Polymerization of Three Hemoglobin A2 Variants Containing Val66 and Inhibition of Hemoglobin S Polymerization by Hemoglobin A2*  

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To understand determinants for hemoglobin (Hb) stability and Hb A2 inhibition of Hb S polymerization, three Val66 Hb A2 variants (Hb A2, dE6V, Hb A2, dE6V,dQ87T, and Hb A2, dE6V,dA22E,dQ87T) were expressed in yeast, and stability to mechanical agitation and polymerization properties were assessed. Oxy forms of Hb A2 and Hb A2, dE6V, dQ87T were 2- and 1.6-fold, respectively, less stable than oxy-Hb S, while the stability of Hb A2, dE6V,dA22E,dQ87T was similar to that of Hb S, suggesting that Ala22 and Gln87 contribute to the surface hydrophobicity of Hb A2. Deoxy Hb A2, dE6V polymerized without a delay time, like deoxy Hb F, dE6V, while deoxy Hb A2, dE6V,dQ87T and deoxy Hb A2, dE6V,dA22E,dQ87T polymerized after a delay time, like deoxy Hb S, suggesting that Thr87 is required for the formation of nuclei. Deoxy Hb F, dE6V, dQ87T showed no delay time and required a 3.5-fold higher concentration than deoxy Hb S for polymerization, suggesting that Thr effects on Val66 Hb A2 and Val66 Hb F variants are different. Mixtures of deoxy Hb S/Hb A2, dE6V,dQ87T polymerized, like deoxy Hb S, while polymerization of Hb S/Hb A2, dE6V mixtures was inhibited, like Hb S/Hb F, dE6V mixtures. These results suggest αβδδ Val, Thr87 hybrids and Hb A2, dE6V,dQ87T participate in Hb S nucleation, while only 50% of αβδδ Val, Thr87 hybrids and none of the Hb A2, dE6V participate. These findings are in contrast to those of mixtures of Hb S with Hb F, dE6V or Hb F, dE6V,dQ87T, which both inhibit Hb S polymerization. Our results also suggest participation in nucleation of some αβδγ Val, Thr87 hybrids in A2S mixtures but not αβδγ hybrids in FS mixtures.

Hb F and Hb A2 inhibit hemoglobin (Hb)1 S polymerization. Mixtures of Hb A2 and Hb S, like mixtures of Hb F and Hb S, have a higher solubility than Hb S alone and result in inhibition of Hb S polymerization (1, 2). The primary inhibitory effects of Hb A2 and Hb F in these mixtures are to exclude the asymmetrical αβδδ and αβδγ hybrids, respectively, as well as Hb A2 and Hb F from initiation of polymerization with Hb S (3–11). Studies comparing minimum gel concentrations for naturally occurring hemoglobin variants, including the Lepore (δβ hybrid) hemoglobins, suggested that Gln87 and Ala22 in Hb A2 are important sites potentiating inhibition of polymerization of deoxy Hb S (5). In addition, inhibition of polymerization by Hb A2 and Hb F is primarily in trans to the Val66 contact in Hb S polymers. Gln87 in Hb F is also an important site for inhibition of Hb S polymerization by Hb F (5).

We previously engineered and isolated Hb S βT87Q as well as Hb F γE6V and Hb F γE6V, γQ87T, using a yeast expression system, and characterized the polymerization properties of these variants to clarify the role of Gln at γ87 in Hb F-mediated inhibition of deoxy Hb S polymerization (12, 13). Oversaturated deoxy Hb F, γE6V and Hb F, γE6V, γQ87T polymerized similarly without a delay time at a higher concentration than Hb S, even though studies of mixtures of Hb S and Hb S βT87Q indicated that the Thr → Gln difference at γ87 (F3) in the non-α-globin chain is a key amino acid required for Hb F inhibition of Hb S polymerization. Furthermore, changing Gln to Thr at γ87 in Hb F, γE6V, γQ87T slightly inhibited rather than promoted polymerization compared with Hb F, γE6V (13).

Recent studies on high levels of expression of human Hb A2 with Hb S in red blood cells from transgenic mice showed that overexpressed δ chains interact with red blood cell membranes, which results in drastic modification of their properties (14). To further understand Hb A2- and Hb F-mediated inhibition of Hb S polymerization as well as hydrophobicity of Hb A2, three Hb A2 variants containing Val66 (1) Hb A2, dE6V; 2) Hb A2, dE6V, dQ87T; and 3) Hb A2, dE6V, dA22E, dQ87T were expressed in yeast. We reasoned that since the δ chain is more homologous to the β chain than to the γ chain, then important sites in inhibition of Hb S polymerization by Hb F or Hb A2 could be more readily identified by studying the polymerization properties of these Val66 Hb A2 variants. In this report, we characterized surface hydrophobicity by monitoring stability to mechanical agitation. In addition, we assessed the polymerization properties of Hb A2, dE6V (α2δ2 6 Glu → Val), Hb A2, dE6V, dQ87T (α2δ2 6 Glu → Val, 8γ Gln → Thr) and Hb A2, dE6V, dA22E, dQ87T (α2δ2 6 Glu → Val, 22 Ala → Gln, 8γ Gln → Thr) to understand the role of Ala22 and Gln87 in Hb A2-mediated inhibition of Hb S polymerization as well as the role of these sites in the polymerization of these Val66 Hb A2 variants.

MATERIALS AND METHODS

Full-length human δ-globin cDNA (15) was isolated by reverse transcription-polymerase chain reaction using mRNA from COS-1 cells that had been transfected with the δ-globin expression vector pSVK3 (δSalI-PolI). The β-globin cDNA in the shuttle vector pGS188 was replaced by the δ-globin cDNA to create pGS188 δ (16, 17) after isolation of human δ-globin cDNA. Three δ-globin cDNAs containing 1) Val66, 2) Val66 and Thr87, and 3) Val66, Gln87, and Thr87 were constructed using polymerase chain reaction mutagenesis and subcloned as described previously (17) using the δ-globin cDNA containing Val66 as a template to introduce the Thr87 change. The double mutant δ-chain cDNA containing Val66 and Thr87 was then used as a template to introduce the Gln87 change. The complete coding sequence of wild-type and δ-globin cDNA variants, flanked by the GGAP promoter and MF

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1 The abbreviation used is: Hb, hemoglobin.
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α\textsubscript{2}-UN regions in pGS188 δ, was determined using site-specific primers and fluorescently tagged terminators in a cycle-sequencing reaction in which extension products were analyzed on an automated laser-activated, fluorescence emission DNA sequencer (18). The entire δ-globin cDNA region was then excised by XhoI digestion and used to replace the entire β-globin cDNA region in the expression vector pGS389 (16, 17). The resultant vectors contain full-length human cDNAs coding for α- and δ-globin variants under transcriptional control of dual pGGAP promoters, as well as a partially functional yeast LEU2d gene and the URA3 gene for plasmid amplification and selection in yeast (16, 17, 19).

Expression in yeast and isolation of Hb A\textsubscript{2} variants were as described previously (17, 19). Purified Hb A\textsubscript{2} variants were subjected to electrospray mass analysis (Fisons Instruments, VG Biotech, Altrincham, United Kingdom) using the multiply charged ion peaks from the α-globin chain (M\textsubscript{r} = 15,126.4 Da) as an external reference for mass scale calibrations (20). The Val\textsuperscript{66} amino acid change and the N-terminal amino acid sequence of purified α and δ chains were directly confirmed by Edman degradation with a pulsed liquid protein sequencer (ABI 477A, Applied Biosystems, Inc., Foster City, CA). Native human Hb A\textsubscript{2} was purified from normal hemolysates on a Mono S column (Pharmacia Biotech, Inc.) using fast performance liquid chromatography with slight modification of the method described by Ou et al. (21).

Cellulose acetate electrophoresis of hemoglobins was performed at pH 8.6 using Supre-Heme buffer (Helena Laboratories, Beaumont, TX), and hemoglobin concentration was determined spectrophotometrically on a Hitachi U2000 spectrophotometer using millimolar extinction coefficients of 13.5 at 541 nm for oxyhemoglobin and 13.4 at 540 nm for carbon monoxyhemoglobin (22). Methods for determination of kinetics coefficients of 13.5 at 541 nm for oxyhemoglobin and 13.4 at 540 nm for deoxyhemoglobin in 1.8M phosphate buffers using the temperature jump method as well as the mechanical stability of oxyhemoglobins were as reported previously (23, 24). The temperature jump method using 1.8 M phosphate buffer differs from a simple “salting out” in high phosphate (2.2 M) in which a hemoglobin sample is injected directly into deoxygenated high phosphate buffer and results in rapid formation of amorphous aggregates (13, 25).

### RESULTS

**Characterization of Hb A\textsubscript{2} Variants**—Fast performance liquid chromatographic elution patterns of the two Hb A\textsubscript{2} variants, Hb A\textsubscript{2} δE6V and Hb A\textsubscript{2} δE6V,δQ87T, were very similar; however, elution of the triple variant, Hb A\textsubscript{2} δE6V,δA22E,δQ87T, occurred slightly after the other two variants. Both Hb A\textsubscript{2} δE6V and Hb A\textsubscript{2} δE6V,δQ87T tetramers migrated with less negative charge than native Hb A\textsubscript{2} following cellulose acetate electrophoresis, while the triple variant migrated in a manner similar to that of native Hb A\textsubscript{2} (Fig. 1). Differences in migration between native Hb A\textsubscript{2} and Hb A\textsubscript{2} δE6V,δA22E,δQ87T or between Hb A\textsubscript{2} δE6V and Hb A\textsubscript{2} δE6V,δQ87T are similar to that comparing Hb A and Hb S. These results indicate that Glu→Val or Ala→Glu substituations at the 6th and 22nd positions in Hb A\textsubscript{2} and Hb A, respectively, contribute similarly to surface charge differences.

The absorption spectra of the three Hb A\textsubscript{2} variants in the CO form were virtually identical with those of native CO Hb A\textsubscript{2}. In addition, N-terminal sequence analysis to 15 residues for each globin chain showed identical results compared with native α- and δ-globins except for the Glu→Val\textsuperscript{66} change. Electrospray mass spectrometry analysis of α and δ chains from the three recombinant Hb A\textsubscript{2} variants and native Hb A\textsubscript{2} showed that the α chains from native Hb A\textsubscript{2} and the variants had the same mass as native α-globin (M\textsubscript{r} 15,126 Da), while δ chain analysis of the three variants showed expected mass values (Table I).

**Mechanical Stability**—The oxy form of Hb S denatures ~10 times faster than oxy Hb A during mechanical agitation (23), which is related to differences in amino acid surface hydrophobicity of dimeric and tetrameric hemoglobins (26). The physical basis of mechanical instability is predicated on the fact that mechanical agitation generally increases the rate of unfolding of proteins at the air-water interface and provides mixing to allow new, undenatured material to reach the surface from the bulk solution (2). The rate of precipitation during mechanical agitation also depends on the hydrophobicity of exposed amino acids on the surface as well as the tertiary structure of proteins (2, 23, 26). The oxy form of Hb A\textsubscript{2} is about 3-fold more unstable than Hb A during mechanical agitation (27). Our results show that native oxy Hb A\textsubscript{2} was less stable than oxy Hb A and that the oxy form of the Val\textsuperscript{66} variant was about 7-fold less stable than native oxy Hb A\textsubscript{2} (Fig. 2). These differences in mechanical stability are similar to those comparing Hb S and Hb A and are consistent with our previous findings showing a direct relationship between mechanical instability and hydrophobicity at the βδ position (26, 28). The stability of Hb A\textsubscript{2}, δE6V was 2-fold less than Hb S, while that of the double mutant Hb A\textsubscript{2}, δE6V,δQ87T was 1.6-fold less. The mechanical stability of the triple mutant Hb A\textsubscript{2}, δE6V,δA22E,δQ87T was, however, similar to that of Hb S. These results suggest that Ala\textsuperscript{622} and Glu\textsuperscript{667} in Hb A\textsubscript{2} promote instability to mechanical agitation by increasing surface hydrophobicity.

**Polymerization Properties**—Polymerization of deoxy Hb S in high (<1.8 M) as well as low phosphate buffer using the temperature jump method is characterized by a delay time prior to polymer formation the length of which depends on hemoglobin concentration: the lower the concentration, the longer is the delay time (Fig. 3A) (24). Polymer formation in vitro can be assessed using low and high phosphate buffers, both of which result in nucleation-controlled formation of ordered polymers (29, 30). This process clearly differs from a simple “salting out” in high phosphate (>1.8 M) in which hemoglobin sample is directly injected into deoxygenated high phosphate buffer and results in rapid formation of amorphous aggregates (25). High phosphate buffer conditions facilitate comparative studies of polymerization properties of sickle and non-sickle hemoglobin, since the hemoglobin concentrations required for these studies are much lower than those required in low phosphate buffer. For example, experiments in 1.8 M phosphate buffer require about 100 mg/dl for Hb S and Hb A\textsubscript{2} variants, which is less than 1/100 of the hemoglobin concentration required for studies in low phosphate buffer. The relative order of inhibition of Hb S polymerization by Hb A, Hb F, and Hb A\textsubscript{2} in 1.8 M phosphate buffer is also similar to that in low phosphate buffer (4, 5, 9–11).

**TABLE I**

| Mass spectrometric analysis of Hb A\textsubscript{2} and Hb A\textsubscript{2} variants |
|-----------------------------------|
| Hb A\textsubscript{2} | Hb A\textsubscript{2} δE6V | Hb A\textsubscript{2} δE6V,δQ87T | Hb A\textsubscript{2} δE6V,δA22E,δQ87T |
| δ-Globin chain (observed) | 15,922.1 | 15,892.3 | 15,866.8 | 15,925.4 |
| δ-Globin chain (expected) | 15,924.3 | 15,894.3 | 15,867.3 | 15,926.3 |

**Fig. 1.** Cellulose acetate electrophoresis of Hb A\textsubscript{2} variants. Mobilities of purified Hb A\textsubscript{2} δE6V (lane 4), Hb A\textsubscript{2} δE6V,δQ87T (lane 5), and Hb A\textsubscript{2} δE6V,δA22E,δQ87T (lane 6) were compared with native Hb A (lane 1), Hb S (lane 2), and Hb A\textsubscript{2} (lane 3) on cellulose acetate electrophoresis at pH 8.6 using Supre Heme buffer (Helena Laboratories).
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13), and those studies show that one-half of AS hybrids and no
FS hybrids participate in the initiation of Hb S polymerization
(10, 11, 13). Recent comparative studies of deoxy Hb S polymer
structures made in high phosphate buffer with those made in
low phosphate buffer using electron microscopy showed that
oversaturated deoxy Hb S first formed fibers, which then began
to form bundles, macrofibers, and crystals. These structures
had the same appearance in low or high phosphate buffers (31),
indicating that deoxy Hb S fibers formed in high phosphate
buffer are similar or identical with those formed in low phos-
phate buffer. Furthermore, contact sites of deoxy Hb S crystals
made in polyethylene glycol or high phosphate buffer corre-
spond to those of deoxy Hb S polymers in low phosphate buffer
(32, 33).

Polymerization of the three Val$^{56}$ Hb $A_2$ variants alone was
also performed using the temperature jump method in 1.8 M
phosphate buffer. Hb $A_2$ δE6V in the deoxy form polymerized at
slightly higher hemoglobin concentrations than deoxy Hb S,
while the other two variants polymerized at concentrations
similar to that of deoxy Hb S. Polymerization of Hb $A_2$ δE6V,
like Hb F γE6V, however, was not accompanied by a delay time
before polymerization (Fig. 3B) (13) and may be explained by a
linear polymerization mechanism (12, 29). In contrast, polymer
formation by Hb $A_2$ δE6V,δQ87T or Hb $A_2$ δE6V,δA22E,δQ87T
was accompanied by a delay time before polymerization like
that of Hb S (Fig. 3, C and D). The length of the delay time for
these two variants depended on hemoglobin concentration, and
logarithmic plots of delay time versus concentration for Hb $A_2$
δE6V,δQ87T or Hb $A_2$ δE6V,δA22E,δQ87T showed a straight
line which was identical with the line for Hb S (Fig. 4). These
results indicate that Thr$^{87}$ in Hb $A_2$ δE6V,δQ87T facilitates
the formation of nuclei as evidenced by a delay time prior to
polymerization, while Ala$^{22}$ in Hb $A_2$ δE6V,δQ87T has a min-
imal or no effect on the formation of nuclei.

Total polymer formation as a function of hemoglobin concen-
tration was also determined at the plateau of the polymeriza-
tion curves to define critical concentrations required for polymer-
ization of Hb $A_2$ δE6V, Hb $A_2$ δE6V,δQ87T, and Hb $A_2$
δE6V,δA22E,δQ87T. These results were then compared with
our previously reported results for polymerization of Hb S and
Hb F γE6V,γQ87T (Fig. 5). Polymer formation increased linearly
with increases in hemoglobin concentration. Critical concen-
trations for all three Val$^{56}$ Hb $A_2$ variants were slightly

Fig. 2. Mechanical stability of the oxy form of the Hb $A_2$ variants. The oxy forms of Hb $A_2$ δE6V (●), Hb $A_2$ δE6V,δQ87T (▲), and Hb $A_2$ δE6V,δA22E,δQ87T (■) in 0.1 M phosphate buffer, pH 8.0, at room temperature were mechanically agitated for varying times, and the percentage of soluble hemoglobin was deter-
mined after centrifugation and compared with native Hb $A_2$ (□—□), Hb A (○—○) and Hb S (●—●).

Fig. 3. Kinetics of polymerization of Hb $A_2$ variants. Time courses for polymerization of deoxy Hb $A_2$ δE6V (B: 190 (c), 153 (d), and 107 (e) mg/dl) in comparison with deoxy Hb S (A: 92.3 (a) and 75.4 (b) mg/dl), deoxy Hb $A_2$ δE6V,δQ87T (C: 140 (f) and 95 (g) mg/dl), and deoxy
Hb $A_2$ δE6V,δA22E,δQ87T (D: 127 (h), and 83 (i) mg/dl) were performed in 1.8 M phosphate buffer, pH 7.4, at 30 °C by the temperature jump
method.
the non-explained by differences in the amino acid at position 87 (F3) in polymerization (Fig. 6). About 2-fold greater than that required for deoxy Hb S polymerization with a delay time when the concentration was one-fourth of the total hemoglobin concentration (3, 10). These results were then compared with those for Hb S, Hb S/Hb A2, and Hb S/Hb F mixtures. Polymer formation increased linearly with increases in hemoglobin concentration (Fig. 8). Critical concentrations for polymerization of Hb S/Hb A2, δE6V, δQ87T mixtures were slightly higher than that of Hb S, while critical concentration for Hb S/Hb A2, δE6V, δQ87T mixtures was similar to that of Hb S. Critical concentrations for polymerization for Hb A2 and Hb F, determined by extrapolation of the lines to zero, are 2.8- and 3.3-fold higher than that of deoxy Hb S, respectively. These results reinforce the contention that exclusion of ΔS hybrids from Hb S polymerization in Hb S/Hb A2 mixtures is primarily in trans to the ValE6 contact and that ThrQ87 near the EF helix hydrophobic acceptor pocket made by Leu88 and Phe85 in the F helix affects the formation of nuclei and polymers.
DISCUSSION

Polymerization of Hb A2 Variants Containing Val66—Hb A2 represents about 2% of the total hemoglobin in adult erythrocytes, and like Hb F, Hb A2 inhibits polymerization in vitro of deoxy Hb S (5). The primary sequence of the δ chain of Hb A2 differs from that of the β chain of Hb A in only 10 of 146 residues. Previous studies comparing naturally occurring hemoglobin variants suggested that 887-Gln and 822-Ala in Hb A2 were important amino acids for inhibition of Hb S polymerization (5). Our previous studies on polymerization of the double mutant deoxy Hb S βT87Q also suggested that Gln87 is a key amino acid involved in exclusion of A2S as well as FS hybrids from Hb S nucleation (12). Our present results show that the critical concentration for polymerization of Hb A2 δE6V was 1.3-fold higher than that of deoxy Hb S and that critical concentrations for Hb A2 δE6V,Q87T and Hb A2 δE6V,Q87T similarly to that deoxy Hb S. These results are similar to those comparing Hb S βT87Q and Hb S and indicate that Gln at position 87 in Hb A2 δE6V or Hb S βT87Q play comparable roles in the inhibition of Hb A2 δE6V and Hb S βT87Q polymerization, respectively. These results in low and high phosphate buffers using recombinant and naturally occurring Hb variants reinforce the role of Gln at position 87 as a key amino acid involved in Hb A2 inhibition of Hb S polymerization.

Recently, recombinant human Hb A containing Gln87 and Ala822 was produced in transgenic mice (30). Evaluation of polymerization of mixtures (25:75) of this variant and Hb S in a high phosphate buffer showed that polymer formation was the same as that of FS mixtures containing 25% Hb F. These results suggested that this Hb A variant, which contains Gln87 and Ala822 like Hb A2, inhibits Hb S polymerization as effectively as Hb F and Hb A2 (30). These findings also corroborate our previous results on polymerization of Hb S/Hb S βT87Q mixtures which showed that substitution of Gln for Thr at β87 is sufficient to promote exclusion of hemoglobin tetramers (12).

Although the critical concentrations for polymerization of the three Hb A2 variants in the deoxy form were similar to that of deoxy Hb S, the kinetics of polymerization are different: Hb A2 δE6V polymerized without a delay time; while polymerization of Hb A2 δE6V,Q87T and Hb A2 δE6V,A22E,Q87T was accompanied by a delay time. These results suggest that Hb A2 δE6V and the two other variants (e.g., Hb A2 δE6V,Q87T and Hb A2 δE6V,A22E,Q87T) polymerize by different mechanisms, the former by a linear and the latter two by a nucleation-controlled polymerization mechanism like that for deoxy Hb S (29). Our recent results with Hb F variants containing Val66 show that both deoxy Hb F γE6V and Hb F γE6V,γQ87T polymerized similarly without a delay time at a higher concentration than deoxy Hb S in low and high phosphate buffers (13). In addition, changing Gln to Thr at γ87 in Hb F γE6V (Hb F γE6V,γQ87T) slightly inhibited rather than promoting polymerization compared to Hb F γE6V (13). These results suggest that the effect of Thr at position 87 in δ and γ chains of the Val66-Hb A2 and Val66-Hb F variants in promoting nucleation-controlled polymerization is different.

McCune et al. (30) explained the anti-sickling effect of Hb A containing Gln87 in mixtures with Hb S using a computer graphic simulation and proposed that the larger side chain of Gln compared with Thr at β87 prevents insertion of Val66 into the acceptor pocket of the F helix. However, our results show that the critical concentration for polymerization of deoxy Hb A2 δE6V was only about 1.3-fold higher than that of deoxy Hb A2 δE6V,Q87T, which is similar to the difference in critical concentrations comparing deoxy Hb S βT87Q and deoxy Hb S. Our previous results also showed that inhibition of polymer formation by substitution of larger bulky amino acids like Phe668 for Leu in the acceptor pocket resulted in a 10-fold increase in the concentration required for polymerization compared with deoxy Hb S (34). In addition, Hb S/Hb Quebec-Chori (Hb βT87I) mixtures, in which the larger side chain of Ile is substituted for Thr at β87, actually accelerates nucleation and polymerization (35), suggesting that substitution of a larger side chain than Thr at β87 can, in fact, promote nucleation and polymerization. We speculate that the EF helix acceptor site for Val66 in Hb S βT87Q has a similar conformation to the EF helix of deoxy Hb A2. Even though Hb S βT87Q polymer formation was preceded by a delay time prior to polymerization, the kinetic progress curve of polymerization after the delay time was linear and not sigmoidal like deoxy Hb S (12). These results suggest that Gln at position 87 in the δ or β chains of Hb A2 δE6V or Hb S βT87Q, respectively, affects the environment of the acceptor pocket for Val66 during polymerization, inhibits the formation of nuclei and polymers, and may also affect...
protein-protein interactions between δ87 or β87 and other amino acids during polymerization.

Thr667 in 1β-δ of deoxy Hb S is not a direct contact site for Val667 but is involved in lateral contacts with Ser9, Ala116, and Ala613 in 1β-δ of Hb S polymers. Thr667 in Hb S is involved in interactions between parallel double strands in crystals or fibers and also forms strong hydrogen bonds with 1α1-139Lys and 2α2-S1Ser (32, 33). From these results, we propose that Val667 in Hb A2 δ667 and Val667 in Hb S β87Q are able to insert into the acceptor pocket made by Phe665 and Leu688, even though this F helix contains Gln at δ87. Previous results with deoxy Hb S crystals indicated participation of Glu622 in an axial contact to form an ionic bond with His B1 (20) α2 (32). Even though differences between Glu and Ala at position δ22 in Hb A2 δ667, δ87Q or Hb A2 δ667, δA22E, δQ87T were expected to affect polymerization properties, our results showed minimal effect if any. These results suggest that Hb B1 (20) α2 at neutral pH did not affect axial contacts of the polymers and/or that Alaδ22 was not involved in protein-protein interactions in polymers of Hb A2 δ667, δQ87T or Hb A2 δ667, δA22E, δQ87T. Other amino acid differences between Hb A2 and Hb A such as Ser9 versus Thr9, His116 versus Arg116 and Val9126 versus Met1126, which may be involved in contact sites (34), may also be contributing to differences in polymerization properties between Hb S and Hb A2 δ667, δQ87T.

Our present results with the Val667 Hb A2 variants suggest that changing Thr to Gln at δ87 of Hb A is critical for anti-sickling, while changing Glu to Ala at β22 has little or no effect on exclusion of hybrid hemoglobins in mixtures with Hb S. Therefore, it may not be necessary to substitute Ala for Glu at β22 when preparing anti-sickling hemoglobins for gene therapy of sickle cell disease (30). In fact, Asp is present in the γ chain of Hb F instead of Glu, and Hb F inhibits polymerization of deoxy Hb S like Hb A2. Furthermore, the rate of assembly of α and non-α chains to form αβ or αβ-variant dimers depends on electrostatic attraction (36). More negatively charged β chains are expected to have a higher affinity than βδ chains for α chains; therefore, hemoglobin with Glu rather than Ala at β22 may, in fact, be a more efficient anti-sickling hemoglobin for gene therapy for sickle cell disease. X-ray analysis of these Hb A2 variants is now required to evaluate the structural effects of the Gln for Thr substitution in the Val667 acceptor pocket as well as at other interaction sites.

Inhibition of Hb S Polymerization by Hb A2—Our previous results using mixtures of Hb S/Hb S β87Q suggested that Hb S β87Q and one-half of the asymmetrical αδβδδβ87Q hybrids were excluded from the formation of nuclei with Hb S (12). Logarithmic plots of delay time versus concentration for the Hb S/Hb A2 δ667 mixture showed a straight line shifted right ~0.25 unit from the line for Hb S, which was similar to the line for Hb S/Hb S β87Q mixtures (Fig. 6). In contrast, the line for the Hb S/Hb A2 δ667, δQ87T mixture was similar to that of Hb S. Equal mixtures of Hb S and Hb A2 δ667 contain 25% Hb S, 50% asymmetrical αδβδδβ87Q hybrid, and 25% Hb A2 δ667. Nucleation in these mixtures is controlled to be 56% of the total hemoglobin concentration based on the 0.25-unit difference comparing the lines for Hb S and Hb S/Hb A2 δ667 mixtures. These results suggest that Hb S and about one-half of the asymmetrical αδβδδβ87Q hybrid contribute to the formation of nuclei with Hb S in these mixtures and that Val667 can interact with an EF acceptor pocket containing Thr667 in hybrid hemoglobins as well as Hb S, just like Val667. These results are consistent with our previous findings on polymerization of Hb S/Hb S β87Q mixtures (12).

The primary inhibitory effect of Hb A2 on Hb S polymerization is to exclude both the asymmetrical hybrid αδβδδβδδ in A2S mixtures like αδβδδγ in FS mixtures from the Hb S polymer (5, 6). Thus, inhibition of polymerization by Hb A2 is primarily in trans to the βδ Val contact of Hb S polymers. The 0.1-unit difference in left shift on the X axis of the line for A2S mixtures compared with that of FS mixtures in the experiments in a high phosphate buffer (Fig. 7) indicates that Hb A2 inhibits Hb S polymerization slightly less than Hb F, which corresponds to results in 0.1 M phosphate buffer reported previously by Benesch et al. (9). These differences can be explained by participation of some αδβδδδ in the formation of nuclei with Hb S at about one-eighth the efficiency of Hb S. Copolymerization with Hb S occurs because Thr667 in βδ in the αδβδδδ hybrid can interact with Val667 in Hb S. In contrast, αδβδδδ hybrid in FS mixtures are completely excluded from Hb S nucleation. Our results also offer an explanation of the slight differences between the effects of Hb A2 and Hb F on the inhibition of Hb S polymerization. These results again suggest that hydrophobic interactions between βδ donor/acceptor sites as well as communication of other interaction sites with Thr667 are critical for the formation of nuclei. These findings also reinforce our conclusions from polymerization studies of mixtures of Hb S and the Val667 Hb A2 variants, suggesting differences in inhibition of deoxy Hb S polymerization by Hb A2 and Hb F.

Even though Hb A2 inhibits polymerization of Hb S like Hb F in vitro, recent studies in transgenic mice show that expression of high levels of δ-globin with βδ-globin chains resulted in severe red blood cell shape abnormalities. These findings suggest that when overexpressed, δ chains can interact with red blood cell membranes, drastically modify their properties, and generate red blood cell membrane abnormalities (14). However, Hb A2 is more resistant to thermal denaturation than Hb A because of an additional contact at the αδδδ interface (37). Our present results showed more instability to mechanical agitation for Hb A2 δ667 and Hb A2 compared with Hb S and Hb A, respectively. These results suggest that high hydrophobicity of δ-globin chains may promote increased hydrophobicity of the αδβδδδ hybrid, which may result in acceleration of interaction of the hybrid hemoglobin with red blood cell membrane proteins. These results discourage the use of the anti-sickling properties of δ chains in gene therapy even though Hb A2 inhibits Hb S polymerization like Hb F. Although deoxygenation-induced sickling is the most easily demonstrated property of sickle erythrocytes, numerous membrane abnormalities have also been described (38). It seems likely that some of these membrane and red blood cell abnormalities in transgenic mice expressing high levels of δ and βδ chains may be related to the instability of oxy Hb S and Hb A2 that becomes apparent during mechanical agitation.

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