for amino acids represent water/octanol partion coefficients for the corresponding *N*-acetyl amino acid amides [23]. Amino acids are indicated by one-letter symbols.

when undenatured hemoglobin reaches the surface at the air/water interface during mechanical agitation that formation of dimeric hemoglobin and unfolding may be accelerated thus promoting denaturation and precipitation. Substitution of Trp and Leu for Val at the β6 position may further accelerate unfolding of dimeric hemoglobin by increasing hydrophobicity of the A helix of the N-terminal portion of the β-globin chain.

It is interesting to note that oxy Hb San Jose (β7 Glu→Gly) has the same mechanical instability as oxy Hb S [12]. The mutation in Hb San Jose is close to the β6 position in the A helix of β chain, and is also located on the surface of the hemoglobin molecule, as with Hb S. The difference, however, in hydrophobicity for the β7 Glu→Gly change (0.64) in Hb San Jose is about 3 times less than that for the β6 Glu→Val change (1.90) in Hb S [26]. Since Hb San Jose and Hb S have similar mechanical instabilities, these results suggest that β7 Glu contributes more to overall stability of hemoglobin than does β6 Glu. Thus, hemoglobin stability during mechanical agitation may depend on both hydrophobicity and location of amino acids on the surface of the hemoglobin molecule. Space-filling models show that a β-bend structure in the A helix in Hb S involves β3 Leu, β4 Thr, β5 Pro and β6 Val, and that β2 His and β7 Glu are close enough to participate in electrostatic interactions responsible for the pK shift of β2 His [33]. This structure may be stabilized by hydrogen bonds formed by the peptide backbone of amino acid side chains of the β-bend. The β7 Glu→Gly substitution in Hb San Jose may affect this interaction by disrupting the first turn of the A-helix, affecting stability during mechanical agitation without causing any functional consequences [12]. The substitution of hydrophobic amino acids like valine, leucine and tryptophan at the β6 position may also possibly affect the A-helix of the N-terminal portion of hemoglobin, thus promoting surface denaturation and precipitation of hemoglobin molecules during mechanical agitation.

3.2. *Solubility of hemoglobins modified at the β6 position*

It is assumed that the low solubility of deoxy-Hb S can be attributed to the presence of the strong hydrophobic β6 Val in Hb S [34]. Recent studies show that the solubilities of recombinant β6 Hb Ile ($\alpha_2\beta_2^{6Ile}$) [16] and Hb Leu ($\alpha_2\beta_2^{6Leu}$) [18] were 80% and 50%, respectively, that of native Hb S, and that both substitutions facilitated tetramer polymerization in the deoxy form. These results indicate that polymerization is not specific for valine at the β6 position, and that an abrupt decrease in deoxyhemoglobin solubility may depend, in general, on hydrophobic amino acids at the β6 position. To further understand the relationship between β6 amino acid hydrophobicity and deoxyhemoglobin solubility, we compared solubilities in high phosphate buffers for β6 Trp and Ala hemoglobins with those of β6 Glu (Hb A), Val (Hb S), Leu ($\alpha_2\beta_2^{6Leu}$), Ile ($\alpha_2\beta_2^{6Ile}$) and Gln (Hb Machida) [17,18,21] (Table I).

Although deoxy Hb Leu and deoxy Hb S polymerized in 1.8 M phosphate buffer after a 20–30 min delay time, deoxy Hb Trp ($\alpha_2\beta_2^{6Trp}$) and deoxy Hb Ala ($\alpha_2\beta_2^{6Ala}$) at the same concentrations did not polymerize even after a 24-h incubation. Lack of polymerization of deoxy Hb Trp was previously reported to be possibly due to β6 Trp steric hindrance at a potential acceptor site for a lateral contact [17]. Further studies are required to determine whether tryptophan at the β6 position is excluded from the acceptor site possibly like β6 Glu in Hb A and β6 Ala in Hb Ala.

Table I

Deoxyhemoglobin solubility (g/dl) for β6-containing hemoglobins

β6 Amino acid	Phosphate buffer concentration	
	1.8 M	2.1 M
Glu	1.9	0.35
Ala	N.D.	N.D. (>0.15)
Gln	1.0	0.144
Trp	N.D. (>0.065)	0.041
Val	0.035* 0.044 (0.08)**	0
Leu	0.028	0
Ile	(0.04)**	

Hemoglobin solubility in 1.8 and 2.1 M phosphate buffers, pH 7.4, was measured at 30°C. Results for the naturally-occurring Hb variant β6 Gln (Hb Machida) have been described previously [21]. Solubility values for native deoxy-Hb S (*) in our experiments and for native Hb S and recombinant Hb Ile ($\alpha_2\beta_2^{6Ile}$) (**) reported by Baudin-Chich et al. [16] were determined in 1.8 M phosphate buffer, pH 7.2. N.D., solubility not determined, since hemoglobin concentration (noted in parentheses) was not sufficient for polymerization under these conditions.

Since the low yield of Hb Trp was not sufficient to directly measure solubilities in 1.8 M phosphate buffer, we used higher phosphate buffer concentrations (2.1 M) in order to determine solubility properties for Hb Trp. Hemoglobin solubility decreases exponentially with increased phosphate concentration, and there is a linear relationship between the log of the solubility and phosphate concentration [35,36]. The solubilities for deoxy Hb Trp and deoxy Hb Ala in 2.1 M phosphate were 41 mg/dl and more than 150 mg/dl, respectively; while under the same conditions the solubilities for deoxy Hb A and deoxy Hb Machida were 350 mg/dl and 144 mg/dl, respectively [21]. The solubility of deoxy Hb Trp is about 8 times less than that of deoxy Hb A. Our results [18] and those for recombinant Hb Ile ($\alpha_2\beta_2^{6Ile}$) [16] show that hemoglobin solubility in high phosphate buffer increases in the order of Ile, Leu, Val, Trp, Gln, Ala and Glu, which does not directly correlate with decreased amino acid hydrophobicity at the β6 position. Lowest solubility occurred with hydrophobic substitutions like Ile and Leu, indicating an important role for hydrophobicity in determining solubility properties of hemoglobin in the deoxy form. However, the results with Trp, which contains the bulkiest side chain and the most hydrophobic β6 substitution, show that Hb Trp solubility was in fact higher than Hb S, but much lower than Hb A. Furthermore, even though Trp is bulkier than Glu, Gln and Ala, its high hydrophobicity may contribute to Hb Trp having a lower solubility than these neutral and charged β6-substituted hemoglobin tetramers.

These results suggest that both hydrophobicity and stereospecificity of the amino acid side chain at the β6 position are important determinants for hemoglobin solubility in the deoxy form. Furthermore, our findings indicate that hydrophobic interactions between β6 Val and the hydrophobic pocket formed by E and F helices of a neighboring deoxy Hb S molecule [37] appear to accommodate some degree of flexibility compared with hydrogen and ionic bonds. Further studies on the effect of these β6 amino acid substitutions on polymer structure of deoxyhemoglobin are now required to test this hypothesis.

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