# Peptide Inhibitors of Sickle Hemoglobin Aggregation: Effect of Hydrophobicity†

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ABSTRACT: Thirty-three peptides have been synthesized which have the ability to inhibit aggregation of sickle hemoglobin which occurs upon deoxygenation. Evidence is presented which indicates that the hydrophobicity of the side chains is the predominant factor. A linear correlation exists between the capacity of peptides to inhibit gelation and the additivity of the side-chain hydrophobic contributions. Small di- and tripeptides are more responsive to changes in their nonpolar content than are larger oligopeptides whose antigelling activity is a slowly varying function of hydrophobicity. With few exceptions, charged substituent groups on the side chain do not appear to contribute to the inhibitory process. Thus, if hydrogen bonding or ionic interactions are involved with these residues, they appear to play only a secondary role in the molecular interactions responsible for inhibiting or delaying the deoxy-HbS gelation.

Many different chemical agents have been shown to inhibit deoxy-HbS gelation through noncovalent interactions. Some representative compounds are amino acids (Noguchi & Schechter, 1977), alkylureas (Elbaum et al., 1974), arylalkanes (Ross & Subramanian, 1977), and oligopeptides (Kubota et al., 1976; Kubota & Yang, 1977; Votano et al., 1977). The inhibitory mechanism lies in the ability of these agents to increase the solubility of monomeric deoxy-HbS and thereby delay the gelation process. This is consistent with studies (Hofrichter et al., 1976) on the kinetics of gelation.

Previously, we reported initial findings for several small peptides which exhibited antigelling activity (Votano et al., 1977). These peptides not only prevented HbS gelation in test tubes but they also prevented the sickling of reconstituted deoxygenated normal erythrocytes containing HbS. Because they are peptides whose degradative products are well-known, it is useful to explore these as potential therapeutic agents for sickle cell anemia, a disease produced by the aggregation of deoxy-HbS molecules. In this paper, we explore various molecular features which enhance or diminish the ability to inhibit HbS aggregation. This activity is related to features intrinsic in the peptides as well as features on the surface of the HbS molecule where they bind. Here we report the inhibitory activity of 33 peptides. In our initial publication (Votano et al., 1977), the peptides were characterized as bifunctional in that a hydrophobic residue, usually phenylalanine, was at one end and a hydrogen-bonding side chain, either arginine or lysine, was at the other end. However, there was no positive indication of which stereochemical features of these peptides influenced their antigelling activity. In the present study, we attempt to address this question by correlating peptide activity as it relates to the number and types of side-chain residues. We find that hydrophobicity of the side chains is the most important feature.

## Materials and Methods

*Hemoglobin S Preparation.* HbS was prepared from heterozygous AS blood as previously described (Votano et al., 1977). The sample was dialyzed for 16 h at 4 °C against deoxygenated buffer [120 mM sodium phosphate (pH 7.2) and 150 mM NaCl]. HbS was concentrated to 35.5 g/dL by Amicon ultrafiltration using an XM-50 membrane and then stored at 77 K. The percentage of met-HbS never exceeded 4% as determined by the method of Hegesh & Gruener (1970).

*Peptide Synthesis.* Peptides were prepared by stepwise synthesis using *N-tert*-butyloxy or *N*-carbobenzoxy amino acids and coupling them to free amino acids or peptides by using appropriate *N*-succinimide esters according to Anderson et al. (1964). Deblocking of peptides was done either by 30% HBr in acetic acid or by catalytic hydrogenation. Peptide purity was determined by thin-layer silica chromatography. Plates were run in two different solvents: BuOH–HOAc–$H_2O$ (4:1:1) and 2-BuOH–3.3% $NH_4OH$ (15:6). Purity was ascertained by both UV fluorescence and ninhydrin staining. Peptides were dissolved in $H_2O$, titrated to pH 7.2, and lyophilized to dryness.

*Gelation Test.* Determination of the peptide/HbS minimum inhibitor mole ratio (MIMR) value necessary to inhibit HbS gelation over a chosen temperature–time span was evaluated by a simple test-tube gelation technique. The test tube was inverted after a given time period, and the flow or lack of flow of a deoxygenated HbS solution was determined by making duplicate measurements. A number of studies were carried out by measuring the time between deoxygenation and gelation (delay time) as a function of peptide concentration. The results showed that at low concentrations of peptide, there was a very weak dependence upon concentration; however, at higher peptide concentrations, there was a sharp rise in the delay time over a narrow range of peptide concentration. Small changes ($\leq$15%) in the mole ratio of peptide to HbS increased the delay time over 100-fold, from minutes to hours. Thus, the assay is reasonably sensitive to changes in the stoichiometric ratio of peptide to HbS.

An initial series of gelation tests determined the approximate peptide concentration necessary for inhibition over a chosen temperature–time span. The second determination was made by varying in small increments the peptide/HbS mole ratio near the first value found in the initial trials with the exception that testing for sample flow was carried out only at the end of a 20-min time period. Samples of oxy-HbS were placed in 6 × 50 mm test tubes followed by the addition of a stoichiometric amount of peptide dissolved in the sample buffer. The test tubes were stoppered, flushed with $N_2$ (gas), agitated gently, and placed in ice. $Na_2S_2O_4$ buffered in 120 mM sodium phosphate (pH 7.2) was then added anaerobically to the

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Table I: Inhibitor Peptides and Their Hydrophobicity

| peptide no. | peptide | MIMR[a] | $-\Delta F_h$[b] (kcal/mol) |
|---|---|---|---|
| 1 | L-Phe-L-Phe-L-Arg | 17.0 ± 1.0 | 5.75 |
| 2 | Suc-L-Phe-L-Phe-L-Arg | 17.0 ± 1.0 | 5.75 |
| 3 | Suc-L-Phe-L-Phe-ε-$NO_2$-L-Arg | 16.0 ± 1.0 | 6.00 |
| 4 | Suc-L-Phe-L-Phe-ε-$NO_2$-L-Arg-$OCH_3$ | 16.0 ± 1.0 | 6.25 |
| 5 | L-Lys-L-Phe-L-Phe | 15.5 ± 1.0 | 6.25 |
| 6 | $Suc_2$-L-Lys-L-Phe-L-Phe | 14.5 ± 1.0 | 6.50 |
| 7 | Suc-L-Phe | 26.5 ± 0.5 | 2.5 |
| 8 | Suc-L-Trp | 22.5 ± 0.5 | 3.4 |
| 9 | Suc-L-Phe-L-Phe | 15.5 ± 0.5 | 5.0 |
| 10 | Suc-L-Phe-D-Phe | 15.5 ± 0.5 | 5.0 |
| 11 | Suc-L-Trp-L-Phe | 12.5 ± 0.5 | 5.9 |
| 12 | Suc-L-Trp-L-Trp | 10.0 ± 0.5 | 6.8 |
| 13 | Suc-L-Phe-L-Phe-L-Phe | 9.5 ± 0.5 | 7.5 |
| 14 | Suc-L-Phe-Gly-L-Phe | 15.5 ± 0.5 | 5.0 |
| 15 | Suc-L-Phe-Gly-L-Nle | 16.0 ± 0.5 | 5.1 |
| 16 | Gly-L-Arg | >30 | 0.75 |
| 17 | Gly-Gly-L-Arg | >30 | 0.75 |
| 18 | Gly-L-Phe | 23.0 ± 1.0 | 2.5 |
| 19 | Gly-Gly-L-Phe | 23.0 ± 1.0 | 2.5 |
| 20 | L-Phe-Gly-Gly | 22.0 ± 0.5 | 2.5 |
| 21 | Gly-Gly-L-Trp | 21.0 ± 1.0 | 3.4 |

[a] The minimum mole ratio of peptide to deoxy-HbS was determined at a final hemoglobin concentration of 27.6 g/dL, $T = 37$ °C, and in a 50 mM $Na_2S_2O_4$ solution. Peptide concentration inhibited gelation for a minimum time of 20 min. Molecular weight of HbS is taken as 64 500. [b] $\Delta F_h$ values were computed for residue side chains only.

solution to give a final concentration of 50 mM. The tube was then agitated and placed again in ice for 5 min, after which it was placed in a water bath at a given temperature. Sample flow can be determined rapidly by a simple visual examination of the inverted tube under the dissecting microscope. Failure to flow upon repeated inversion indicated gelation of the sample.

## Results

In Tables I and II are listed 33 peptides and 2 amino acids. Column 3 in each table contains the minimum inhibitor mole ratio (MIMR) of peptide to deoxy-HbS necessary to delay gelation up to 20 min at a concentration of HbS = 27.6 ± 0.3 g/dL at 37 °C. Succinylation of the $\alpha$-$NH_2$ terminus of many peptides served to enhance their solubility, to change the net charge, or to do both. The peptides were composed of at most three different amino acids out of a pool of six (Arg, Gly, Lys, Nle, Phe, and Trp).

*Charge Effects.* Many variations in charge were examined, especially for those peptides containing two adjacent phenylalanyl residues and a terminal basic amino acid residue. Peptides with charged side chains, lysyl or arginyl (**1–6** in Table I) showed no significant MIMR dependency on charge even up to a maximum net charge difference of 3. This was maintained whether the charge alteration occurred on a side chain, on the backbone, or on both. It is especially interesting that reversal of the amino acid sequence as well as the charge had only a minor influence on changes in inhibitory activity for these peptides. Diminished inhibitory activity was found upon succinylation of the $\alpha$-amino terminus of L-Phe-Gly-L-Arg (compare compounds **23** and **25** in Table I). In this case, location of a negatively charged carboxyl group on the peptide backbone increased the required peptide concentration by 23%.

*Variation in Terminal Residue Separation.* Glycine and glycyl-containing peptides (di- to tetrapeptides) were found to be devoid of antigelling activity. Thus, it was possible to assess the effect of increased separation between the flanking

Table II: MIMR Values as a Function of Terminal Residue Separation and Hydrophobicity[a]

| peptide no. | peptide | MIMR | $-\Delta F_h$ (kcal/mol |
|---|---|---|---|
| 22 | L-Phe-L-Arg | >26 | 3.25 |
| 23 | L-Phe-Gly-L-Arg | 17.5 ± 0.5 | 3.25 |
| 24 | L-Phe-Gly-D-Arg | 16.0 ± 0.5 | 3.25 |
| 25 | Suc-L-Phe-Gly-L-Arg | 21.5 ± 0.5 | 3.25 |
| 26 | L-Phe-Gly-Gly-Gly-L-Arg | 18.0 ± 0.5 | 3.25 |
| 27 | Suc-L-Phe-Gly-Gly-L-Phe | 16.5 ± 0.5 | 3.25 |
| 28 | L-Phe-Gly-Gly-L-Phe | 17.5 ± 0.5 | 3.25 |
| 29 | D-Phe-Gly-Gly-L-Phe | 17.0 ± 0.5 | 3.25 |
| 30 | L-Phe-Gly-Gly-L-Trp | 16.5 ± 0.5 | 3.25 |
| 31 | L-Trp-Gly-L-Trp | 16.0 ± 0.5 | 3.25 |
| 32 | L-Phe-Gly-Gly-Gly-L-Phe | 16.0 ± 0.5 | 3.25 |
| 33 | L-Phe-Gly-Gly-Gly-D-Phe | 16.0 ± 0.5 | 3.25 |
| 34 | L-Phe-Gly-Gly-Gly-L-Trp | 15.5 ± 0.5 | 3.25 |
| 35 | L-Trp-Gly-Gly-Gly-L-Trp | 15.5 ± 0.5 | 3.25 |

[a] Terminal residue separation was varied by introducing intervening glycine residues.

nonglycyl amino acid residues as compared to their parent dipeptide compounds. Table II contains peptides which vary in chain length and configuration. Addition of an internal glycyl residue can increase the terminal separation by 3.7 Å. It is interesting that the effect of glycyl insertions on peptide inhibitory activity produced either a negligible or a relatively large change in the MIMR value relative to that of the parent dipeptide depending upon the residues. For example, Suc-Phe-Phe (**9** in Table I) and Suc-Phe-Gly-Phe (**14** in Table I) have the same activity. However, substantial changes in activity took place in going from the di- to the tetrapeptide stage, as seen in the Phe-Arg series (**22–24** and **26** in Table II). Small changes are seen in those oligopeptides containing Phe or Trp or both (**28–35** in Table II). In the latter group there is a continued small decrease in activity up to the tetrapeptide stage as opposed to a dramatic enhancement in activity in going from L-Phe-L-Arg to L-Phe-Gly-L-Arg. In the latter case an increase greater than 50% is observed, whereas in the former series, neglecting effects of succinylation, a maximum decrease of 50% in activity occurs in some cases between the parent dipeptides and their homologues. However, further chain length increase above the tetrapeptide stage has only a small negative or positive influence on peptide activity irrespective of whether one terminal residue is hydrophobic or hydrophilic. It is interesting that these trends in the inhibitory capacity of these peptides seem to be independent of the configuration of the specific peptide. This is apparent when comparing the DL diastereoisomers **24**, **29**, and **33** and the appropriate peptides containing L-amino acids, **23**, **28**, and **32**, in Table II.

*Correlation of Hydrophobicity and Peptide Antigelling Activity.* The hydrophobic effect arises primarily from the strong attractive interactions among water molecules via hydrogen bonding. Theoretical models (Tanford, 1973) predict that nonpolar molecules containing large nonpolar substitutent groups disrupt this local solvent ordering and tend to reorder it even more strongly at the hydrocarbon–water interface. This leads to a positive free energy increase mainly due to a decrease in entropy rather than being totally unfavorable in an energetic sense. Such solute molecules will associate with themselves, leading to decreased solubility, or with other nonpolar groups that are present to minimize the free energy. Since solvent–solute interactions are short range, the solvent-dependent chemical potential of the solute molecule can, to a good approximation, be considered as the sum of independent contributions from the hydrophilic and hydrophobic groups. Thus,

Table III: Correlation of Peptide Hydrophobicity and MIMR Value

| peptide class(es)[a] | peptide no.[d] | population no. [$N(\Delta F_h$, MIMR)] | correlation coeff ($\gamma$) | probability of accidental occurrence ($P$) |
|---|---|---|---|---|
| $R_1$-$R_5$[b] | **1-35** | 32 | 0.840 | $P << 0.001$ |
| $R_1$-$R_5$ | **1-35** | 19[c] | 0.877 | $P < 0.001$ |
| $R_2$ | ⟨**9-10**⟩, **11, 12, 18** | 4 | 0.987 | $0.02 > P > 0.001$ |
| $R_3$ | ⟨**1-6**⟩, **13,** ⟨**14-15**⟩, ⟨**18-20**⟩, **21,** ⟨**23-25**⟩ | 6 | 0.945 | $0.01 > P > 0.001$ |
| $R_4$ | ⟨**17-19**⟩, **30, 31** | 3 | 0.999 | $0.02 > P > 0.01$ |
| $R_5$ | **26,** ⟨**32-33**⟩, **34, 35** | 4 | 0.940 | $0.10 > P > 0.05$ |
| $R_1$-$R_2$-$R_3$ | ⟨**1-6**⟩, **7, 8,** ⟨**9-10**⟩, **11, 12, 13,** ⟨**14-15**⟩ | 8 | 0.965 | $P < 0.001$ |
| $R_4$-$R_5$ | ⟨**27-29**⟩, **30, 32,** ⟨**32-33**⟩, **34, 35** | 6 | 0.574 | $P > 0.10$ |

[a] Members of peptide classes (dipeptide, tripeptide, etc.) are those listed in Tables I and II. [b] Subscript refers to the number of amino acid residues for a given class. [c] Difference in the number of peptides and the population number, $N$, is a result of averaging $\Delta F_h$ and MIMR values for peptides in the same class whose $\Delta F_h$ values were equal to or less than 0.50 kcal/mol. This procedure was used in all computations in Table III, with the exception of the first row. [d] Angular brackets denote averaged peptides.

the hydrophobicity of a peptide is simply additive, determined by the number and type of side chains. This, of course, assumes no intramolecular side-chain interactions. At the point of attachment of polar and nonpolar groups, the $-CH_2-$ group proximal to the hydrophilic group makes only a negligible nonpolar contribution, as has been shown with fatty acids (Smith & Tanford, 1973). In the present case, this eliminates the $-(CH_2)_2-$ group of succinate as a contributor to the hydrophobicity. The free energy, $\Delta F_h$, which is a measure of the hydrophobicity of a given side chain, is given by Nagaki & Tanford (1973) from solubilities of amino acids in water, dioxane, and ethanol relative to glycine. $\Delta F_h$ values for the residues $-(CH_2)_3-$ and $-(CH_2)_4-$ of Arg and Lys, respectivly, are not available and must be estimated. It has been shown (Cohn & Esdall, 1943) that $\Delta F_h$ for a straight hydrocarbon chain is a linear function of the number of $-CH_2-$ groups; therefore, a similar relation may be expected to hold to good approximation for the Arg and Lys methylene residues. With an average $\Delta F_h$ value of 0.5 ± 0.1 kcal/mol per C atom from leucine, norleucine, and valine, the values of $\Delta F_h$ are likely to lie in the range 0.5–1.0 kcal/mol for Arg and 1.0–1.5 kcal/mol for Lys. A reasonable estimate of 0.75 and 1.25 kcal/mol was used for the arginyl and lysyl residues, respectively, plus an additional 0.25 kcal/mol if the $\epsilon$-amino group was blocked. The $\Delta F_h$ values are tabulated in column 4 of Tables I and II.

A simple linear relation was found between $\Delta F_h$ and MIMR values. Table III gives the relations for the various peptides and some individual and mixed classes (dipeptide, tripeptide, etc.). Only Gly-L-Arg, Gly-Gly-L-Arg, and L-Phe-L-Arg are excluded since their MIMR values are greater than 26. Column 3 in Table III gives the number ($N$) of pairs of variables, $\Delta F_h$ and MIMR, in the correlations. Column 4 lists the linear correlation coefficients between these $N$ pairs of variables. Finally, column 5 expresses the probability, $P$, that these variables could be accidentally related in a linear manner if they were random variables based on a bivariate normal population (Young, 1962). As seen in the first row of Table III, consideration of all 33 compounds resulted in a very high degree of correlation, $\gamma = 0.840$. While a reasonably high degree of correlation was expected, a $P \ll 0.001$ was not. Therefore, peptides in the same class whose $\Delta F_h$ values differed by only 0.5 kcal/mol were averaged and correlation ($\gamma$) values were computed by using an unweighted least-squares fit. Although the population, $N$, was reduced in this treatment by 41%, a very high degree of correlation between $\Delta F_h$ and MIMR is still evident for all peptides as seen in the second row of Table III. Subsequently, the remaining $\gamma$ values were based on this averaging procedure. Since the criterion, $P \leq$ 0.05, is considered a significant correlation, each individual class of peptides (di- to pentapeptides) also has a high linear correlation between $\Delta F_h$ and MIMR for their respective members, as seen in rows 3–6 in Table III. Further examination of various combinations of peptide classes gave a similar result except for the tetra- and pentapeptides composed of terminal aromatic amino acids. A poor correlation, bottom row of Table III, exists for this mixed class. This reflects the previously mentioned fact that there is a negligible incremental response in antigelling activity at and above the tetrapeptide stage.

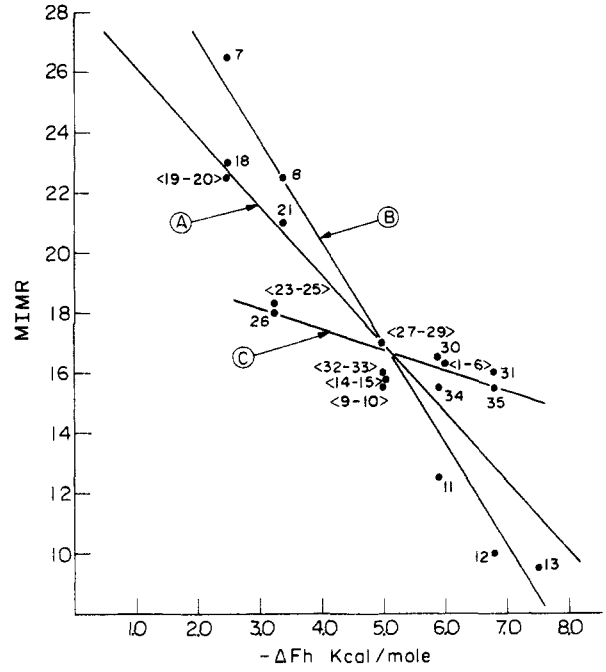


FIGURE 1: Variation in the minimum inhibitor mole ratio (MIMR) of peptide/HbS as a function of peptide hydrophobicity, $\Delta F_{h_i}$, for peptides listed in Tables I and II. $\Delta F_h = \sum_i \Delta F_{h_i}$ where the summation is taken over all peptide side chains. $\Delta F_{h_i}$ is the observed standard free energy per mole upon transfer of a given side chain from an aqueous to an organic solvent (see text). Peptides in angular brackets are those whose $\Delta F_h$ values differed by only 0.5 kcal/mol or less and are represented by a single average value in MIMR and $\Delta F_h$. Curves A, B, and C are the least-squares fits of MIMR and $\Delta F_h$ for peptides **1–35**, **1–15**, and **23–35** in Tables I and II.

In Figure 1 are plotted the MIMR vs. $\Delta F_h$ values for all individual peptides and those averaged as designated by angular brackets. The overall trend of these two variables is given by curve A with a slope equal to $-2.31$ kcal$^{-1}$ mol. It is apparent that curve A is a composite of two varying responses of peptide activity with respect to their hydrophobicity. One

mixed class of peptides, **1–15** in Table I, whose activities can best be described as a rapidly varying function of their hydrophobic content, is given by curve B with slope = −3.39 $kcal^{-1}$ mol. Excluded from this group are Gly-L-Phe and Gly-L-Trp since their succinylated amino acids were included. A second mixed class of peptides, **23–35** in Table II, is a more slowly varying function of $\Delta F_h$ in terms of antigelling activity and is given by curve C with slope = −0.69 $kcal^{-1}$ mol. Peptides associated with curves B and C have correlation coefficients of 0.965 and 0.902, respectively, as given in rows 7 and 8 of Table III. The fivefold difference in the rate of activity between curves B and C demonstrates that peptides associated with curve B are the major contributors to the generalized correlation curve, curve A. There are several peptides such as L-Phe-$Gly_2$-L-Phe and Suc-L-Phe-$Gly_2$-L-Phe which may belong to either curve B or C. Thus, an additional stereochemical criterion was used to aid in separating these two peptide groups. The predominant feature, accounting for 75% of the members in curve B, is the existence of two or more adjacent aromatic side chains. This is to be contrasted with peptides associated with curve C which have two aromatic rings separated by two or more glycyl residues or, in the case of L-Phe-$Gly_2$-L-Arg, one aromatic side chain with a terminal hydrophilic side chain.

## Discussion

In the initial study (Votano et al., 1977), peptides L-Phe-L-$Phe_{n\geq 2}$-L-Arg or -L-Lys were thought to be bifunctional in that the charged terminal hydrophilic side chain was considered to be a contributing factor in peptide activity. The present investigation with its considerably expanded number of peptides indicates that a charged substituent is not needed. In terms of the capacity to inhibit gelation, a basic residue in some cases is detrimental, as seen in the comparison of the much smaller MIMR value of Suc-L-Phe-Gly-L-Nle as opposed to Suc-L-Phe-Gly-L-Arg. If the arginine guanido group is involved in hydrogen bonding or in an ionic interaction, it is not apparent in the peptides studied. Substituent groups (charged or uncharged) on aromatic side chains may influence peptide antigelling activity to varying degrees. In the present study no attempt was made to examine the possibility. However, several studies (Ross & Subramanian, 1977; Nogachi & Schechter, 1978) centered around single phenyl-containing compounds indicate that substituent groups can have a positive or negative effect on deoxy-HbS solubility.

In the majority of peptides solubilized by succinylation, the effect of such chemical modification appears to be negligible in terms of the peptide inhibitory capacity. A comparison of MIMR values of L-Phe-L-Phe-L-Arg, L-Lys-L-Phe-L-Phe, and L-Phe-$Gly_2$-L-Phe with those of their succinylated analogues shows little difference. The only exception is the large detrimental change in activity for L-Phe-Gly-L-Arg upon introduction of the succinate group.

Competitive binding studies centered around peptides **1–15** in Table I indicate that they bind to the same localized surface region of the hemoglobin molecule with negligible competition from the oligopeptides **23–25** in Table II and from the homologous series L-Phe-$Gly_{n\geq 1}$-L-Arg (unpublished experiments). This reinforces the classification of peptides into two groups, as those whose activity is either a fast or a slowly varying function of hydrophobicity [Figure 1, curves B (fast) and C (slow)].

The question of whether such a dichotomy is a general feature or is specific to oligopeptides with consecutively spaced glycyl units has been answered somewhat. Kubota & Yang (1977) have examined the antigelling activity of sickle β-chain peptides $\beta^s\langle$**1–8**$\rangle$, $\beta^s\langle$**1–6**$\rangle$, and $\beta^s\langle$**3–6**$\rangle$. These were essentially equivalent in inhibitory activity to those of two enkephalin members, Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu. Hydrophobicities of these latter five compounds are 6.95, 5.7, 3.7, 6.1, and 6.6 kcal/mol, respectively, with the only unknown being the contribution to $\Delta F_h$ from the proline residue. These various oligopeptides demonstrate a diminished MIMR dependency on $\Delta F_h$ for four or more amino acid residues irrespective of the presence of intervening glycyl residues. The lack of a response in activity with an increase in $\Delta F_h$ of the oligopeptides may be the result of steric effects. In the case of oligopeptides with glycyl residues, the backbone itself may reduce the overall free energy of protein–peptide association since the backbone seeks an aqueous environment. It is difficult to separate these factors, but the fact that Gly-L-Phe and Gly-Gly-L-Phe both have similar MIMR values suggests that this latter consideration is only of secondary importance. Steric effects, commencing at the tetrapeptide stage, seem to be a plausible explanation for the diminished influence of hydrophobicity. It is possible that tetrapeptides and larger oligopeptides have limited access to one or more hydrophobic areas on the surface of the hemoglobin molecule whereas the smaller peptides can bind there. Crystallographic structure analysis of the monoclinic form of the bromine salt of L-Lys-L-Phe-L-Phe (A. Wang, J. Votano, and A. Rich, unpublished experiments) shows that the two adjacent phenyl side chains have a center–center distance of 4.42 Å and are in the cis position with respect to the peptide backbone. It is quite possible that a similar configuration exists in solution for the di- and tripeptides associated with curve B and thereby produces a rather high nonpolar density in the molecule. In the case of the large oligopeptides with intervening glycyl residues, it is sterically possible for the terminal aromatic side chains to approach to within 4 Å of one another. However, the bulk of the peptide may restrict the extent of the intermolecular interactions involved with the hemoglobin molecule by limiting either the approach of the terminal side chains or possibly their orientation.

The high efficiency of nonpolar peptides in inhibiting deoxy-HbS gelation and the absence of such activity by hydrophilic moieties (Votano et al., 1977) indicate that nonpolar interactions between the protein and the peptide may be the predominant mechanism for destabilization or delay of deoxy-HbS fiber formation rather than interference with ionic or hydrogen-bonding interactions which are also known to be of some importance (Benesch et al., 1976, 1977). The hydrophobic scale used to evaluate $\Delta F_h$ for a given side chain is a measure of the tendency for such a group to seek a nonpolar environment, i.e., for forming a hydrophobic bond. Theoretical and experimental findings (Wishnia & Pinder, 1966; Bracha & O'Brien, 1970) applicable to such hemoglobin–peptide complexes indicate that the free energies of such bonds are probably less than −10 kcal/mol. However, such small values may be of the appropriate magnitude to compete with similar intermolecular protein interactions involved in the gelation process. This is reinforced by the reported values of Ross et al. (1975) of −3 kcal/mol, 0, and 10 cal/(mol deg) for the standard free energy, enthalpy, and entropy, respectively, for the deoxy-HbS gelation at 37 °C.

It is not clear whether all peptides investigated bind to the same surface region of the hemoglobin molecule. An area capable of nonpolar interactions is the V-shaped nonhelical EF region of the β chain. X-ray diffraction analysis at 3-Å resolution on deoxy-HbS (Wishner et al., 1975) strongly indicates that β85 (Phe) and β88 (Leu), both located in this

region, are the complementary sites for the β6 (Val) interaction which exists as a side to side contact between adjacent strands in the crystal. Residues 78–88 in one arm of the pocket contain an abnormally high number of closely packed hydrophobic side chains. From model building, this series of residues appears as a hydrophobic patch roughly 100–200 Å$^2$ in area. Interestingly enough, this region is accessible to small molecules approaching from either the aqueous or the protein (toward the twofold molecular axis) side of the molecule in the deoxygenated state. At present, this area is a good candidate for hydrophobic bond formation by these inhibitory peptides.

The overall binding of these peptides to HbS is moderately weak; nonetheless, they are able to prevent gelation at high stoichiometric ratios. This does not necessarily mean that they possess no potential therapeutic value. These experiments were carried out under complete deoxygenation, while venous blood is usually 70% oxygenated in most organs. Furthermore, delay times for HbS aggregation of only 20–40 s are required for in vivo activity, while our assay was for a longer period of time (20 min). Thus, the therapeutic potential of these agents has yet to be determined.

This applicability of hydrophobic peptides for the treatment of sickle cell disease has been hindered by their lack of permeability into the erythrocyte cell. A potential solution to this problem appears more promising in light of the fact that nonpolar amino acid side chains are the major factor in inhibiting deoxy-HbS gelation and charge per se does not appear to have a significant role. Furthermore, smaller peptides are more effective inhibitors than the larger oligopeptides. Thus, with suitable modification it is possible that such small peptides could be transported into the cell.

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## References

Anderson, G. W., Zimmerman, J. E., & Callahan, F. M. (1964) *J. Am. Chem. Soc. 86*, 1839.

Benesch, R. E., Yung, S., Benesch, R., & Mack, J. (1976) *Nature (London) 260*, 219.

Benesch, R. E., Kwong, S., Benesch, R., & Edalji, R. (1977) *Nature (London) 269*, 772.

Bracha, P., & O'Brien, R. D. (1970) *Biochemistry 9*, 741.

Cohn, E. J., & Edsall, J. T. (1943) *Proteins, Amino Acids, and Peptides as Ions and Dipolar Ions*, p 196, Reinhold, New York.

Elbaum, D., Nagel, R. L., Bookchin, R. M., & Herskovits, T. T. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4718.

Hegesh, E., & Gruener, N. (1970) *Clin. Chim. Acta 36*, 679.

Hofrichter, J., Ross, P., & Eaton, W. A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3035.

Kubota, S., & Yang, J. T. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5431.

Kubota, S., Change, C. T., Samejima, T., & Yang, J. T. (1976) *J. Am. Chem. Soc. 98*, 2677.

Nagaki, Y., & Tanford, C. (1973) *J. Biol. Chem. 248*, 2211.

Noguchi, C. T., & Schechter, A. N. (1977) *Biochem. Biophys. Res. Commun. 74*, 637.

Noguchi, C. T., & Schechter, A. N. (1978) *Biochemistry 17*, 5455.

Ross, P., & Subramanian, S. (1977) *Biochem. Biophys. Res. Commun. 77*, 1217.

Ross, P., Hofrichter, J., & Eaton, W. (1975) *J. Mol. Biol. 96*, 239.

Smith, R., & Tanford, C. (1973) *J. Biol. Chem. 248*, 2211.

Tanford, C. (1973) *The Hydrophobic Effect*, p 12, Wiley, New York.

Votano, J. R., Gorecki, M., & Rich, A. (1977) *Science 196*, 1216.

Wishner, B. C., Ward, K. B., Latterman, E. E., & Love, W. E. (1975) *J. Mol. Biol. 98*, 179.

Wishnia, A., & Pinder, T. W., Jr. (1966) *Biochemistry 5*, 1534.

Young, H. D. (1962) *Statistical Treatment of Experimental Data*, p 126, McGraw-Hill, New York.