Inhibition of Sickle Hemoglobin Gelation by Amino Acids and Related Compounds†

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ABSTRACT: The effects of amino acids, several aromatic compounds, and peptides on the gelation and solubility of deoxyhemoglobin S have been studied. The aromatic amino acids (tryptophan, phenylalanine, and possibly tyrosine) significantly inhibited the rate of gel formation and increased solubility. The dipeptide L-Thr-L-Phe, the tripeptide L-Lys-L-Phe-L-Phe, and various phenylalanine analogues (hydrocinnamic acid, phenethylamine, benzamine, and amphetamine) also inhibited gelation. However, aromaticity is not a sufficient condition for inhibiting gelation as shown by the fact that several aromatic compounds (acetylsalicylic acid, salicyclic acid, aniline, and phenol) enhanced gelation. Surprisingly, several oligopeptides (β^S1-12, β^S4-8, β^S3-13, and β^S4-10) also enhanced gelation. All of these additives follow the supersaturation relationship that the delay time for gelation is proportional to the ratio of the total hemoglobin concentration to the solubility of deoxyhemoglobin S to the nth power ($n \sim 35$). A possible mechanism for the action of these inhibitors is considered in terms of a specific site of interaction on the hemoglobin molecule. Although none of these compounds may prove to be efficacious in treatment of sickle cell anemia, they should yield information about the structure and process of formation of the deoxyhemoglobin S gel.

Sickle hemoglobin under physiological conditions and concentration aggregates upon deoxygenation to form a viscous gel composed of long fibers consisting of filaments of stacked rings (Finch et al., 1973; Dykes et al., 1978), of which several detailed structures have been proposed. The gel is sensitive to changes in temperature, pH, hemoglobin S concentration, solvent, and presence of other hemoglobins. For a given concentration, lowering the temperature will melt the gel. By warming the sample to the original gel temperature the hemoglobin S will again aggregate after a delay period (t_d) (Hofrichter et al., 1974; Malfa & Steinhardt, 1974; Moffat & Gibson, 1974; Ross et al., 1975). At a given temperature, the delay time is related to the solubility of deoxyhemoglobin S $[HbS]_0$ to the nth power (Hofrichter et al., 1974)

$$\frac{1}{t_d} \propto ([HbS]/[HbS]_0)^n$$

([HbS] is the total hemoglobin S concentration).

Much recent work has been done with compounds, such as cyanate salts (Cerami & Manning, 1971), dimethyl adipimidate (Lubin et al., 1975), and nitrogen mustard (Roth et al., 1972) which react to covalently modify the hemoglobin molecule. An alternate approach is the use of less reactive molecules which would be expected to interact reversibly with the hemoglobin molecule (for a review see Dean & Schechter, 1978). We have previously reported studies on amino acids as inhibitors of gelation which demonstrated that phenylalanine increased deoxyhemoglobin S solubility and increased the corresponding delay time (Noguchi & Schechter, 1977). The other amino acids that were included in that study did not significantly inhibit gelation. The increase in deoxyhemoglobin S solubility by phenylalanine was found to be about 2.5 times that of urea on a molar basis.

We report here our results on the effects of the 20 amino acids and homoserine on hemoglobin S solubility and gelation kinetics. We have found that only the aromatic amino acids (phenylalanine, tryptophan, and possibly tyrosine) significantly inhibit gelation, with tryptophan being the most effective. Several simple aromatic compounds and oligopeptides from the β^S sequence surprisingly enhanced gelation. However, we did find that a number of phenylalanine analogues and a di- and tripeptide containing phenylalanine inhibited gelation, with about the same efficacy as phenylalanine alone. The inhibiting effects of the phenylalanine containing peptides and phenylalanine analogues may indicate competitive interference with an intermolecular contact site in the deoxyhemoglobin S gel, but other mechanisms cannot now be excluded.

Materials and Methods

Hemoglobin S was prepared from whole blood obtained from donors homozygous for hemoglobin S. Washed, packed red blood cells were lysed with distilled water. The NaCl concentration of the lysate was increased to 0.5 M, the lysate was spun at 30 000g for 30 min at 5 °C, and the pellet containing the cell membranes was discarded. For experiments using hemolysate (tri- and dipeptides) instead of chromatographed hemoglobin S, the lysate was spun at 100 000g for 30 min at 5 °C before the membrane pellet was discarded. Hemoglobin S was further purified by ion-exchange chromatography on DEAE-Sephadex A-50 as described by Huisman & Dozy (1965). Hemoglobin S was then concentrated by ultrafiltration and vacuum dialysis, and dialyzed into 0.15 M potassium phosphate buffer, pH 7.35.

The L-amino acids alanine, cysteine, glutamic acid, glutamine, glycine, isoleucine, leucine, lysine hydrochloride, phenylalanine, proline, and valine were obtained from Fox Chemical Co.; aspartic acid, methionine, serine, threonine, tyrosine, and homoserine were from Calbiochemicals; histidine hydrochloride and tryptophan were from Sigma; arginine was from Mann Research Laboratory; asparagine was from Eastman Organic. The D-amino acids (phenylalanine, tryptophan, and tyrosine) and dipeptides (L-threonyl-L-phenylalanine and L-phenylalanyl-L-alanine) were purchased from Vega Fox Biochemicals. Stock solutions of the L-amino acids, D-amine acids, dipeptides and other additives (aniline, phenol,

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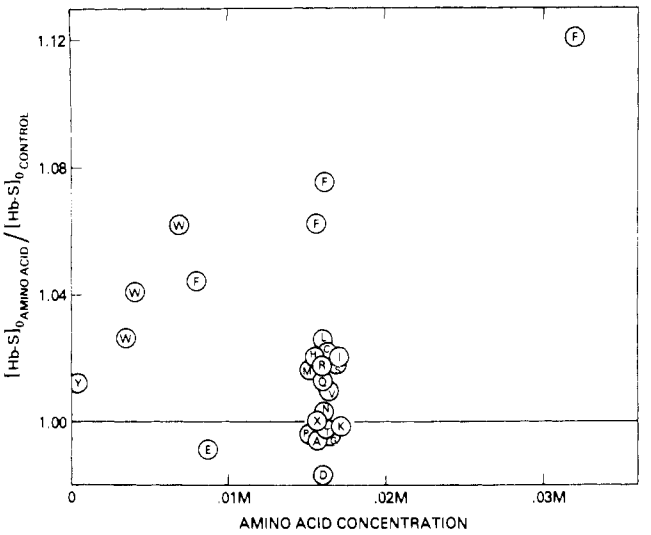

FIGURE 1: Effects of L-amino acids on deoxyhemoglobin S solubility at 30 °C. The data are expressed as ratios of the solubility with each amino acid to the control solubility determined simultaneously for each set of data. Each data point represents duplicate samples (except for one phenylalanine point at 1 mM). The standard one-letter amino acid abbreviations are used (X represents homoserine).

acetylsalicylic acid, salicylic acid, phenethylamine, hydrocinnamic acid, benzylamine, *d*-amphetamine sulfate (Sigma), and colchicine (Aldrich)) were made by dissolving the additives in potassium phosphate buffer, 0.15 M, pH 7.35, and readjusting the final pH with concentrated KOH or HCl when necessary.

The β^S4–8, β^S3–13, and β^S4–10 peptides were synthesized following the Corley et al. (1972) modification of the Merrifield procedure as outlined by Eastlake et al. (1976) and were a gift from A. Eastlake Dean. The tripeptide L-Lys-L-Phe-L-Phe, a gift from J. W. H. Sutherland and A. Eastlake Dean, was similarly synthesized.

The tripeptide was purified by a gel filtration (Bio-Gel P2, Bio-Rad Laboratories) and the amino acid analysis of the final product hydrolyzed with constant boiling HCl (5.7 N) for 20 h at 110 °C yielded a lysine:phenylalanine ratio of 1:2.05. The β^S1–12 peptide was synthesized following the Merrifield solid phase methodology and was a gift from Drs. R. Shepherd and M. F. Perutz.

The sample tubes, kinetic measurements, and solubility determinations were made following the procedure described by Hofrichter et al. (1976a). The sample tubes (quartz electron paramagnetic resonance tubes [PQ 701 Wilmad Glass Co.] cut to 45 mm) were loaded under nitrogen with predetermined amounts of concentrated hemoglobin S solution, buffer, and additive stock solution. Deoxygenation of the sample was achieved by addition of a similarly buffered solution of sodium dithionite (Vine Chemicals Limited, Manchester, England) resulting in a final dithionite concentration of 0.05 M. The tubes were sealed with pressure caps (521PC, Wilmad), and placed in an ice bath with occasional mixing to obtain a homogeneous deoxyhemoglobin S solution. To improve oxygen impermeability, the caps were then coated with varnish (Glyptal 1201 Red Enamel, General Electric Co.). The sample tubes were stored in nitrogen filled vacutainers at 5 °C on a rotating wheel to ensure homogeneity of the sample. The final deoxyhemoglobin S concentration in the tubes was determined spectrophotometrically in a Cary 17 using optical density readings at 910 and 1090 nm ([Hb-S] = (OD 910 to 1090 + 0.04) 24.56 g/dL). Teflon spacers were used to center the tubes in a rectangular quartz cuvette. Water was used to fill the void between the cylindrical sample tube and the rectangular cuvette. Samples with spectra which deviated from the expected deoxyhemoglobin S spectrum in the near-infrared region (1300 to 700 nm), indicating the presence of methemoglobin, were discarded.

Kinetic measurements were performed by transferring the samples from an ice bath into a 30 °C temperature bath for 1 min, putting the sample tubes into a thermally controlled Gilford 2000 spectrophotometer equilibrated to the same temperature, and monitoring turbidity at 800 nm. Again, Teflon spacers were used to center the tubes in the rectangular cuvettes and water was used to fill the void between the tube and cuvette. The time between the temperature jump and the onset of turbidity change is defined as the delay time (t_d).

The solubility of deoxyhemoglobin S ($[HbS]_0$) was determined by spinning the gelled sample at 100 000*g* for 3 h at 30 °C temperature. After ultracentrifugation, the tubes were vigorously shaken to obtain homogeneity of the supernatant. The solubility or supernatant concentration was determined spectrophotometrically using optical densities at 910 and 1090 nm as described above.

In the process of testing the effect of numerous additives on gelation, we found that hemolysates from homozygous S individuals could be used in the Hofrichter-Ross-Eaton assay. Extra precautions were taken to minimize membrane debris and the hemolysate was checked to contain less than 2% fetal hemoglobin. Delay times could be monitored on these samples, were well-behaved, and corresponded to the relative shifts in solubility. Although the actual deoxyhemoglobin solubility was higher than pure deoxyhemoglobin S due to small amounts (less than 2%) of deoxyhemoglobin A_2 and F present in the hemolysate, the relative increases and decreases in solubility were comparable and screening of a large number of additives was facilitated. Results for the tri- and oligopeptides are given for SS hemolysate samples.

Results

The total hemoglobin concentration in the sample tubes varied from 21.7 g/dL to 27.7 g/dL. For 27 control samples (no additive) the solubility of pure deoxyhemoglobin S at 30 °C was found to be 16.40 ± 0.26 g/dL. Additives taken from concentrated stock solutions were added to the test samples to yield a final additive concentration of 16 mM (lower final concentrations were used for less soluble additives as noted), approximately equal to the concentration of heme-containing monomer. The effect of L-amino acids on the solubility of deoxyhemoglobin S at 30 °C is shown in Figure 1. The data are expressed as the ratio of the solubility with additive to the control solubility. A ratio greater than 1 represents an increase in deoxyhemoglobin S solubility and an inhibition of gelation, whereas a ratio less than 1 represents a decrease in solubility and enhancement of gelation. The addition of L-phenylalanine increased the solubility by 7% at 16 mM and by 12% at 32 mM, levels which are significantly higher than those observed for the controls. The inhibition effect of L-tryptophan is even greater, increasing the solubility by 6% at 7 mM. In fact, the changes in solubility due to tryptophan appear to be at least twice that of phenylalanine. L-tyrosine also appears to inhibit gelation, although concentration studies were limited due to low solubility of tyrosine.

Although the effects of the other L-amino acids are not as great as the effects of the aromatic amino acids, some small perturbations in solubility do appear. At 16 mM leucine, cysteine, histidine, isoleucine, and to a lesser extent arginine tend to increase deoxyhemoglobin S solubility, while the negatively charged amino acids (aspartic acid and at 8 mM glutamic acid)

TABLE I: Effects of D-Amino Acids, Di-, Tri-, and Oligopeptides, and Some Aromatic Compounds on Deoxyhemoglobin S Solubility.

additive	[additive][a] (mM)	$[HbS]_{0\ Add}/[HbS]_{0\ Cont}$[b]
(A) D-aromatic amino acids		
D-Phe	15.8	1.046
D-Trp	8.3	1.068
		1.071
D-Tyr	0.3	1.007
		1.003
(B) Phe-containing peptides		
L-Lys-L-Phe-L-Phe	43.6	1.129
		1.132
	32	1.113
		1.113
L-Thr-L-Phe	17.4	1.074
		1.081
L-Phe-L-Ala	2.5	1.003
		1.006
(C) β^S peptides		
β^S1-12	5.5 (7.0 mg/mL)	0.989
		0.994
β^S3-13	10.9 (12.5 mg/mL)	0.954
		0.960
β^S4-8	15.4 (9.3 mg/mL)	0.967
		0.960
β^S4-10	59.2 (45 mg/mL)	0.866
		0.839
(D) Phe-like compounds		
hydrocinnamic acid	7.8	1.033
		1.033
phenethylamine	16	1.062
		1.061
benzylamine	16	1.049
		1.048
amphetamine	16	1.106
		1.099
(E) aromatic compounds		
aniline	16.3	0.923
		0.934
phenol	16.3	0.940
		0.944
acetylsalicylic acid	4.4	0.957
		0.966
salicylic acid	4	0.949
		0.938

[a] [Additive] is the final additive concentration. [b] $[HbS]_{0Add}/[HbS]_{0Cont}$ is the ratio of the deoxyhemoglobin S solubility at 30 °C in the presence of additive to the solubility at 30 °C in the absence of additive. A ratio greater than 1 indicates an increase in solubility or inhibition of gelation and a ratio less than 1 indicates a decrease in solubility or enhancement of gelation.

tend to decrease solubility. At the same concentration the other nonaromatic amino acids do not significantly alter deoxyhemoglobin S solubility. These data suggest that aliphatic side chains and charged groups on L-amino acids affect deoxyhemoglobin S solubility, but not as significantly as aromatic side chains.

The D-isomeric forms of the aromatic amino acids increase deoxyhemoglobin S solubility to the same extent as the L isomers (Table I). Hence the interactions between the aromatic amino acids and the deoxyhemoglobin S gel do not depend on the isomeric form of the amino acid. Two phenylalanine containing peptides corresponding to residues 84–85 (L-Thr-L-Phe) and 85–86 (L-Phe-L-Ala) of the β^S chain were also assayed. The β^{85} region constitutes an intermolecular contact region for the valine residue at the β^{S6} position in the deoxyhemoglobin S crystal (Wishner et al., 1975, 1976). L-Thr-L-Phe increases deoxyhemoglobin S solubility to a similar extent as phenylalanine, while L-Phe-L-Ala at 2.4 mM does not have any significant effect. (Higher concentrations of Phe-Ala were not tested because it did not readily go into solution.) The phenylalanine dipeptide results are similar to those obtained with phenylalanine alone. The tripeptide L-Lys-L-Phe-L-Phe also increased deoxyhemoglobin S solubility, but was no more effective than phenylalanine alone. Several oligopeptides from the β^{S6} mutation region, β^S1-12, β^S4-8, β^S3-13, and β^S4-10, were assayed for their antisickling potential. Surprisingly, the β^S peptides enhanced gelation, decreasing deoxyhemoglobin S solubility proportionately to the gram concentration added and decreasing the corresponding delay time of gelation. (Assays for β^S4-8, β^S3-13, β^S4-10, and Lys-Phe-Phe were carried out on SS hemolysate containing less than 2% hemoglobins A_2 and F. The effect of L-phenylalanine on the same hemolysate preparation was similar to the effect on chromatographically pure hemoglobin S.)

Aromaticity is not a sufficient condition for an inhibitory agent of gelation as indicated by the results obtained from singly substituted benzene compounds (aniline and phenol) and doubly substituted compounds (salicylic acid and acetylsalicylic acid) as shown in Table I. Acetylsalicylic acid and salicylic acid at 4 mM decrease deoxyhemoglobin S solubility by 3.8% and 5.6%, respectively, while aniline and phenol at 12 mM decrease solubility by 7.2% and 5.9%, respectively. The results obtained from testing various phenylalanine analogues are also shown in Table I. Phenethylamine and hydrocinnamic acid increased deoxyhemoglobin S solubility to the same extent as phenylalanine at the concentrations tested. Benzylamine increased the solubility to a lesser extent. The increase in solubility of 10.6% with 16 mM amphetamine is about 1.5 times the increase obtained with phenylalanine at the same concentration. Thus, by replacing the carboxylic acid group on phenylalanine with a methyl group, a more effective inhibitor of gelation is obtained.

Figure 2 shows the relationship between the kinetic data or delay time (time between temperature jump from 0 to 30 °C and the onset of gelation) and deoxyhemoglobin S solubility at 30 °C. Included are data both with and without additives. The data can be fit by a straight line with a slope of about 35. This agrees with the analysis by Hofrichter et al. (1974, 1976a) of the relationship of delay time to solubility in the presence and absence of noncovalently interacting gelation inhibitors.

Colchicine, known to inhibit microtubule assembly, was also tested at 16 mM and found to increase deoxyhemoglobin S solubility to a degree similar to that of phenylalanine.

Discussion

We have presented a systematic study of the effects of amino acids on solubility and gelation kinetics of deoxyhemoglobin S. The Hofrichter et al. (1976a) assay was chosen because both solubility, an equilibrium measurement, and rate of gelation of deoxyhemoglobin S, a kinetic measurement, are monitored. The data obtained from amino acids and related aromatic compounds followed the empirical supersaturation law, $1/t_d \propto S^n$, i.e., the delay time (t_d) is inversely proportional to the supersaturation ratio (S = total hemoglobin S concentration/deoxyhemoglobin S solubility) to the nth power, with $n \simeq 35$. This suggests that the perturbation of solubility and gelation kinetics occurs without fundamentally changing the mechanism of gelation. Similar results have been obtained from analyses of the effects of protons, CO, and urea on the

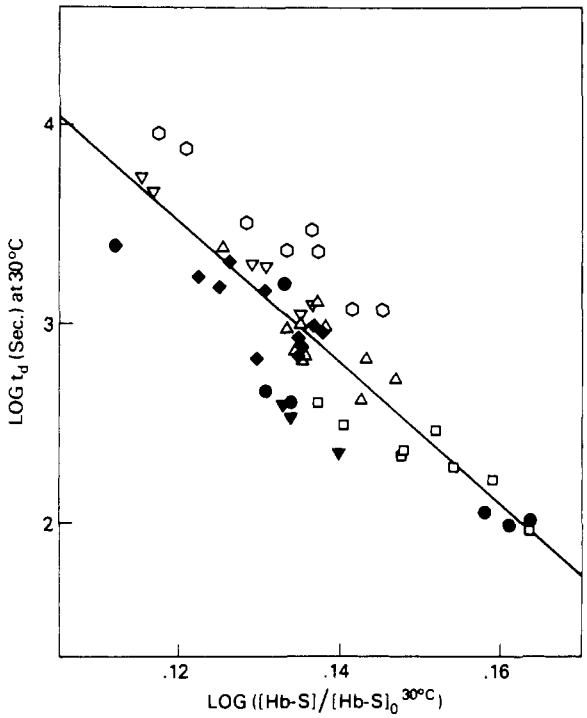

FIGURE 2: Relationship between delay time (t_d) and the supersaturation ratio (S = [HbS]/[HbS]$_0$) at 30 °C. The straight line (log t_d = 7.75 − 35.4 log S) is determined by a linear regression analysis of the data (r^2 = 0.73). The results indicate that the relationship $1/t_d = \gamma S^n$ holds for the additives tested, and that $n \approx 35$. Each symbol represents a group of additives assayed simultaneously: (●) L-homoserine and L-Phe; (□) L-Ile, L-Lys, and L-Tyr; (♦) L-Gly, L-Met, L-Pro, L-Ser and L-Trp; (△) L-Ala, L-Arg, L-Asp, L-Leu, and L-Thr; (○) L-Cys and L-Trp; (▽) L-Phe-L-Ala and L-Thr-L-Phe; (▼) acetylsalicylic acid and salicylic acid.

gelation reaction (Hofrichter et al., 1976a,b). (The value of 35 for n in the supersaturation equation is probably significantly higher than the value of the kinetic order of the reaction, due to the effects of nonideality (Ross & Minton, 1977).)

Several amino acids, asparagine, glutamine, homoserine (Rumen, 1975a,b; Kang & Benjamin, 1975), arginine, lysine, aspartate, glutamate, and arginine aspartate (Sophianopoulous et al., 1976) have been previously studied for their therapeutic effects on erythrocyte sickling and deoxyhemoglobin S gelation. However, we found that none of these amino acids inhibited gel formation as significantly as the aromatic amino acids (Figure 1).

Although the differences among the nonaromatic amino acids are of secondary significance, the differences do indicate the importance of aliphatic side chain length and side chain charged groups. The importance of side chain composition is also illustrated by the increasing effect of the phenylalanine analogues: amphetamine > phenethylamine > benzylamine. Similar dependence on aliphatic chain length has been reported for the alkylureas (Elbaum et al., 1974) and for some alcohols and other aromatic compounds (Ross & Subramanian, 1977).

Aromaticity is not a sufficient condition for inhibition of gelation as demonstrated by the decrease in deoxyhemoglobin S solubility and enhancement of gelation observed with phenol, aniline, salicylic acid, and acetylsalicylic acid. 5-Iodosalicylate has also been reported to decrease solubility (Ross & Subramanian, 1977). The enhancement of gelation by these aromatic compounds is consistent with the ability of aniline, phenol, toluene, and related compounds to facilitate crystallization of hemoglobin S in 1.9 M phosphate, pH 7.0 (Farnell & McMeekin, 1973). These results may be due to excluded volume effects or to the higher affinity of these additives for the polymer state.

To determine if longer peptides would be more effective inhibitors of gelation, di- and tripeptides containing phenylalanine were assayed (Table I). L-Thr-L-Phe and L-Phe-L-Ala are the dipeptides from the β^{85}-Phe region, suggested as a complementary binding site of the β^{S6}-Val region by X-ray crystallography data (Wishner et al., 1975, 1976). The tripeptide L-Lys-L-Phe-L-Phe has previously been reported to be many times more effective than phenylalanine in inhibiting gelation (Votano et al., 1977). We found that L-Thr-L-Phe and L-Lys-L-Phe-L-Phe could increase the solubility of deoxyhemoglobin S to a similar extent as phenylalanine. However, these phenylalanine containing di- and tripeptides were no more effective than phenylalanine alone.

Several peptides from the $\beta^{S}1$–6 and $\beta^{A}1$–6 regions were reported to increase MGC (Kubota et al., 1976; Kubota & Yang, 1977). Surprisingly, the β^{S} peptides which we assayed ($\beta^{S}1$–12, $\beta^{S}4$–8, $\beta^{S}3$–13, and $\beta^{S}4$–10) decreased deoxyhemoglobin S solubility and the corresponding delay time. The enhancement of gelation by the peptides from the $\beta^{S}1$–13 region may be due to the hydrophobic character of these peptides resulting in excluded volume effects.

From our own work it seems likely that only small differences may exist among various classes of inhibitors of gelation and that a reliable, precise assay is essential for studies of any of the numerous amino acids, amino acid derivatives, and peptides that may be considered. The present study demonstrates that a specific group of compounds, aromatic amino acids and phenylalanine analogues, can inhibit gelation and increase the solubility of deoxyhemoglobin S. Specifically, compared with urea on a molar basis, phenylalanine is about 2.5 times and tryptophan 5 times more effective. If these effects continue to be linear at higher concentrations, then 60 mM of phenylalanine or 30 mM of tryptophan would increase the delay time for gelation by 100-fold, a condition possibly sufficient to improve the clinical manifestation of sickle cell anemia (Hofrichter et al., 1976b; Sunshine et al., 1978). However, low erythrocyte membrane permeability and high required dosages might limit the use of the amino acids in treatment of the disease. For example, phenylalanine in the millimolar concentration range is toxic in individuals with phenylketonurea (Knox, 1972), although in unpublished observation (Noguchi & Noguchi), 60 mM of L-phenylalanine did not have any observable effect on human fibroblasts in tissue culture. If the aromatic amino acids are toxic at the concentrations required to inhibit gelation sufficiently, then a suitable therapeutic agent with low toxicity and high erythrocyte uptake might be found among their analogues.

Currently studies are underway to investigate the inhibitory effects of longer oligopeptides corresponding to the β^{85} contact region as well as other contact regions in the high resolution crystal structure (Wishner et al., 1975, 1976). It is likely that information about the relative inhibitory effects of the various synthetic peptides—coupled with determination of their binding sites—could be used to ascertain the energetically significant interactions in the polymer. Although none of these compounds may ever become practical therapeutic agents, a systematic study of the effects of peptide additives on gelation kinetics and solubility of sickle hemoglobin could yield information on the structure and mechanism of formation of the gel.

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