Role of γ87 Gln in the Inhibition of Hemoglobin S Polymerization by Hemoglobin F

(Received for publication, October 6, 1993, and in revised form, January 3, 1994)

Kazuhiko Adachi, Patrick Konitzer, and Saul Surrey

From the The Children's Hospital of Philadelphia, Division of Hematology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Previous studies suggested that γ87 Gln in hemoglobin (Hb) F is an important site for promoting inhibition of Hb S (αβγδ6  Gln → Val) polymerization by Hb F. We engineered and isolated the double mutant (Hb αβγδ6  Gln → Val, Thr → Gln) using a yeast expression system and characterized polymerization properties of this modified tetramer in an effort to clarify the role of Gln at position 87 in inhibiting Hb S polymerization. Electrophoretic mobility and absorption spectra of this double mutant were the same as that of Hb S, while oxygen affinity was higher, and effects of organic phosphates on oxygen affinity were reduced. The deoxy form of the double mutant showed a characteristic delay time prior to polymerization in vitro. The critical concentration for polymerization of the double mutant was about 1.5 times higher than Hb S, and delay and polymerization times were much longer than Hb S at the same hemoglobin concentrations. The logarithmic plot of delay time versus hemoglobin concentration for the double mutant showed a straight line that was intermediate between lines for AS and FS mixtures. These results and those of kinetics of polymerization of Hb S/double mutant mixtures indicate that substitution of Gln for Thr at position 87 in Hb S prolongs delay time and inhibits polymerization, although the double mutant forms polymers like Hb S.

Sickle cell disease results from a Glu to Val change at the sixth position in the β-globin chain of hemoglobin (Hb). This substitution of a hydrophobic for a polar residue at the β6 position on the Hb surface results in polymer formation after deoxygenation. It is clear that Hb F has a marked inhibitory effect on polymerization. It is evident that Hb F is effective in inhibiting Hb S polymer formation (4). In contrast, hybrid hemoglobin in mixtures of Hb A and Hb S (αβγδ6  Gln → Val, Thr → Gln) appears to be incorporated into Hb S polymers (4, 10). Thus, amino acid differences between these non-α globin chains facilitate inhibition of Hb S polymerization. Previous studies suggested that γ87 Gln and γ80 Asp in Hb F are important sites for inhibition of Hb S polymerization (3). β87 Thr is located in the translateral contact area of Hb S polymers, whereas β80 Asp is not one of the proposed contact points (11).

Hb F and Hb Aβ are equally effective in promoting inhibition of Hb S polymerization in vitro (2, 5, 12). Since δ and β chains share high amino acid homology, potential sites facilitating inhibition of Hb S polymerization can be more readily implied. Therefore, proper studies with naturally occurring hemoglobin variants including Hb Lepore (6G β80) showed that 622 Asp and 887 Gln are important inhibitory sites (3). Thus, differences at position 87 between Thr in β and Gln in γ or δ chains may be crucial to understanding the inhibition of Hb S polymerization by Hb F. In this report, we present studies using a recombinant DNA-engineered hemoglobin tetramer containing Gln → Val at the β6 position as well as Thr → Gln at the β87 position. We have characterized polymerization properties of this modified tetramer and performed polymerization studies of mixtures of Hb S and this double mutant in an effort to clarify the role of γ or 887 Gln in promoting inhibition of Hb S polymerization.

MATERIALS AND METHODS

The expression vector pGS389 was engineered to contain the full-length human α- and β-globin cDNAs under transcriptional control of dual GGAP promoters, as well as a partially functional yeast LEU2d gene and the URA3 gene for selection in yeast (13, 14). The plasmid pGS188 β contains a single GGAP promoter and the β-globin cDNA, and was constructed by mutagenesis and subsequent screening of the Xhol fragment containing the β-globin cDNA from pGS188β into pBluescript SKII as described previously (14).

We used our previously described PCR-based mutagenesis protocol involving homologous recombination in the shuttle vector pGS188β as a template for mutagenesis to engineer a β87 Thr → Gln mutation into β-globin cDNA (14). Primers used for recombination PCR were as follows: primer 1 (5'-ctcaagagttggtgggtagaaag-3'), primer 2 (5'-CTCAATGGACGtattgagtc-3'), primer 3 (5'-ctcaggtcctttctccc-3'), and primer 4 (5'-CTCACTCAAGttggaagtttctt-3'). Capital letters indicate complementary overlapping regions of primers 2 and 4. Primers 1 and 2 were used for PCR with Ssrldigested pGS188, while primers 3 and 4 were used with Ssp1-digested pGS188. The two PCR products were mixed and directly transformed into BRL Max competent Escherichia coli DH5α cells. Uptake of PCR products followed by direct recombination in vivo results in formation of complete circles with the desired induced mutation (14, 15).

β8 Val/β87 Gln-containing colonies were screened for by loss of Ban3I and DraIII sites and then subjected to DNA sequence analysis of the β-globin cDNA using site-specific primers and fluorescently tagged terminators in a cycle-sequencing reaction in which extension products were analyzed on an automated DNA sequencer (14, 16). The mutated β-globin cDNA region was then excised by Xhol digestion and subcloned back into the Xhol site of the expression vector pGS3889.

* This research was supported by National Institutes of Health Grants HL32908 and P60 HL38832 and by a grant from the American Heart Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: The Children's Hos-
pital of Philadelphia, Division of Hematology, University of Pennsylvania School of Medicine, 34th St. & Civic Center Blvd., Philadelphia, PA 19104. Tel.: 215-590-3576; Fax: 215-590-3623.

• The abbreviations used are: Hb, hemoglobin; PCR, polymerase chain reaction.

9562
Inhibition of Hb S Polymerization by Thr → Gln Change at β87

Yeast growth, plasmid transformation, and induction of Hb were described previously (14). Native Hb S was purified from hemolysates from AS and SS individuals using CM-52 cation exchange chromatography (17). Hemoglobinogens made in yeast were further purified by fractionation on a Mono S column using fast protein liquid chromatography, and the β mutation was directly confirmed by N-terminal amino acid sequence analysis purified β chain using a Pharmacia LKB Biotechnology Inc. HR 5/10 Pro RPC prepacked column (C5/C18, 5-μm particle size support) as described previously (14, 17). The β-globins were digested with trypsin (TPCK-treated, Sigma) in 50 mM NH₄HCO₃, with 2 mM urea at 37 °C for 4 and 8 h, and the β87 Thr → Gln substitution was confirmed by mass spectral analysis of tryptic peptides using a LASER-MAT, matrix-assisted laser desorption/ionization mass analyzer (Finigan, San Jose, CA).

Cellulose acetate electrophoresis of Hb solutions was performed at pH 8.6 using Supro-Heme buffer (Helena Laboratories, Beaumont, TX), and Hb concentration was determined spectrophotometrically on a Hitachi U2900 spectrophotometer using millimolar extinction coefficients of 13.5 at 541 nm for oxy-Hb and 13.4 at 540 nm for carbonmonoxy-Hb (18). Oxygen dissociation curves were determined in 0.1 mM phosphate buffer, pH 7.1, at 20 °C using a Hemox Analyzer (TCS Medical Co., Huntington Valley, PA) (14, 17). Kinetics of polymerization of deoxy-HbS was performed in 1.8 mM phosphate buffer, pH 7.4, at 30 °C as described previously by the temperature jump method from 0 to 30 °C (19).

RESULTS

PCR-based Mutagenesis—After generation of PCR products with overlapping homologous ends and transformation into E. coli, β Val-β87 Gln-containing colonies were screened for by loss of a Bsu36I site, which is destroyed by the β6 Val-β87 Gln-containing colonies with overlapping homologous ends and transformation into E. coli. β Val-β87 Gln-containing colonies were screened for by loss of a Bsu36I site, which is destroyed by the (β6 Val) (14). DNA from plasmid mini-preps containing the appropriate fragment pattern were then subjected to automated DNA sequence analysis to directly confirm the changes and to ensure that no additional PCR-induced errors were present in the rest of the β-globin cDNA. Sequence analysis revealed no undesired mutations. Hb tetramers containing the β6 and β87 substitutions were then expressed in yeast, and to ensure that no additional PCR-induced errors were present in the rest of the β-globin cDNA. Sequence analysis revealed no undesired mutations. Hb tetramers containing the β6 and β87 substitutions were then expressed in yeast, and soluble hemoglobin was purified as described previously (14, 17).

Characterization and Oxygen Binding Properties of the Double Mutant—The purified double mutant, αβS Val-β87 Gln (Hb β6 Val, β87 Gln), migrated as a single band and had the same electrophoretic mobility as normal human Hb S on cellulose acetate electrophoresis at pH 8.6 (Fig. 1). Elution profiles after fast protein liquid chromatography for heme as well as α- and β-globin chains from the double mutant were identical to that of Hb S (14). Sequence of the 10 N-terminal amino acids for the double mutant was also confirmed by Edman degradation using an automated protein microsequencer after separation of individual globin chains (14, 17). Both α- and β-globin chains had correctly processed N termini, and their 10 N-terminal sequences were identical to α- and β-globins. Mass spectral analysis of tryptic peptides from the double mutant compared with native Hb S showed a similar pattern except for fragment TpX-TpXI (Fig. 2). Under these conditions, TpX-TpXI is not completely cleaved by trypsin. The signal at mass (m/z) 2526.5 in β corresponding to TpX-TpXI (expected value, 2528.8; fragment β83–104) is increased to 2556.4 in the double mutant (expected value, 2555.8). This mass increase corresponds to that expected for substitution of Gln for Thr at β87. The absorption maximum and extinction coefficient of the double mutant in the UV-visible range for the ox, carbonmonoxy, and deoxy forms were the same as the corresponding values for recombinant Hb S (14, 18). Oxygen binding properties of the double mutant, however, differed from native and recombinant Hb S (Table I). The $P_{50}$ value for the double mutant was 3.5 compared with 6.5 for Hb S. In addition, 2,3-diphosphoglycerate at 1 mM had no effect on the $P_{50}$ value for the double mutant, while the $P_{50}$ for Hb S increased 20%. Inositol hexaphosphate at 1 mM also increased $P_{50}$ for the double mutant only 1.4-fold compared with 5.4-fold for Hb S.

Kinetics of Polymerization for the Double Mutant—Deoxy Hb S polymer formation is characterized by a delay time prior to polymerization in vitro (19). The length of the delay time depends on hemoglobin concentration; the higher the hemoglobin concentration, the shorter the delay time (Fig. 3a). The kinetics of polymerization of the double mutant also showed a characteristic delay time prior to polymer formation with the length of the delay time depending on hemoglobin concentration (Fig. 3b). More than twice the hemoglobin concentration was required for the double mutant to exhibit a delay time similar to that of Hb S. Furthermore, the double mutant required more time than Hb S to complete polymerization. For Hb S, polymerization time is similar in length to the delay time, while for the double mutant, polymerization time was always longer than delay time. For example, delay and polymerization times for Hb S at 80 mg/dl in 1.8 mM phosphate buffer were 22 and 18 min, respectively (A in Fig. 3a), while corresponding values for the double mutant at 180 mg/dl were 25 and 100 min, respectively (A in Fig. 3b). The logarithmic plot of delay time versus hemoglobin concentration for the double mutant showed a straight line that was shifted right from the line of Hb S (Fig. 4). The line for the double mutant was intermediate between those of AS and FS mixtures (20, 21). In addition, the slope of the line for the double mutant (2.5) was less than that for Hb S (3.5). These data suggest that substitution of Gln for Thr at β87 in β inhibits nuclei formation and polyionization, although the double mutant clearly forms polymers in vitro like deoxy Hb S.

Kinetics of Polymerization of Mixtures of Hb S and the Double Mutant—Polymerization of 1:1 mixtures of Hb S and the double mutant was also evaluated and compared with 1:1 Hb S/Hb F mixtures in order to further understand the role of β87 Gln in inhibiting Hb S polymer formation. Hb S/Hb F mixtures form FS hybrids, which have high solubility and a prolonged delay time prior to polymerization when compared with Hb S (6, 21).
Inhibition of Hb S Polymerization by Thr → Gln Change at β87

Figure 2. Mass spectral analysis of tryptic digest from βS and β-chain from the double mutant. Mass of tryptic peptides (2 μM) from native βS (A) and the β-chain from the double mutant (B) after 8 h of digestion were analyzed on a Lasermat mass spectrometer.

Table I

<table>
<thead>
<tr>
<th>Hb</th>
<th>αβGlu-Val, 87 Thr-Gln</th>
<th>αβGlu-Val, 87 Thr-Gln</th>
</tr>
</thead>
<tbody>
<tr>
<td>βS</td>
<td>6.5</td>
<td>2.3-DPG</td>
</tr>
<tr>
<td>β7</td>
<td>3.5</td>
<td>3.5</td>
</tr>
</tbody>
</table>

2,3-DPG: 2,3-Diphosphoglycerate

The logarithmic plots of delay time versus hemoglobin concentration for the FS mixture showed a straight line like deoxy Hb S; however, the line for the PS mixture was right shifted ~0.55 units on the x axis from the line for deoxy Hb S (Fig. 4).

When Hb S and the double mutant in the oxy state are mixed, tetramers no doubt rapidly dissociate into dimers, which then reassociate randomly to form hybrid hemoglobin (αβGlu-Val, 87 Thr-Gln). Less than 50% of the total hemoglobin in the mixture is expected to form hybrid, since the oxygen affinity of the double mutant is higher than that of Hb S. This difference could result in differences in dissociation rates of αβ dimers for the double mutant. Polymerization studies of the mixture revealed a delay time whose length also depended on hemoglobin concentration.

Figure 3. Kinetics of polymerization. Time course of polymerization for Hb S (A) and the double mutant (B) were performed using 1.8 M phosphate buffer, pH 7.4, at 30 °C by the temperature jump method (6). a, Hb S, 80 mg/dl (A) and 98 mg/dl (B); b, the double mutant, 180 mg/dl (A) and 225 mg/dl (B).

Figure 4. Relationship between log of reciprocal delay time and Hb concentration. Polymerization studies of hemoglobins at different concentrations were performed in 1.8 M phosphate buffer, pH 7.4, at 30 °C. Symbols O, ■, ▲, and □ refer to native Hb S, the double mutant, equal mixtures of Hb S and the double mutant, and equal mixtures of Hb S and Hb F, respectively.

Polymerization times for the mixture were longer than those of deoxy Hb S and not delayed like the double mutant (Fig. 5). Logarithmic plots of delay time versus hemoglobin concentration of the mixture showed a straight line that shifted right 0.26 units from the line for deoxy Hb S (Fig. 4). Under the same conditions, the logarithmic plots of delay time versus hemoglobin concentration of equal mixtures of Hb A and Hb S shifted right 0.3 units from the Hb S line (20), suggesting that like the AS mixture, about half of the total hemoglobin in the Hb S/double mutant mixture is important in determining delay time.

Total polymer formed as a function of hemoglobin concentration was also determined by monitoring turbidity at the end of polymerization. Turbidity of mixtures of the double mutant and Hb S as well as the double mutant alone increased linearly with increases in initial hemoglobin concentration (Fig. 6). The slope
of the line for the double mutant alone, however, was 35% that of Hb S. The line for the Hb S/double mutant mixture shifted right from the Hb S line, but its slope was the same as that of Hb S. The critical concentrations for polymerization (measured by extrapolation of the line to zero turbidity) for Hb S, the double mutant, and equal mixtures of these two hemoglobins were 40, 60, and 90 mg/dl, respectively. The line for 1:1 FS mixtures shifted farther right from the line for the Hb S/double mutant mixture, and the slope of the line for the FS mixture was 55% that of Hb S alone.

**DISCUSSION**

**Oxygen Affinity of the Double Mutant**—Our previous studies showed that after fast protein liquid chromatography purification, the oxygen affinity of Hb A and Hb S made in yeast was similar to native human Hb A (14). The double mutant (α2β2 Val* Glu*) made in yeast had a higher oxygen affinity and was less affected by organic phosphates than Hb S (Table 1). It is not readily obvious why the change at position β87, which is located on the exterior of the hemoglobin molecule (22), should increase oxygen affinity of the double mutant. Hb A8, which has the same β87TQ mutation, has a slightly higher oxygen affinity than Hb A (23). Furthermore, Hb Lepore Boston, which has the same amino acid content as the δ chain from its N terminus to position 115 and Glu at position 87, has a higher oxygen affinity than Hb A, and its P50 value is ~55% that of Hb A (23).

Oxygen affinity of Hb F is similar to that of Hb A, although effects of organic phosphates on Hb F are less than that of Hb A (24). Effects of organic phosphates on Hb Lepore were also less than those of Hb A (23).

Although β87 Thr in Hb A is not an organic phosphate binding site, it is located near β82 His, which is a 2,3-diphosphoglycerate binding site (22). Proximity of β87 Thr to this binding site could explain the altered affinity for organic phosphates. β87 Thr on the F helix (F3) is located near the heme pocket but does not directly interact with heme (22). Since β88 Leu (F4) does directly contact the side chain of the heme group, substitution of Glu for Thr at position 87 may affect F helical structure. Hemoglobin Quebec-Chori, a naturally occurring variant with a Thr to Ile change at β87, shows similar oxygen affinity and effects of 2,3-diphosphoglycerate compared with Hb A (25). Furthermore, Hb Valletta, a naturally occurring variant with a Thr to Pro change at β87, is quite stable, while Hb Santana with a Leu to Pro change at β88 is very unstable, presumably because of tertiary structural changes induced by the β88 Pro substitution (26). Further studies are now needed to define the molecular basis for reduced oxygen affinity and altered response to organic phosphates by the β T87Q substitution.

**Effect of β T87Q Substitution on Polymerization of Hb S**—Our present studies show that substitution of Glu for Thr at β87 in Hb S inhibits polymerization, even though the double mutant does polymerize like Hb S. The β87 position in 1-β is involved in a lateral contact with β9 Ser, β10 Ala, and β13 Ala in 1-β in Hb S fibers (11). Furthermore, the β87 position in 2-β is also involved in a lateral contact with β10 Ala and β13 Ala in 1-β. Previous studies showed that higher hemoglobin concentrations were required for gelation of mixtures of Hb S and Hb D Ibadan (Hb β T87K) than for mixtures of Hb A and Hb S, suggesting that substitution of Lys for Thr at β87 inhibits polymerization (3). In contrast, mixtures of Hb Quebec-Chori (Hb β T87L) and Hb S accelerate polymerization compared with mixtures of Hb A and Hb S, suggesting that substitution of Ile for Thr at β87 accelerates polymerization (25). These results are in agreement with x-ray analyses of Hb S polymers, which define β87 as a critical residue for polymerization (11). Our results on the kinetics of polymerization of the double mutant also suggest that nucleation and polymerization processes are inhibited compared with Hb S. Substitution of Glu for Thr at β87 in Hb S may directly affect lateral interactions, which then result in inhibition of polymerization. Whether this substitution also leads to structural perturbations at β88 Leu, which affect interactions between β6 Val in 2-β and β88 Leu in 1-β, is not clear.

Studies on gelation of deoxygenated mixtures of sickle and non-sickle hemoglobins have provided important information about contact points between neighboring molecules in sickle polymers. Gelation studies of mixtures of Hb S and a variety of β-chain variants show that all eight known variants with changes at intermolecular contact sites on polymers show altered polymerization compared with mixtures of Hb S and Hb A (27, 28). These studies, however, often fail to provide an unambiguous structural interpretation, since it is not clear whether the contact is in cis or in trans to β6 Val. Furthermore, these mixtures contain hybrid tetramer (αβββ) as well as the two parent hemoglobin, and effects of the β-chain substitution on dimer/tetramer interactions in the hybrid during polymerization of Hb S are not clear. More definitive information is now attainable studying polymerization of Hb S with second site mutations at important polymer contact sites.

Studies with several naturally occurring sickle double mutants, such as Hb C_Harlem (α2β2 Glu* Val*, 73 Asp* Asp*), Hb S Antilles (α2β2 Glu* Val*, 23 Val* Ile*), and Hb S Osman (α2β2 Glu* Val*, 121 Glu* Lys*) show dramatically different effects on polymerization (29-33). Hb C_Harlem inhibits, while Hb S inhibits.
Antilles, Hb S Osman, and the recombinant double mutant (αβγδ Gla - Val, 23 Val - Ile) accelerate polymerization (30-34). Kinetics of polymerization in the 1.8 M phosphate buffer for the recombinant double mutant (αβγδ Gla - Val, 23 Val - Ile) and Hb C Harlow showed a straight line for the logarithmic plots of delay time versus hemoglobin concentration like deoxy Hb S (30, 34). The line for the double mutant was, however, left shifted ~0.3 units on the x axis from the line of deoxy Hb S, while the line for Hb C Harlow was right shifted more than 0.5 units (30, 34), β23 Val and β73 Asp are involved in axial and lateral contacts, respectively, in Hb S fibers (11), and changes at these sites obviously influence polymerization.

The line for the double mutant (αβγδ Gla - Val, 87 Thr - Gln) in our studies was shifted right from the previously defined line for AS mixtures (20). Earlier studies showed the minimum gel concentration for mixtures of Hb S and C Harlow was the same as that for mixtures of Hb A and Hb S but was lower than that of Hb C Harlow alone (33). These general findings are analogous to our results in which the line for the logarithmic plot of delay time versus hemoglobin concentration for the double mutant (αβγδ Gla - Val, 87 Thr - Gln) mixture was similar to AS mixtures but was more left shifted than the line for the double mutant alone. These results suggest that only one β87 position in the hybrid tetramer (αβγδ Gla - Val, 87 Gln) is involved in nucleation prior to polymerization. Our results using mixtures of Hb S and the double mutant indicate that polymerization of the hybrid (αβγδ Gla - Val, 87 Thr β2 Val, 87 Gln) is intermediate between Hb S and the double mutant alone. X-ray diffraction studies suggest that only one of the two β6 Val positions in any tetramer participates in an intermolecular contact in the polymer (35). Therefore, β6 Val, 87 Thr in the β1 chain of the hybrid, which is located in trans to the double mutant β2 chain (β2 Val, 87 Gln), might behave like Hb S upon polymerization. β6 Val in the β2 chain of one hybrid tetramer could insert into the hydrophobic pocket created by a β1 chain of an adjacent tetramer upon deoxygenation or β87 Thr in the β1 chain could participate in a lateral polymer contact. In contrast, β6 Val, 87 Gln in the hybrid, which is located in trans to the active β6 Val, might behave like the double mutant in that β87 Gln would inhibit nucleation prior to polymerization. Our results using equal mixtures of Hb S and the double mutant also indicate that the length of the delay time prior to polymerization is determined by ~55% (10^-26) of the total hemoglobin concentration (Fig. 4). Therefore, in these mixtures, Hb S and one-half of the hybrid would then contribute similarly to initiate polymerization.

In contrast, the line for the delay time versus hemoglobin concentration for FS mixtures is shifted right ~0.55 from the Hb S line, suggesting that only 28% (10^-34) of the total hemoglobin in solution contributes to length of the delay time. Equal mixtures of Hb S and Hb F contain 25% Hb S, 50% hybrid (αβγδ), and 25% Hb F. Therefore, in FS mixtures, delay time length can be totally attributed to Hb S, which represents ~25% of the total hemoglobin concentration. These results suggest that nuclei formation in FS mixtures is dependent on the concentration of Hb S and that FS hybrids as well as Hb F appear to be excluded from nuclei formation. Our results on polymerization of the double mutant alone as well as mixtures of Hb S and the double mutant suggest that Gln at position 87 in the γ chain in Hb F is a key amino acid required for inhibition and exclusion of FS hybrids from Hb S nucleation prior to polymerization.

The slope of total polymer formation as a function of hemoglobin concentration for the Hb S/double mutant mixture was the same as that for Hb S alone. This result suggests that polymer in the mixture includes the total amount of hybrid as well as Hb S. This interpretation appears to conflict with our conclusions based on delay time determination in which only half of the hybrid is thought to be involved in nucleation.

If half of the hybrid were totally excluded from polymer in the mixture, then in studies of total polymer formation as a function of hemoglobin concentration, the slope of the line for the mixture should be half that of Hb S alone. Our finding that Hb S alone and the mixture lines have the same slope suggests that a portion of Hb S/double mutant hybrid as well as the double mutant participate in polymer formation after nucleation.

Similarly, the slope of the line for the FS mixture should be a quarter of that for Hb S alone, if polymer were formed from Hb S alone in FS mixtures. Hb S represents 25% of total hemoglobin in the mixture. Under these conditions, the FS hybrid and Hb F in the mixture should be completely excluded from polymer. However, the slope of the line for the FS mixture was 55% of Hb S alone, again suggesting that some of the FS hybrid also contributes to polymer formation. Our previous studies in high phosphate buffer and other studies in low phosphate buffers indicate that cross-linked FS hybrid hemoglobin can indeed copolymerize with Hb S (5, 30). Results from measurements of delay time length, which is a measure of time required for nuclei formation prior to polymerization, however, suggest exclusion of FS hybrids from nuclei formation.

Results on total polymer formed as a function of hemoglobin concentration suggest inclusion of some of the FS hybrid in polymer formation. This apparent discrepancy between exclusion and inclusion of FS hybrid hemoglobin in polymer could be possibly reconciled, if homogeneous nucleation and polymerization were treated as distinctly separable steps (27, 37). Monomers predominate during the nucleation phase, and after sufficient nuclei form, monomers then associate with nuclei to form polymers (27). Initiation of polymerization would then be dependent solely on Hb S concentration in the FS mixture; however, once polymerization started, then some of the FS hybrid tetramers could participate in and also lead to a partial inhibition of polymer formation. Detailed structural analyses of polymers formed in these mixtures are now required to test this hypothesis.

Acknowledgments—We thank Dr. Eric Rapoport and members of the Nucleic Acid/Protein Core at the Children's Hospital of Philadelphia for oligonucleotide syntheses, protein characterization, and automated DNA sequence analysis. We also thank Dr. D. Speicher at the Wistar Institute for mass spectral analysis of tryptic peptides.

REFERENCES

Inhibition of Hb S Polymerization by Thr → Gln Change at β87