Inhibition of Deoxyhemoglobin S Polymerization by Biaromatic Peptides Found To Associate with the Hemoglobin Molecule at a Preferred Site†

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ABSTRACT: Association of three succinylated biaromatic peptides with deoxyhemoglobin has been measured. These peptides composed of indolyl or phenyl rings were found to have ΔG values for their binding to deoxyhemoglobin between −2.9 and −3.4 kcal/mol at 23 °C. Binding experiments among these peptides demonstrate one preferred site, one of strongest binding of the peptide to the Hb molecule, as well as the existence of one or more weaker binding sites. Both aromatic side chains and at least one of the terminal carboxyl groups of the succinylated peptides are involved in the interactions with the hemoglobin (Hb) side chains at the preferred binding site. The latter also was found to be capable of binding monocyclic moieties of sufficient hydrophobicity, i.e., indolyl ring compounds. Increases in deoxyhemoglobin S (deoxy-HbS) solubilities in the presence of these three biaromatic peptides show a strong correlation between the values of their dissociation constants and their ability to destabilize deoxy-HbS aggregation. The symmetric site to which the peptides bind must be located at or near a contact site needed to stabilize the deoxy-HbS polymer.

One rational approach toward the development of therapeutic agents for sickle cell disease has been to identify noncovalent chemical agents that bind to the HbS tetramer in order to partially destabilize deoxy-HbS[1] aggregation. Such a process can lead to an increase in deoxy-HbS solubility, C_s, and thereby to larger delay times (Hofrichter et al., 1974) for deoxy-HbS gelation. Studies on amino acids (Noguchi & Schechter, 1977), peptides (Votano et al., 1977), and aryl-substituted alanines (Poillon, 1982), to name a few, have revealed a structural prerequisite of compounds likely to have a greater efficacy in their antigelling activity than those without them. Compounds containing bicyclic or multiaromatic residues will have a higher activity than those that carry a single aromatic or aliphatic side chain. Also, an increase in the apolar content of the aromatic residue (i.e., indolyl vs. a phenyl ring) and ring polarizability will further enhance antigelling activity of such compounds.

Although insight has been gained into what type of moieties are likely to be suitable antigelling agents, little is known concerning whether such compounds bind to more than one site on the Hb molecule or the strength of binding and its relationship to antigelling activity. In this study, we chose small oligopeptides as a class of such compounds to address these questions. Small peptides with aromatic side chains are good candidates to use since, once succinylated at their α-amino position, they achieve a high degree of solubility needed in rapid equilibrium dialysis as well as deoxy-HbS solubility measurements.

Materials and Methods

Hemoglobin A and S Preparations. HbA was prepared as previously described (Gorecki et al., 1980) except final dialysis was done against 50 mM sodium phosphate, pH 7.2, and 150 mM NaCl. HbS was obtained from homozygous sickle cell blood and treated the same as above except dialysis was done in the absence of NaCl. The HbF content of the prepared HbS solution was <4% as determined by the FTEST (Isolab, Akron, OH), and the percent of met-Hb for both hemoglobins was less than 2% by the method of Hegesh & Gruener (1970).

Peptide Synthesis. All peptides were purchased from Vega Biochemicals (Tucson, AZ) except L-Phe-(Gly)$_n$-L-Phe peptides where n = 1 or 2. The latter were synthesized and purified by methods previously described (Gorecki et al., 1980). Succinylation of peptides and amino acids was accomplished by use of succinic anhydride (Klotz, 1967). Peptide purity was determined by thin-layer silica chromatography (TLC) in the solvent system BuOH–HOAc–H_2O (4:1:1), followed by UV fluorescence and ninhydrin staining. Radioactive homologous peptides and amino acids were prepared by using ^{14}C-labeled succinic anhydride (New England Nuclear) and purified by TLC.

Deoxy-HbS Solubility Measurements. At 4 °C, a 0.4-mL solution of HbS containing the dissolved compound was placed in a 0.8-mL cellulose tube that was then flushed with N_2(g) and stoppered, and finally, 20% (w/v) $Na_2S_2O_4$ in 0.35 M NaOH was added anaerobically. The final concentrations of HbS and $Na_2S_2O_4$ were 28.0 ± 0.2 g % and 50 mM, respectively, at pH 7.2. The stoppered tube was inverted continually for 5 min and allowed to stand for 4–5 min, and then, the deoxy-HbS solution was overlaid by mineral oil. Centrifugation to separate the polymerized and nonpolymerized deoxy-HbS was done at 240000g at 28 ± 1 °C for 75 min. After centrifugation, the supernatant concentration of deoxy-HbS, C_s, was determined with Hb in the cyanomet form with a millimolar extinction coefficient of 11.0 cm^{-1} per heme at 540 nm. The C_s values at a given compound concentration were determined for two or three samples and at least one control sample (untreated HbS solution) for each centrifugation run.

Determination of Dissociation Constants and Competitive Binding to Deoxygenated Hemoglobin. Binding determinations were carried out by a rapid dialysis technique and chamber configuration described elsewhere (Colowick &

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[1] Abbreviations: SP_2, Suc-L-Phe-L-Phe; SPGP, Suc-L-Phe-Gly-L-Phe; ST_2, Suc-L-Trp-L-Trp; SPG_2P, Suc-L-Phe-Gly-Gly-L-Phe; Hb, hemoglobin; EDTA, (ethylenedinitrilo)tetraacetic acid disodium salt.

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Womack, 1969). The volumes of the upper and lower dialysis chambers were 3.0 and 1.2 mL, respectively, with a 2.4-cm diameter membrane separating them. The dialysis membrane (Union Carbide Co.) was prepared by boiling it in deionized water containing 10 mM $NaHCO_3$ plus 0.5 mM EDTA, followed by extensive rinsing with deionized water. This procedure was repeated 2 more times and the membrane equilibrated in the dialysis buffer (50 mM sodium phosphate, pH 7.2, and 150 mM NaCl) for 12 h or more before use.

Prior to obtaining binding curve data, the upper chamber was fitted with a rubber cap that contained an N_2(g) inlet and outlet and flushed with N_2(g), and into the upper chamber was placed a 2.0-mL solution of deoxy-HbS at pH 7.2 containing 20 mM $Na_2S_2O_4$. During the course of a binding experiment, the dead space of the buffer reservoir and upper dialysis chamber was continually flushed (3–4 cm^3/min) with N_2(g). Both the upper and lower chambers were stirred continually during a run (usually 30 min or less), and a flow rate of 6.0 mL of buffer (50 mM sodium phosphate, pH 7.2, 150 mM NaCl, 20 mM $Na_2S_2O_4$) through the bottom chamber was used to collect free labeled and unlabeled substrate from the lower chamber every 24 s. Initially, the concentration of Hb in the upper chamber was determined, followed by addition of 35 to 45 μL of ^{14}C-labeled compound to give (1.0–1.4) × 10^7 cpm in the upper chamber. Either five or six sequential additions of unlabeled ligand were made to the upper chamber to eventually displace all the bound ^{14}C-labeled homologue. Between each addition of unlabeled compound at least six 2.4-mL fractions were collected from the bottom chamber. This allowed at least three determinations to be made of the equilibrium concentration of unbound ligand prior to the next addition of unlabeled compound. At the conclusion of the run, the final mole ratio of compound to Hb always exceeded 200 to ensure that all the labeled ligand was free, >99.8%. The total volume of unlabeled compound added was <0.10 mL, which resulted in only a 0.2–0.3 g % change in the Hb concentration. In any run, the percentage change or loss in initial counts per minute added did not exceed 1.2%. A 2.0-mL aliquot from each fraction collected was counted in 10 mL of Aquasol (New England Nuclear) at an efficiency of 67% at a counter preset error of 1.0%.

In the competitive binding experiments, the concentration and volume of all substances were the same for all runs. A 1.0-mL volume of deoxy-Hb at 0.71 mM was added to the upper chamber to which was added 13 μL of ^{14}C-labeled compound to give a concentration of 0.151 mM. A single displacement of bound radioactive compound was achieved by addition of 52.0 μL of unlabeled competitor to give a competitor concentration of 14.9 ± 0.05 mM. This resulted in a mole ratio of approximately 100, of unlabeled to labeled ligand. Such a ratio was sufficient to displace about 90% or more of a bound labeled compound by its unlabeled homologue.

Results

Evaluation of the dissociation constant, K_d, for a peptide–Hb complex involved determining the fraction of unbound peptide at five to seven equilibrium positions on their binding curve. The fraction of free ligand was determined by measuring the ^{14}C activity of its labeled homologue. In Figure 1 is a set of typical binding curves for the bicyclic peptides ST_2, SP_2, and SPGP. Arrows in Figure 1 indicate additions of unlabeled compound to Hb solution and the dotted boxes the equilibrium position of freely diffusible labeled peptide. At any equilibrium position on the curve, the fraction of free compound is simply the average cpm per milliliter value at that position divided by that of the last position on the curve that corresponds to

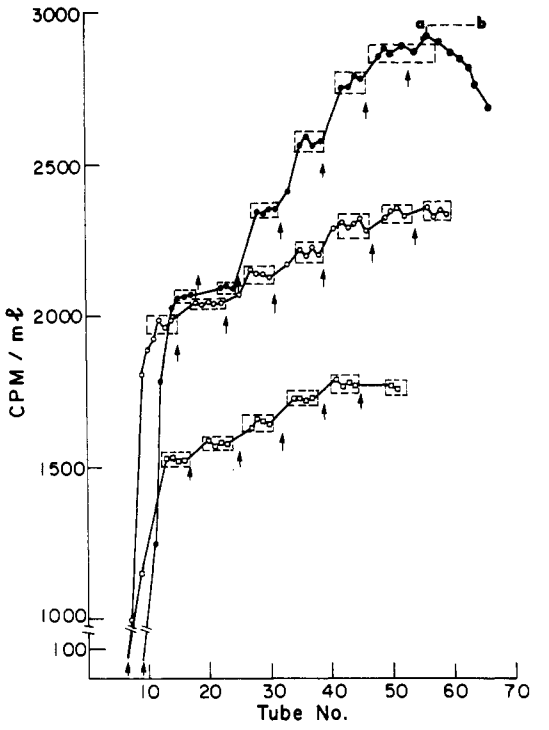

FIGURE 1: Measurements of the binding of three bicyclic peptides at various concentrations to deoxyhemoglobin: (●) Suc-L-Trp-L-Trp (ST_2); (○) Suc-L-Phe-L-Phe (SP_2); (□) Suc-L-Phe-Gly-L-Phe (SPGP). To the upper dialysis chamber containing the Hb solution was added ^{14}C-labeled peptide, followed by additions of unlabeled peptide to displace the bound radioactive homologue. The total concentrations of peptide after each addition were 0.21, 1.1, 2.7, 6.0, 12.4, 26.5, and 45.3 mM ST_2, 0.16, 2.1, 6.0, 13.7, 28.8, 42.4, and 10.2 mM SP_2, and 0.18, 2.2, 6.4, 15.1, 32.0, and 46.6 mM SPGP. The initial concentrations of Hb were 0.76, 0.84, and 0.87 mM for ST_2, SP_2, and SPGP. Changes in Hb concentration due to additions ranged from 0.2 to 0.3 g %. Line ab for the ST_2 binding curve is the corrected final per milliliter value (see Results) at 100% unbound labeled peptide.

100% free labeled compound. However, for ST_2 a small correction had to be made to the final observed cpm per milliliter value on the binding curve. As seen in Figure 1, there was a retardation in ST_2 diffusion through the membrane above 30 mM in ST_2. This effect may have been due to aggregation of ST_2 on the surface of the membrane since in solution ST_2 is quite soluble (~0.75 mol/μL). To find a corrected final equilibrium position (line ab in Figure 1) on the binding curve for ST_2, the negative slope was treated as linear and was used to extrapolate backward to the cpm per milliliter value for the third or fourth fraction after the last addition of compound. Either of these collected fractions would represent the equilibrium of labeled ligand between the upper and lower dialysis chambers. Such corrections amounted to less than 3% of the final observed cpm per milliliter volume in ST_2 binding experiments.

Results in Figure 1 are shown in Scatchard plots in Figure 2. C_b and C_f are the concentrations (mM) of bound and free peptide expressed in the Scatchard equation

$$C_b = nC_p - K_d(C_b/C_f) \quad (1)$$

where n and C_p are the number of apparent binding sites on the protein and its concentration (mM), respectively. All the data in Figure 2 fall within C_b/C_f values <0.6 and were fitted by least squares given their apparent linearity. The ordinate intercepts, 2.2, 1.8, and 1.8, for site SP_2, ST_2, and SPGP, respectively, are larger than the Hb concentration (<0.9 mM), indicating more than one binding site exists for each of the bicyclic peptides.

Table I: Dissociation Constants, Number of Binding Sites, and ΔG Values for Peptide with Deoxyhemoglobin[a]

compd	K_d ($\times 10^3$ M)[b]	n	T (°C)[c]	ΔG (kcal/mol)
Suc-L-Phe-L-Phe	7.7 ± 0.3	2.5 ± 0.3	23	−2.86
Suc-L-Phe-Gly-L-Phe	7.2 ± 0.3	2.3 ● 0.2	23	−2.90
Suc-L-Trp-L-Trp	3.0 ± 0.2	2.3 ± 0.2	22	−3.40

[a] Values were determined in 50 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl and 20 mM $Na_2S_2O_4$. [b] Average of three runs with [Hb] ranging from 0.74 to 0.88 mM. [c] Average temperature rounded off to nearest degree.

In Table I are the average values of n and K_d for three peptides, obtained from three sets of binding curves including that of Figure 2. Both phenylalanyl peptides, SP_2 and SPGP, have very similar K_d values around 7.5×10^{-3} M, whereas the K_d of ST_2 with its indolyl residues is approximately 2.5 times smaller. All peptides have about the same number of apparent binding sites, 2.3.

To test for commonality among the binding sites for SP_2, SPGP, and ST_2 as well as with several other compounds, competitive binding determinations were made. In the competitive runs, a fixed mole ratio of 99, unlabeled (the displacer) to labeled compound was used to enhance the possibility that weaker binding by a competitor might be detected. In Table II the competitive results are expressed as the percent fractional increase, Y, in the observed activity (cpm/mL) of free labeled compound after addition of an unlabeled compound and its normalized form RD = Y_i/Y_0, where subscripts i and 0 are for a given compound and the nonlabeled homologous peptide, respectively. RD is the relative displacement of the labeled ligand by the unlabeled ligand.

Examination of the RD values of SP_2 and SPGP in Table II shows that these peptides bind to the same sites. Whether SP_2 displaces SPGP or the reverse, either one fully displaces the other within the error (<8%) in RD. On the other hand, ST_2 can totally displace SP_2 or SPGP, but in reverse situations SP_2 or SPGP can displace, on the average, only 66% of ST_2 even though these competitors are 99 times more concentrated than labeled ST_2. To explain these differences and to determine if ST_2 binds only to SP_2 or SPGP sites, one can use the data of Table I to predict their Y values in Table II.

The number of moles of each ligand, for example, peptides

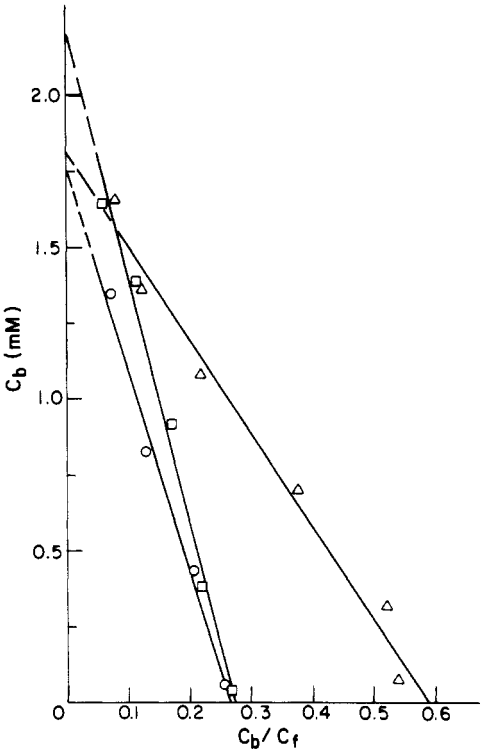

FIGURE 2: Scatchard plots of the binding of three bicyclic peptides to deoxyhemoglobin. Plots were constructed from the data in Figure 1 after small corrections were made to the measured per milliliter values to account for small dilution effects due to added volume of unlabeled peptides. (△) ST_2; (□) SP_2; (○) SPGP. Slopes for the curves are 3.04, 8.07, and 7.12 mM for ST_2, SP_2, and SPGP, respectively.

1 and 2, bound to a common site on protein P, is given by (Steinhardt & Reynolds, 1969)

$$V_1 = \frac{n_1 K_1 C_1}{1 + K_1 C_1 + K_2 C_2} \qquad (2)$$

$$V_2 = \frac{n_2 K_2 C_2}{1 + K_1 C_1 + K_2 C_2}$$

where V_1 and V_2 are the moles of peptides 1 and 2 bound per mole of protein, K_1 and K_2 are their association constants, and

Table II: Competitive Binding among Bicyclic Peptides and Other Compounds to Deoxyhemoglobin

^{14}C-labeled peptide displaced[a]	added unlabeled compd[b]	obsd % increase, Y, in free unlabeled peptide[c]	% relative displacement, RD[d]
Suc-L-Phe-L-Phe (SP_2)	Suc-L-Phe-L-Phe	15.8	100.0
	Suc-L-Trp-L-Trp	14.4	91.1
	Suc-L-Phe-Gly-L-Phe	16.5 (15.5)	104.4
	L-Phe	1.1	7.0
	Suc-L-Phe	4.5	28.5
Suc-L-Trp-L-Trp (ST_2)	Suc-L-Trp-L-Trp	49.3	100
	Suc-L-Phe-L-Phe	33.2 (32.5)	67.3
	Suc-L-Phe-Gly-L-Phe	31.4 (34.0)	63.7
	Suc-L-Trp	15.5	31.4
	Gly-Gly-L-Trp	18.6	37.7
Suc-L-Phe-Gly-L-Phe (SPGP)	Suc-L-Phe-Gly-L-Phe	17.0	100.0
	Suc-L-Phe-L-Phe	16.1 (15.2)	94.7
	Suc-L-Trp-L-Trp	15.7	92.4
	Suc-L-Phe-$(Gly)_2$-L-Phe	9.6	56.5
	L-Phe-$(Gly)_2$-L-Phe	3.6	21.2
	Gly-Gly-L-Phe	0.9	5.3
	Gly-Gly-L-Trp	10.5	62.0

[a] Total concentration of unlabeled peptide was 0.151 mM in 1.0 mL of 0.771 mM deoxy-Hb. [b] Final concentration of unlabeled compound was 14.9 ● 0.05 mM. [c] $Y = [(cpm/mL)_1 - (cpm/mL)_0]/(cpm/mL)_0$ where subscripts 1 and 0 stand for after and before addition of unlabeled compound. $(cpm/mL)_1$ is the observed value at equilibrium times 1.051 to account for the dilution effect due to added volume of unlabeled compounds. [d] RD = Y_i/X_0 where subscript i is for peptide and 0 that for the homologous peptide.

C_1 and C_2 are their respective free ligand concentrations. If the labeled peptide is peptide 1, then the percent displacement, Y, in Table II is

$$Y = \frac{(V_1^0 - V_1)C_p}{C_{t1} - V_1^0 C_p} \quad (3)$$

where the superscript signifies the absence of competitor and subscript, t, total concentration of ligand. If the labeled and unlabeled peptide binds only to a common site, then eq 2 must be obeyed, and the V_1 value determined from it, when substituted into eq 3, should predict very closely the observed Y value for the labeled peptide in the presence of its competitor in Table II. Conditions of the competitive peptide binding runs, $C_2 > C_1$ and $C_{t2} > C_{t1}$, and the magnitudes of the K values for any two peptides allow a good approximation of V_1 to be obtained from eq 2. V_1 can be solved for by using the K_d and n values of Table I, C_1 and C_2 from eq 1, and its Y values in Table II. The predicted Y values from substitution of V_1 into eq 3 are given in parentheses in column 3 of Table II. As can be seen, the estimated values for competition by SP_2 or SPGP for the ST_2 site are in very good agreement with those observed, which confirms that ST_2 also binds to the same sites as does SP_2 and SPGP.

Since the Hb molecule is symmetric, the n values in Table II must represent one major symmetric site and at least one or more additional minor sites. Since the dialysis technique is suitable for K_d values <0.01 M, a computerized fit using multiple binding equations of Steinhardt & Reynolds (1969) was used to reproduce to within 7% the Scatchard plots of all three bicyclic peptides (ST_2, SP_2, SPGP) from a two-site model with independently varied K_d values for each site. The results indicate that, in order to account for the apparent n values in Table I, a minimum value for K_d for one secondary site would be 0.04 M while the K_d values for the primary site, the stronger binding site, would average approximately 6% less than those given in Table I. Therefore, the biaromatic peptides can be considered to associate much more strongly with one symmetric site on the Hb molecule.

There are several stereochemical features involved in the binding of these peptides as seen in their site competition with the remaining compounds in Table II. Blockage of the positive α-amino group on the peptide backbone by the succinate group contributes to the binding of the peptide at the symmetric site. This is evident, for example, by the 3–4-fold increase in RD values of succinylated vs. unsuccinylated homologues of PG_2P and L-Phe in Table II. Another feature of the peptides is that the terminal aromatic rings act in concert to give stronger binding than the simply additive effects of each ring system. Neither Suc-L-Phe and $(Gly)_2$-L-Phe nor Suc-L-Trp and $(Gly)_2$-L-Trp additively give RD values of their analogous dipeptides. Lastly, the most important stereochemical feature is the hydrophobicity of the aromatic ring itself. We see this not only in the smaller K_d value of ST_2 as compared to SP_2 but with the RD value differences for monocyclic phenyl or indolyl compounds in Table II. Suc-L-Trp can displace ST_2 1.5 times more than Suc-L-Phe can, as estimated by using the latter's RD value for SP_2. Gly-Gly-L-Trp has a RD value 12 times that of Gly-Gly-L-Phe in competing with SPGP, indicating again how the 36% larger nonpolar content of the indolyl vs. phenyl side chain (Nozaki et al., 1971) enhances the former's ability to bind to the Hb tetramer.

A reduction in the amount of deoxy-HbS polymerization due to the interaction of a chemical agent with the HbS molecule can be indexed by the measured deoxy-HbS solubility ratio, C_s/C_s^0. The latter is the concentration of HbS in its monomer phase in the presence and absence of a chemical agent. C_s/C_s^0 values are given in Figure 3 as a function of inhibitor concentration for L-Phe, SP_2, SPGP, SPG_2P, and ST_2. Here, L-Phe was used as a control, and its slope, 3.2 M^{-1}, is in good agreement with that found by Poillon (1982). The slopes of the C_s/C_s^0 curves in Figure 3 for SP_2, SPGP, and SPG_2P are 6.9, 7.3, and 4.3 M^{-1}, respectively. In contrast, the values of C_s/C_s^0 for ST_2 showed nonlinear behavior with an initial slope of 18.5 M^{-1}, and that above 20 mM in ST_2 was 5.3 M^{-1}, where the latter portion of the ST_2 curve was treated as being linear.

Inasmuch as all three bicyclic peptides bind with moderate strength to the same site, at least one site of this pair must be involved in deoxy-HbS aggregation. Therefore, a correlation would be expected between the K_d or RD value of a compound and its C_s/C_s^0 values. Indeed, this is just what is seen in the descending values of the association constants, $K_a(1/K_d)$, for the peptides, $ST_2 > SP_2 \sim SPGP > SPG_2P \gg$ L-Phe, and a similar trend in their initial slope of the C_s/C_s^0 curves in Figure 3.

Discussion

Binding of a noncovalent, nonpolar compound to the Hb molecule must involve a decrease in its total entropy in solution, which has to be more than compensated for by a net decrease in the enthalpy (ΔH) for the formation of the complex. Decreases in ΔH can be brought about by a hydrophobic interaction, hydrogen bonding, or a combination of both types of interactions between a suitable aromatic moiety and the hydrophobic side chains on the Hb molecule. It is just such interactions that are present at the symmetric binding site of the bicyclic peptides in this study. The energies involved are highly dependent on the number and orientation of the interacting groups. Since the number of interacting groups is limited in these peptides, it is not too surprising that ST_2, SP_2, and SPGP have moderate negative free energies of interaction with the deoxy-Hb (ΔG values in Table I) of −3.4, −2.9, and −2.9 kcal/mol, respectively. Nevertheless, these free energies of binding are of sufficient magnitude to compete with those in the deoxy-HbS gelation process. The ΔG value of the latter has been shown to be −3.0 kcal/mol at 37 °C (Ross et al., 1975). Therefore, a small change in the ΔG of binding for these bicyclic peptides to their symmetrical binding site should have a significant change in the initial slope, $C'_s = d(C_s/C_s^0)/dC$, of the solubility curves in Figure 3. This is exactly what is found for ST_2 and SP_2. A 19% change in ΔG for ST_2 in comparison to SP_2 results in a 1.7-fold increase in the initial antigelling activity of ST_2.

A further feature of the solubility curves in Figure 3 for SP_2, SPGP, and SPG_2P and that initially for ST_2 is the constant value of C'_s within the error (<4%) in the C_s/C_s^0 values. The linearity of most of these curves in Figure 3 is not unique to these peptides but is a very common feature of aromatic antigelling agents in the same concentration ranges as in Figure 3. What is surprising from this study is that the magnitude of C'_s appears to depend predominately on the association constant K_a $(1/K_d)$ rather than on C_b, the concentration of bound peptide to the Hb tetramer. If we define $C'_k = dC'_s/dK_a$ and use the data in Table I and Figure 3, we find that C'_k is 0.057 and 0.058 in comparing the initially linear C'_s value of ST_2 to those of SP_2 and SPGP, respectively. In addition, three estimated values of C'_k were determined between these three peptides and SPG_2P to see if this rate of change, C'_k, held for a compound with a smaller K_a value than that of either SP_2 or SPGP. Although SPG_2P binds too weakly to obtain a suitable Scatchard plot, its K_a value of 90 ± 5 (K_d = 0.0111)

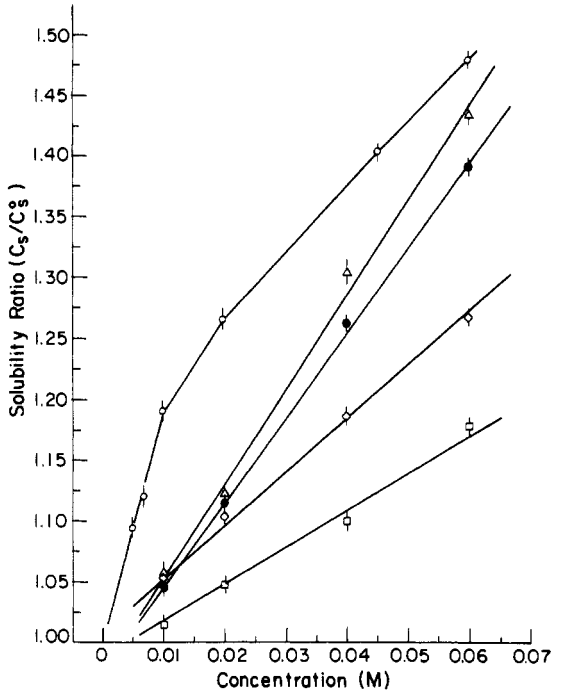

FIGURE 3: Equilibrium solubility ratio, C_s/C_s^0, of deoxy-HbS as a function of peptide concentration. Concentration of deoxy-HbS for all samples was 28 ± 0.5 g %, and solubilities were determined at 28 ± 1 °C. (○) ST_2; (△) SPGP; (○) SP_2; (◇) SPG_2P; (□) L-Phe.

was determined with the competitive binding equations mentioned above and its Y value in Table II. Use of this K_a results in C'_k values of 0.061, 0.065, and 0.058 between SPG_2P and SPGP, SP_2, and the initial C'_s value of ST_2, respectively. An average value for all five C'_k values is 0.06 ± 0.003, which indicates that on the average a 6% increase in the initial linear slope of the deoxy-HbS solubility curve occurs per unit change in K_a for binding of these peptides to their preferred site. As of yet, models (Minton, 1975) for deoxy-HbS antigelling agents cannot explain this dependency of C'_s on K_a and what appears to be a very weak dependency on C_b. However, a dependency of C_s/C_s^0 on C_b does show up in stronger binding as in the case of ST_2 but only when C, the total inhibitor concentration, is in excess of the Hb concentration. Such dependency would be expected with stronger binding of a compound since dC_b/dC changes much more rapidly for smaller K_d values as the concentration of the binding agent exceeds that of the substrate. At inhibitor concentrations less than that of Hb, the C'_k value of 0.06 for these biaromatic peptides indicates how sensitive deoxy-HbS solubility is to K_d. For example, if ST_2 in Figure 3 had a K_d = 0.001 M rather than K_d = 0.003 M, a C_s/C_s° value of 1.2 would be reached at 1 mM rather than 11 mM as found experimentally.

As found elsewhere (Ross & Subramanian, 1977; Noguchi & Schechter, 1978; Beth & Englander, 1979; Poillon, 1982) and in this study, both apolar and hydrogen-bonding interactions have been identified with antigelling agents in which the aromatic ring was attached to a backbone having one or more polar terminal groups (i.e., CO_2^-, NH_3^+). With respect to the small peptides of this study, what is important is not that they are peptides but biaromatic compounds that seek a preferred binding site on the Hb molecule. Small peptides themselves have poor permeability toward the red cell. However, recently we have shown (Votano et al., 1984) that a simple substitution of the terminal succinylated L-Phe group of SP_2 with a phenyl ring to give N-(phenylacetyl)-L-Phe (PAP) results in good permeability and antisickling activity even at low concentrations under venous O_2 tensions even though the estimated binding strength of PAP ($K_d > 10^{-3}$ M) is weak. Both PAP and SP_2 have a very similar conformation in terms of their terminal phenyl rings (unpublished results), but PAP has 50% greater antigelling than SP_2, which may reflect the absence of fewer carbonyl groups in PAP in contrast to SP_2. It seems likely that PAP probably binds to the same site on the Hb molecule as SP_2 since both terminal groups of biaromatic peptides are involved in binding.

In a previous report (Gorecki et al., 1980) it was suggested that a likely site for binding of these biaromatic peptides was in the U-shaped region formed by the E and F helixes of the β chain, especially near the apolar residues β85 (Phe) and β88 (Leu). The F-helical segment 83–β88 was a logical choice since it has been shown to be involved in the side to side contacts in the basic double strand (Wishner et al., 1975) of the deoxy-HbS polymer, which have been confirmed in mutant hemoglobin solution studies (Nagel et al., 1980). It is interesting that an axial contact site has been found in recent Hb crystal complex studies with noncovalent monocyclic antigelling agents (Abraham et al., 1983).

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