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Mechanism for the Increase in Solubility of Deoxyhemoglobin S due to Cross-Linking the β Chains between Lysine-82β_1 and Lysine-82β_2†

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ABSTRACT: In a previous publication [Walder, J. A., Walder, R. Y., & Arnone, A. (1980) *J. Mol. Biol. 141*, 195–216], we showed that bis(3,5-dibromosalicyl) fumarate reacts selectively with oxyhemoglobin at the 2,3-diphosphoglycerate binding site to cross-link the β chains of the tetramer between Lys-82β_1 and Lys-82β_2. This chemical modification markedly increases the solubility of deoxyhemoglobin S and is therefore of potential clinical value in the management of sickle cell disease. By crystallographic analysis of the cross-linked derivative in the deoxy form, we showed that this modification causes Lys-82β and the neighboring β-chain residues (specifically Phe-85β and Leu-88β which form the acceptor site for Val-6 within the deoxyhemoglobin S fiber) to be pulled inward toward the central cavity of the tetramer. No alterations in the structure were observed in the region of the mutation site in hemoglobin S, residue 6β. These results suggested that there is a direct relationship between the magnitude of the perturbation of the acceptor site and the increase in the solubility of deoxyhemoglobin S which results from cross-linking the two lysine residues. To test this hypothesis, we have compared the structures of deoxyhemoglobin A cross-linked by bis(3,5-dibromosalicyl) succinate (C_4), bis(3,5-dibromosalicyl) glutarate (C_5), and bis(3,5-dibromosalicyl) adipate (C_6) and the solubilities of the corresponding derivatives of deoxyhemoglobin S. As the length of the bridging group is increased, there is a progressive decrease in the movement of Lys-82β toward the central cavity and in the accompanying perturbation of the acceptor site for Val-6. Correspondingly, the solubility of the C_4-cross-linked derivative is increased by the largest amount (nearly 50%), and as the cross-link is increased in length, the solubility decreases, approaching that of native deoxyhemoglobin S. To complete the analysis of the stereochemistry of the reaction pathway, we have determined the effect of the cross-link on the structure in the liganded quaternary state by using CO-β_4 as a model of normal liganded $\alpha_2\beta_2$ tetrameric hemoglobins. The results of these studies indicate that the fumaryl group is able to span the two lysine residues in oxyhemoglobin without perturbing the structure of the protein. The structural correlations arrived at in this work provide important constraints for the design of new antisickling compounds.

The molecular basis for sickle cell disease is a single point mutation within the hemoglobin molecule; glutamic acid at the sixth position of the β chains is replaced by valine. This mutation has relatively little effect on the functional properties of hemoglobin but does markedly reduce its solubility in the deoxy form (Perutz et al., 1951). Under physiological conditions, deoxyhemoglobin S initially precipitates from solution in the form of extended helical fibers. Within the erythrocyte, these fibers tend to align parallel to one another to form large aggregates which distort the morphology of the cell, giving rise to a variety of abnormal shapes, most characteristically the sickled form. These abnormally shaped cells are less deformable than the normal erythrocyte and are responsible for the vaso-occlusive complications of the disease.

Knowledge of the pathogenesis of sickle cell disease has led to many efforts to design reagents that would interfere with the polymerization of deoxyhemoglobin S either by covalently modifying the protein (Cerami & Manning, 1971; Roth et al., 1972; Benesch et al., 1974; Hassan et al., 1976; Lubin et al., 1975; Nigen & Manning, 1977) or by binding noncovalently to some specific site on the hemoglobin molecule (Schoenborn, 1976; Kubota & Yang, 1977; Ross & Subramanian, 1977; Noguchi & Schechter, 1977, 1978; Votano et al., 1977; Schechter, 1980; Poillon, 1980, 1982). The initial observation by Klotz & Tam (1973) that aspirin acetylates amino groups on hemoglobin provided the impetus for the investigation of a large number of related acylating agents as potential antisickling compounds (Zaugg et al., 1975, 1980; Walder et al., 1977, 1979; Wood et al., 1981). Recently, we described in detail the properties of a series of these derivatives which we have shown form a new class of affinity reagents that react with hemoglobin at the 2,3-diphosphoglycerate (DPG)[1] binding site (Walder et al., 1980). The prototype of these reagents, bis(3,5-dibromosalicyl) fumarate, reacts selectively with hemoglobin in the liganded quaternary state to cross-link the β chains between Lys-82β_1 and Lys-82β_2, spanning the DPG binding pocket (Scheme I). As a result of this modification, the solubility of deoxyhemoglobin S is increased by about 36%

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[1] Abbreviations: DPG, 2,3-diphosphoglycerate; $NaDodSO_4$, sodium dodecyl sulfate; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane.

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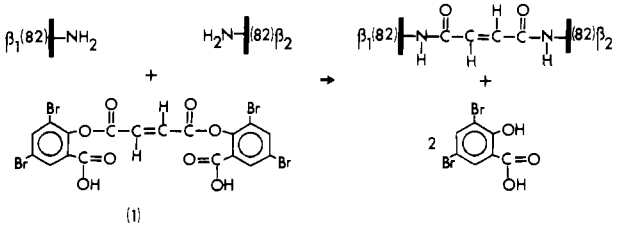
Scheme I



(I)

(Walder et al., 1980), an effect much larger than that thought to be needed to bring about a substantial improvement in the clinical status of patients with sickle cell disease (Hofrichter et al., 1976). This large increase in solubility suggested that the cross-link might alter the structure so as to perturb one of the important intermolecular contacts which stabilize the deoxyhemoglobin S fiber.

Complementary X-ray studies on single crystals (Wishner et al., 1975) and fibers (Magdoff-Fairchild & Chiu, 1979) of deoxyhemoglobin S have revealed the basic structural unit of the fiber: a double-stranded structure in which the two strands of hemoglobin tetramers are related by an approximate 2-fold screw axis running longitudinally between them. Electron microscopic studies of the fiber (Dykes et al., 1979) and of the fiber–crystal transition (Wellems & Josephs, 1979; Wellems et al., 1981), solubility measurements of hemoglobin S double mutants (Benesch et al., 1976, 1979), and estimates of the minimum gelling concentration of mixtures of deoxyhemoglobin S with other mutant hemoglobins (Nagel & Bookchin, 1978) all support this conclusion. In the lateral intermolecular contact between the two strands seen in the crystal, the side chain of Val-6β, the mutation site, extends into a hydrophobic pocket formed by Phe-85β and Leu-88β of an adjacent tetramer. This interaction, which obviously would not be favorable in the case of hemoglobin A with glutamic acid at β6, is presumably the principle basis for the decrease in the solubility of deoxyhemoglobin S.

X-ray crystallographic studies of deoxyhemoglobin A cross-linked by bis(3,5-dibromosalicyl) fumarate showed that there is no effect on the structure in the region of residue 6β (Walder et al., 1980). However, as a result of the cross-link, both the F′ helix (which includes Lys-82β) and the adjacent F helix (residues β85–95) are markedly displaced toward the central cavity of the tetramer. Phe-85β and Leu-88β, the residues which form the acceptor site for Val-6 within the deoxyhemoglobin S fiber, are located at the beginning of the F helix. The difference electron density map for the cross-linked derivative showed very clearly that in addition to the polypeptide backbone the side chains of these residues were shifted toward the central region of the molecule, and hence less exposed to the surface. These results suggested that there is a direct relationship between the magnitude of this perturbation of the structure and the increase in the solubility of deoxyhemoglobin S due to cross-linking the two β82 lysine residues. In order to test this hypothesis, we have now compared the structures of deoxyhemoglobin A cross-linked by bis(3,5-dibromosalicyl) succinate (C_4), bis(3,5-dibromosalicyl) glutarate (C_5), and bis(3,5-dibromosalicyl) adipate (C_6) and the solubilities of the corresponding derivatives of deoxyhemoglobin S. Both the perturbation of the acceptor site and the solubility were found to decrease progressively as the length of the bridging group is increased.

To complete the stereochemical analysis of the reaction pathway, we have determined the effect of the cross-link on the structure in the liganded quaternary state by using CO-β_4 as a model of normal liganded $\alpha_2\beta_2$ tetrameric hemoglobins. The results of these studies show that the fumaryl group, which is fixed in conformation by the trans carbon–carbon double bond, is of the appropriate length and geometry to bridge between the two lysine residues in oxyhemoglobin without perturbing the structure of the protein. This complementary stereochemistry is an important determinant of the specificity of bis(3,5-dibromosalicyl) fumarate and related bifunctional reagents toward the oxy structure.

Experimental Procedures

Materials. Bis(3,5-dibromosalicyl) fumarate and the succinate, glutarate, and adipate analogues were available from previous studies in this laboratory (Walder et al., 1980). Ampholines for analytical and preparative isoelectric focusing were obtained from LKB. Nonidet P40 was purchased from Particle Data Laboratories Ltd. *p*-(Hydroxymercuri)benzoate was obtained from Sigma Chemical Co. All other commercial reagents used were of the highest purity available.

Chemical Modification Studies and Isolation of the Cross-Linked Hemoglobins. Human hemoglobin was obtained from fresh whole blood by the procedure described by Perutz (1968). β chains with sulfhydryl groups blocked were isolated from (carbon monoxy)hemoglobin A treated with *p*-(hydroxymercuri)benzoate according to Bucci & Fronticelli (1965). The sulfhydryl groups were regenerated with 2-mercaptoethanol to reconstitute the β_4 tetramer as described by Waks et al. (1973).

Chemical modification studies as well as preparative reactions were carried out in 0.01 M Bis-Tris buffer, pH 7.2, with the concentrations of hemoglobin and the modifying reagent each equal to 1 mM. Reactions were allowed to proceed for 2 h at 37 °C and then analyzed by isoelectric focusing and $NaDodSO_4$–polyacrylamide gel electrophoresis (Walder et al., 1979, 1980). Cross-linked derivatives of β_4 were purified by preparative flat-bed isoelectric focusing using Ampholines from pH 5 to 7. Electrofocusing was achieved at 29 V/cm for 8 h at 4 °C. Focused bands were removed from the gel and the hemoglobins isolated as described previously (Walder et al., 1980). Cross-linked derivatives of hemoglobin A and of hemoglobin S were purified first by chromatography on DEAE-cellulose by using the buffer system described by Abraham et al. (1976) except that KCN was omitted. Hemoglobins were eluted from the column with a 0.03–0.06 M NaCl gradient for the derivatives of hemoglobin A and with 0.03 M NaCl for hemoglobin S. In each case, the cross-linked derivative was the major modified hemoglobin peak. At this stage of purification, two-dimensional gel electrophoresis (see below) revealed between 5 and 10% contamination with non-cross-linked products. These impurities were removed by gel filtration on Sephacryl S-200 in the presence of 1 M $MgCl_2$ (Macleod & Hill, 1973). All hemoglobins were stored as 10–15% solutions frozen in liquid nitrogen.

The two-dimensional gel electrophoresis procedure used to analyze the hemoglobin derivatives was essentially as described by O'Farrell et al. (1977). In the first dimension, nonequilibrium pH gradient electrophoresis, the gels were prerun according to the sequence (a) 15 min at 200 V, (b) 30 min at 300 V, and (c) 30 min at 400 V. The gels were usually loaded with 20 μg of protein but could be loaded with as much as 40 μg to detect minor impurities. Electrophoresis of the sample was carried out for 3 h at 500 V. At the end of the run, gels were removed and equilibrated with $NaDodSO_4$ sample buffer (5% 2-mercaptoethanol, 2.3% $NaDodSO_4$, and 0.062 M Tris-HCl, pH 6.8) for 50 min. Longer times of equilibration led to a substantial loss of α and β chains from the gel. The second dimension of $NaDodSO_4$ gel electro-

phoresis was carried out on slab gels with a 10–20% acrylamide gradient. The current was maintained at 25 mA until the voltage reached 150 V, after which no further adjustments were made. The gels were run for 6–7 h and were then stained with Coomassie blue.

X-ray Crystallography. Chemically modified derivatives of hemoglobin A were crystallized in the deoxygenated form according to the procedure described by Perutz (1968). Cross-linked derivatives of CO-β_4 were crystallized as described previously for native CO-β_4 (Arnone & Briley, 1978). In both cases, crystallization is carried out at pH 6.5 from concentrated solutions of ammonium sulfate. Crystals selected for data collection measured between 0.5 and 1.0 mm on each edge. Diffraction data were collected to a resolution of 4.4 Å on an Enraf-Nonius CAD4 diffractometer by using the Ω scan mode. One crystal was used for each set of data collected. Degradation due to radiation damage to the crystal never exceeded 5% as determined by the measurement of four standard reflections. Corrections for radiation damage and absorption by the crystal were made as described previously (Ten Eyck & Arnone, 1976; North et al., 1968).

Difference electron density maps were calculated by using the known phases of deoxyhemoglobin A (Ten Eyck & Arnone, 1976) or of CO-β_4 (Arnone et al., 1981) and the difference amplitudes ($|F|_{\text{modified Hb}} - |F|_{\text{Hb}}$) and then symmetry averaged about the molecular 2-fold rotation axis (Muirhead et al., 1967). Difference maps of derivatives of CO-β_4 were symmetry averaged only about the 2-fold axis coinciding with that of hemoglobin A. Stereoscopic drawings were made with a computer graphics program developed by Patrick Briley.

Solubility Studies. The solubilities of deoxyhemoglobin S cross-linked by bis(3,5-dibromosalicyl) succinate, bis(3,5-dibromosalicyl) glutarate, and bis(3,5-dibromosalicyl) adipate, and of native deoxyhemoglobin S, isolated by using the same chromatographic procedures, were determined by measuring the concentration of hemoglobin in equilibrium with the polymer phase after centrifugation for 3 h and 45 min at 130000*g* in a Beckman air ultracentrifuge (Bertles et al., 1970; Magdoff-Fairchild et al., 1976; Hofrichter et al., 1976). In this system, very small sample volumes (75 μL) could be used, minimizing the amount of each derivative required. The solubilities were determined at pH 6.8 in 0.05 M Bis-Tris buffer. After deoxygenation with sodium dithionate (2.5 equiv/heme), the sample was overlaid with mineral oil, and polymerization was allowed to occur for 1 h at 30 °C. The temperature during centrifugation is controlled by the air jet. A water sample was included in each rotor and its temperature measured with a small thermocouple immediately after centrifugation. The temperatures measured ranged between 29 and 31 °C. In five independent determinations, the solubility of native deoxyhemoglobin S was found to be 16.6 ± 0.4 g/dL. The error is reduced even further if the samples compared are centrifuged together in the same rotor. The derivatives were compared in this manner. The values reported in the text are the average of two separate experiments.

Results

Purification of the Cross-Linked Hemoglobins. The two-dimensional gel electrophoresis method described by O'Farrell et al. (1977) was used to analyze the derivatives of hemoglobin A and of hemoglobin S cross-linked by bis(3,5-dibromosalicyl) succinate, bis(3,5-dibromosalicyl) glutarate, and bis(3,5-dibromosalicyl) adipate at each stage of purification. Non-equilibrium pH gradient gel electrophoresis was used rather than isoelectric focusing in the first dimension in order to resolve the α chains, as is generally required for highly basic proteins (O'Farrell et al., 1977). The two-dimensional gel electrophoresis pattern for native hemoglobin A is shown in Figure 1A. The α chains, having three less negatively charged residues than the β chains, migrate much further toward the cathode in the first dimension. In the second dimension, the α chains have a slightly greater mobility than the β chains as is usually observed in $NaDodSO_4$ gel electrophoresis. Figure 1B shows the pattern for oxyhemoglobin A modified with an equal concentration (1 mM) of bis(3,5-dibromosalicyl) succinate. In addition to the α and β chains, there is a prominent spot (arrow) due to the Lys-82 → Lys-82β dimer. Several other very minor cross-linked species as well as a number of monomeric derivatives are also resolved on the gel. The succinate derivative isolated by chromatography on DEAE-cellulose (see Experimental Procedures) is shown in Figure 1C. Native hemoglobin A and nearly all of the other modified hemoglobins in the initial reaction mixture have been removed. At this stage of purification, the derivative appeared as a single band by analytical isoelectric focusing. However, the two-dimensional gel electrophoresis revealed two minor non-cross-linked species representing about 8% of the total protein as estimated by densitometric scanning of the gel. The glutarate and adipate derivatives, and the three corresponding cross-linked derivatives of hemoglobin S, showed similar impurities. The derivatives were purified further by chromatography on Sephacryl S-200 in the presence of 1 M $MgCl_2$. Under these conditions, non-cross-linked hemoblobins are dissociated to αβ dimers (Macleod & Hill, 1973) and are separated from the desired cross-linked product by gel filtration. After this step, the two-dimensional gel electrophoresis of each of the cross-linked hemoglobins showed only free α chains and the β dimer (Figure 1D).

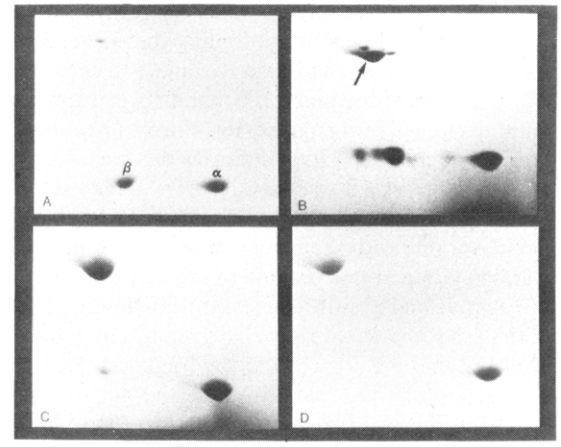


FIGURE 1: Analysis of the cross-linked hemoglobins by two-dimensional gel electrophoresis. (A) Native hemoglobin A. (B) Oxyhemoglobin A modified with bis(3,5-dibromosalicyl) succinate. The arrow marks the position of the Lys-82 → Lys-82β dimer. (C) Hemoglobin A cross-linked by bis(3,5-dibromosalicyl) succinate after chromatography on DEAE-cellulose. (D) The purified succinate derivative after gel filtration through Sephacryl S-200 in the presence of 1 M $MgCl_2$.

Comparison of the Structures of Deoxyhemoglobin Cross-Linked with Bridging Groups of Different Lengths. The structures of deoxyhemoglobin A cross-linked by bis(3,5-dibromosalicyl) succinate, bis(3,5-dibromosalicyl) glutarate, and bis(3,5-dibromosalicyl) adipate were compared by difference Fourier methods. In this series, the maximal span of the bridging group ranges between 6.8 and 9.3 Å. As pointed out previously, the structures of the succinate and fumarate derivatives, both four-carbon cross-links, appear identical at least at 4.4-Å resolution (Walder et al., 1980). The difference map for the succinate derivative vs. native deoxyhemoglobin A is shown in Figure 2B superimposed on the atomic model of the

DPG binding site. The intense band of positive difference electron density (solid contours) joining the β chains is due to the cross-link bridge and is clearly linked to each β chain at Lys-82. An inorganic anion is bound between Lys-82 and the amino terminus in the native structure. The absence of a negative peak at this position indicates that the anion is not displaced in the cross-linked derivative. This suggests that the amide NH group at Lys-82 in the cross-linked hemoglobin interacts favorably with the bound anion in a fashion similar to the amino group at this position in the native protein. The pair of negative and positive peaks associated with His-143β shows that the imidazole side chain is shifted toward the cross-link bridge and is probably hydrogen bonded to the carbonyl group of the amide linkage (the positive density occurs to a much greater extent in the sections shown in Figure 2C). There is a small shift of the amino terminus, and low-intensity features are associated with His-2β and Leu-3β. No significant changes in the structure are observed within the A helix which begins at Thr-4 and includes the mutation site at β6. The most marked alteration in the structure occurs within the F′ and F helices, residues 80–84 and 85–95, respectively. Throughout this region, there is a series of alternating peaks of negative and positive difference electron density indicating a shift of these portions of the molecule toward the central cavity of the tetramer. The extension of this movement to the side chains of Phe-85 and Leu-88, the residues forming the Val-6 acceptor site, is clearly seen in Figure 2C. Smaller changes are observed in the remainder of the F helix, but there does not appear to be any direct perturbation of the heme group. No significant changes in the structure of the protein are propagated beyond the F helix. Overall, the quaternary structure is the same as that of native deoxyhemoglobin A.

The difference maps for deoxyhemoglobin A cross-linked by bis(3,5-dibromosalicyl) glutarate and by bis(3,5-dibromosalicyl) adipate are qualitatively very similar to that for the succinate derivative, indicating all of the same structural perturbations just described. In order to compare quantitatively the differences in structure caused by cross-linking the β chains by bridging groups of varying length, we calculated the difference electron density maps between the glutaryl and succinyl derivatives and between the adipoyl and glutaryl derivatives (panels A and B, respectively, of Figure 3). In each case, there is an alternating series of positive and negative difference electron density peaks within the F′ and F helices similar to that seen in the succinyl – native deoxyhemoglobin A difference map (Figure 2). However, the sign of the peaks is reversed, indicating that with each unit increase in the length of the bridging group the displacement of the F′ and F helices toward the central cavity of the tetramer is less. In both of these difference maps, there is also a peak of negative difference density at the site of the cross-link bridge. This feature probably reflects a greater mobility of the bridging group as the span is increased.

Correlation of the Effect of the Cross-Link on the Structure and the Solubility of Deoxyhemoglobin S. The solubilities of deoxyhemoglobin S cross-linked by bis(3,5-dibromosalicyl) succinate, bis(3,5-dibromosalicyl) glutarate, and bis(3,5-dibromosalicyl) adipate (measured at 30 °C in 0.05 M Bis-Tris-HCl, pH 6.8) were found to be 24.1, 19.8, and 16.8 g/dL, respectively, compared to 16.3 g/dL for native deoxyhemoglobin S. Immediately evident is an excellent correlation between the magnitude of the displacement of the F helix, and hence the perturbation of the Val-6 acceptor site, and the effect of the cross-link on the solubility. The solubility of the succinate derivative is increased to the greatest degree (nearly 50%). Progressively, as the length of the bridging group is increased and the displacement of the F helix becomes less, the solubility decreases, approaching that of native deoxyhemoglobin S. Over this range, the free energy of polymerization varies by about 0.9 kcal/mol.[2]

Structure of CO-β_4 Cross-Linked with Bis(3,5-dibromosalicyl) Fumarate. At the time these studies were begun, there was not yet available a refined high-resolution set of coordinates for any liganded derivative of human hemoglobin. However, X-ray crystallographic studies of CO-β_4, which consists of just the β chains of hemoglobin A, indicated that this structure would be an appropriate model of the liganded quaternary form of normal $\alpha_2\beta_2$ tetrameric hemoglobins (Arnone & Briley, 1978). To compare the structures of CO-β_4 and oxyhemoglobin A within the region of the DPG binding site, we first examined the reactivities of these two proteins with bis(3,5-dibromosalicyl) fumarate and with the corresponding succinate, glutarate, and adipate diesters. Figure 4 shows the $NaDodSO_4$ gel electrophoresis of CO-β_4 and oxyhemoglobin A modified under the same conditions with an equivalent concentration (1 mM) of each of these reagents. With β_4, two distinct cross-linked species are resolved on the gel (Figure 4B). Mixing experiments showed that the lower of the two bands comigrates with the Lys-82 → Lys-82β dimer formed in the reactions with oxyhemoglobin A (arrow), and in the case of the fumarate derivative, in which this is by far the predominant product, the site of the cross-link was also established crystallographically (see below). The fumarate analogue is the most active cross-linking agent in this series toward both CO-β_4 and oxyhemoglobin A, and in both cases, there is a sharp decrease in reactivity at Lys-82 between the C_5 and C_6 derivatives. Moreover, the extent of cross-linking with each reagent at this site is quantitatively very similar for the two hemoglobins. These results provide evidence of the close relationship of the two structures at the DPG binding site.

The derivative of CO-β_4 cross-linked by bis(3,5-dibromosalicyl) fumarate was isolated by preparative isoelectric focusing and the structure determined by X-ray diffraction studies. Figure 5A shows a sketch of the DPG binding site in CO-β_4. Several sections of the difference map for the cross-linked derivative from within this region are shown in Figure 5B superimposed on the native structure. The prominent band of positive difference electron density is due to the cross-link bridge and is clearly seen to join the two β chains at Lys-82. The only other features in the map that appear to be significant are the two negative peaks on either side of the cross-link bridge which arise from the displacement of two symmetry-related inorganic anions seen in the native map. The alternating peaks of positive and negative difference electron density observed within the F′ and F helices of the cross-linked derivatives of deoxyhemoglobin A are conspicuously absent (see Figure 2), indicating that these regions of the molecule are not perturbed in the liganded quaternary state.

Discussion

Conformation of the Cross-Linked Derivative in the Liganded State. In this aspect of the present work, we used CO-β_4 as a model of normal liganded tetrameric hemoglobins to determine the effect of the cross-link on the structure in the liganded quaternary state. Initial X-ray crystallographic studies of CO-β_4 indicated that the arrangement of subunits

[2] Standard free energies of polymerization were calculated with the activity coefficients for hemoglobin given by Ross & Minton (1977).

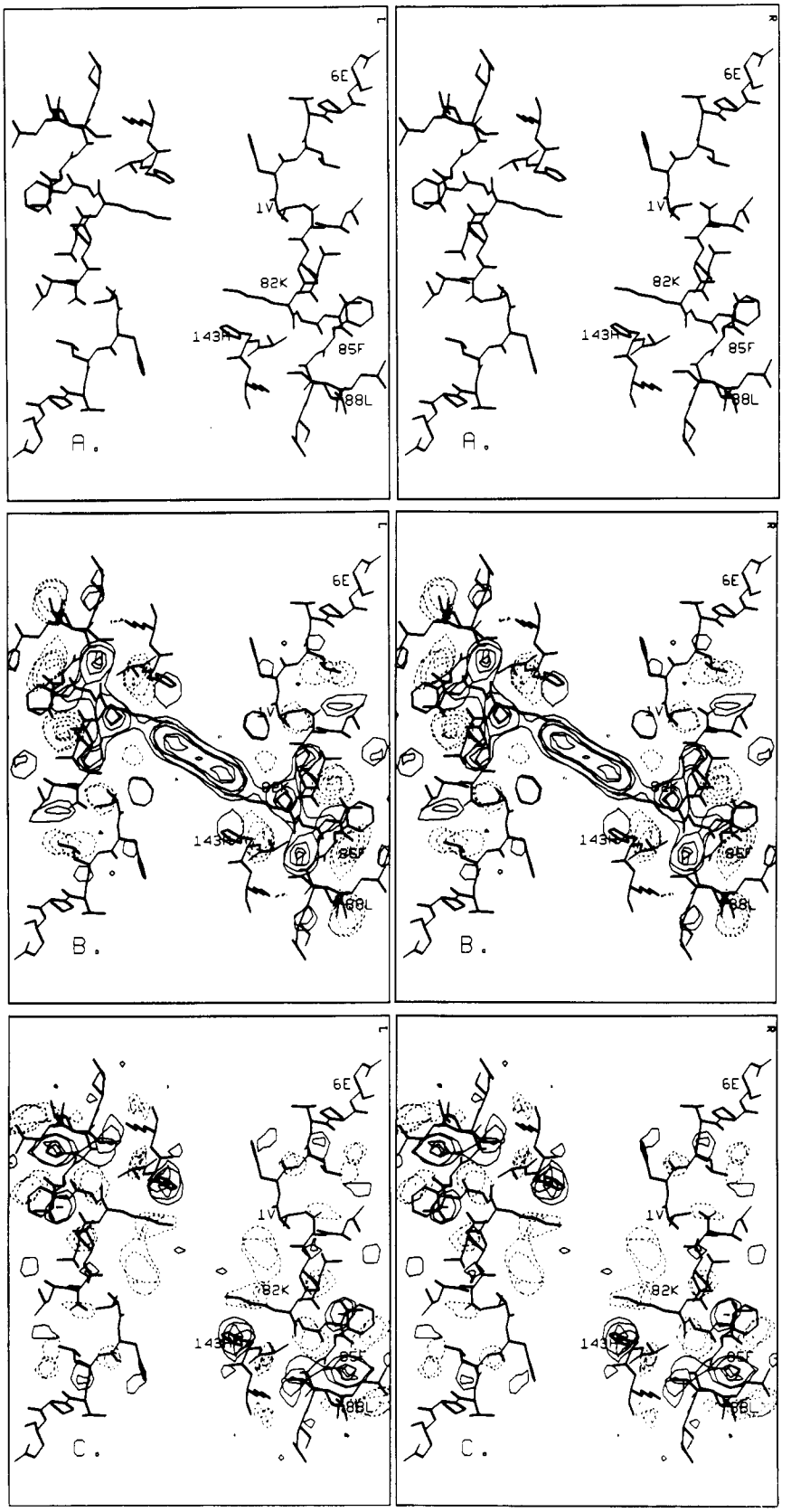


FIGURE 2: Difference electron density maps of deoxyhemoglobin A cross-linked by bis(3,5-dibromosalicyl) succinate – native deoxyhemoglobin A superimposed on the atomic model of deoxyhemoglobin A at the DPG binding site. The region shown includes β chain residues 1 to 6 (A3), 78 (EF1) to 90 (F6), and 142 (HC3) to 144 (HC5). Selected residues are labeled next to their α-carbon atoms. The difference electron density is contoured in two-dimensional sections spaced 1 Å apart with contours starting at $\pm 2\sigma$ and increasing in increments of $\pm 2\sigma$. σ is defined as the root mean square density of the map. Dashed (negative) contours show decreases in electron density relative to deoxyhemoglobin A; solid (positive) contours show increases in electron density. (A) Stereopair drawing of the atomic model. (B) Three contiguous sections of the difference electron density map centered at the α-carbon atom of Lys-82β superimposed on the atomic model. The band of positive difference electron density joining the two Lys-82β residues is due to the cross-link bridge. Pairs of associated positive and negative peaks indicate a displacement of the surrounded structure in the atomic model toward the region of positive difference density. (C) Upper three adjacent sections of the difference electron density map. The negative and positive peaks on either side of Phe-85β and Leu-88β show the displacement of these residues toward the central cavity between the β subunits.

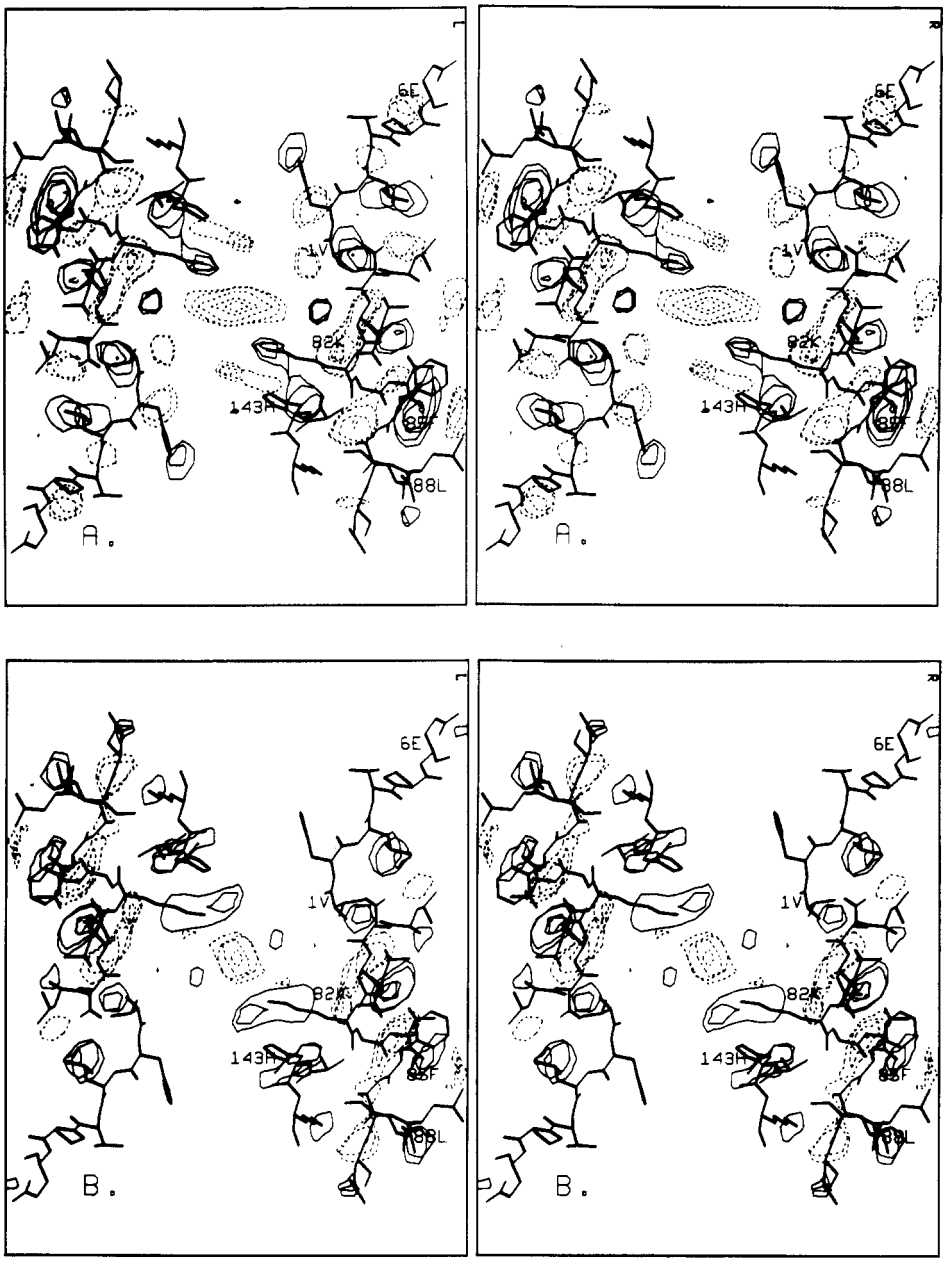


FIGURE 3: Difference electron density maps of the glutaryl – succinyl cross-linked deoxyhemoglobins (A) and the adipoyl – glutaryl cross-linked deoxyhemoglobins (B). The same sections of the difference map are shown as in Figure 2B superimposed on the atomic model of deoxyhemoglobin A. The difference densities were contoured in increments of $\pm 2\sigma$ as in Figure 2. The series of alternating positive and negative difference electron density peaks within the F′ and F helices, residues 80–84 and 85–95, respectively, are reversed in sign from those in Figure 2B. This shows that with each increase in the length of the bridging group, the displacement of the F′ and F helices toward the central cavity is less.

within the tetramer is very similar to that in horse methemoglobin (Arnone & Briley, 1978). Recently, the first high-resolution structure of a liganded derivative of human hemoglobin, the CO form, has been completed, and a refined set of coordinates has become available (Baldwin, 1980). A detailed comparison of CO-β_4 and (carbon monoxy)-hemoglobin A has shown that the quaternary structures of the two proteins and the tertiary structures of the β chains in each are remarkably similar (Arnone et al., 1981). In CO-β_4, the distance between the α-carbon atoms of the two Lys-82 residues on opposing β chains across the DPG binding site is 14.7 Å. In (carbon monoxy)hemoglobin A, this distance is 14.3 Å. This common structural feature as well as similarities in the disposition of other residues within the DPG site provides the stereochemical basis for the similar reactivities of bis-(3,5-dibromosalicyl) fumarate and the series of related cross-linking agents described here toward oxyhemoglobin A and CO-β_4 (Figure 4). In deoxyhemoglobin A, the structure is markedly different within the DPG binding site, and as a result, little cross-linking occurs between Lys-82β_1 and Lys-82β_2 with any of these reagents, regardless of the length of the bridging group (Walder et al., 1980).

The analysis of the structure of CO-β_4 cross-linked with bis(3,5-dibromosalicyl) fumarate shows that the fumaryl group, which is fixed in conformation by the trans carbon–carbon double bond, is complementary to the disposition of the two Lys-82β residues within the DPG site in the liganded quaternary state. Hence, cross-linking of oxyhemoglobin occurs without perturbing the structure of the protein. This favorable stereochemistry is an important determinant of the specificity of the reaction. If rotational freedom is permitted about the central carbon–carbon bond, as in the succinate diester, or if the length of the bridging group is increased beyond the C_5 derivative, the selectivity toward Lys-82, as well

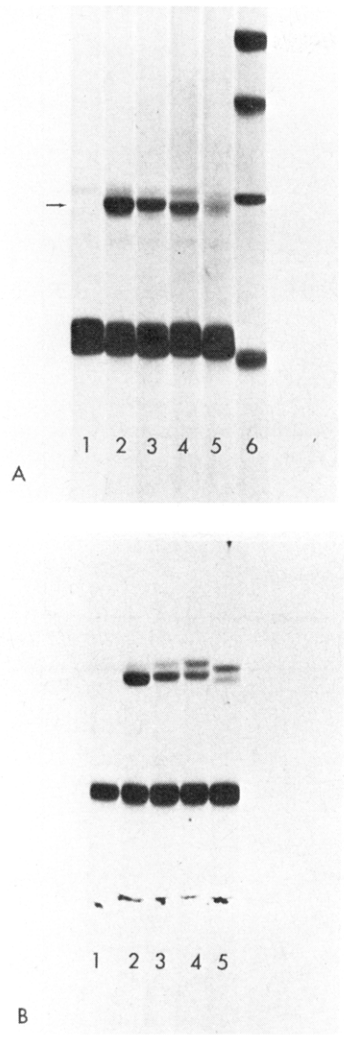


FIGURE 4: NaDodSO$_4$–polyacrylamide gel electrophoresis of oxyhemoglobin A (A) and CO-β_4 (B) modified with analogues of bis(3,5-dibromosalicyl) fumarate. In each reaction, the concentrations of the hemoglobin and the modifying reagent were both equal to 1 mM. The reactions were allowed to proceed for 2 h at 37 °C in 0.01 M Bis-Tris buffer, pH 7.2. (A) Lane 1, native hemoglobin A; lanes 2–5, hemoglobin A modified with bis(3,5-dibromosalicyl) fumarate, bis(3,5-dibromosalicyl) succinate, bis(3,5-dibromosalicyl) glutarate, and bis(3,5-dibromosalicyl) adipate, respectively; lane 6, molecular weight markers—bovine serum albumin, ovalbumin, carbonic anhydrase, and lysozyme. The arrow marks the position of the Lys-82 → Lys-82β dimer. (B) Lane 1, native β_4; lanes 2–5 correspond to those in (A).

as the absolute rate of reaction at this site, decreases markedly (see Figure 4).

Perturbation of the Val-6 Acceptor Site in Deoxyhemoglobin and Its Relation to the Increase in the Solubility of Deoxyhemoglobin S due to Cross-Linking the β Chains between Lys-82β_1 and Lys-82β_2. From the results just described, it is apparent that the displacement of Lys-82β and the F helix toward the central cavity of the tetramer seen in the deoxy structure must arise in the transition of the cross-linked derivative from the liganded to the unliganded state. In this process, the β chains move apart, and the distance between the α-carbon atoms of the two Lys-82β residues increases by more than 5 Å. The constraint imposed by the cross-link bridge apparently limits this movement without preventing the overall change in quaternary structure. As a result, the two lysine residues appear shifted toward the central region of the molecule. The comparison of the structures of the series of cross-linked derivatives shown in Figure 3 is in agreement with this mechanism. Progressively as the length of the bridging group is increased, the constraint on the structure is relaxed, and the displacemnt of Lys-82 toward the central cavity becomes less.

On the basis of the span of the cross-link alone, one would have predicted the constraint on the structure to be fully relaxed once the bridging group had reached six carbon units in length. The maximal span of the adipoyl group is about 9.3 Å, nearly 1 Å greater than the distance required to bridge the β chains at Lys-82 in deoxyhemoglobin. Nonetheless, there is still a significant shift of the F′ helix toward the central region of the molecule in the adipate derivative. It is possible that simply the loss of the positive charge of the modified amino group which occurs when it is converted to an amide also contributes to the perturbation of the structure. This could be rationalized on the basis of a decrease in the repulsive interactions between the cationic residues within the DPG site. However, there is no effect on the structure due to the loss of the positive charge at Lys-82β either by the substitution of a neutral residue as in hemoglobin Providence (Lys-82β → Asn) or by chemical modification with an analogue of bis(3,5-dibromosalicyl) fumarate that cannot cross-link the protein (A. Arnone and J. A. Walder, unpublished results). Therefore, it seems unlikely that this factor plays a significant role in the cross-linked hemoglobins. A more plausible explanation can be put forward on the basis of interactions between the cross-link bridge and other groups on the protein. In each of the cross-linked derivatives, the amide group of the cross-link bridge appears to interact with both His-143β and a tightly bound inorganic anion. Model building studies show that in order for these interactions to occur, the side chain of Lys-82β must adopt a folded conformation. As a result, the distance required to span the two lysine residues is increased and cannot be bridged by the adipoyl group. Since these same interactions occur in the succinate derivative, in this instance, the two lysine residues must be pulled closer together by at least 2.5 Å.

As a result of the linkage between the movement of Lys-82β and the F helix, changes in the conformation of the protein are propagated over a distance of more than 20 Å from the site of modification. However, the changes in the tertiary structure of the β chains do not extend significantly beyond the F helix, and overall the quaternary structure is essentially identical with that of native deoxyhemoglobin. Similarly, the cross-link has only a very small effect on the allosteric properties of the protein. The Hill coefficient for hemoglobin A cross-linked by bis(3,5-dibromosalicyl) succinate is 2.3 compared to 2.8 for native hemoglobin A determined under the same conditions, and there is only a small reduction in the Bohr effect (R. Chatterjee et al., unpublished results) attributable directly to the loss of the contribution of Lys-82 (Perutz et al., 1980).

In contrast to the relatively small effect of the succinyl cross-link on the oxygen binding properties, this modification markedly increases the solubility of deoxyhemoglobin S. Crystallographic studies of the cross-linked derivative give no evidence of a significant change in the structure in the region of the mutation site, residue 6β (Walder et al., 1980). Rather, the results described here show that the increase in solubility is directly related to the perturbation of the acceptor site for Val-6 which is formed by Phe-85β and Leu-88β at the beginning of the F helix. Through the displacement of the F helix, these residues are drawn toward the interior of the molecule, and hence are less accessible to interact with Val-6 at the surface of the tetramer. Increasing the length of the

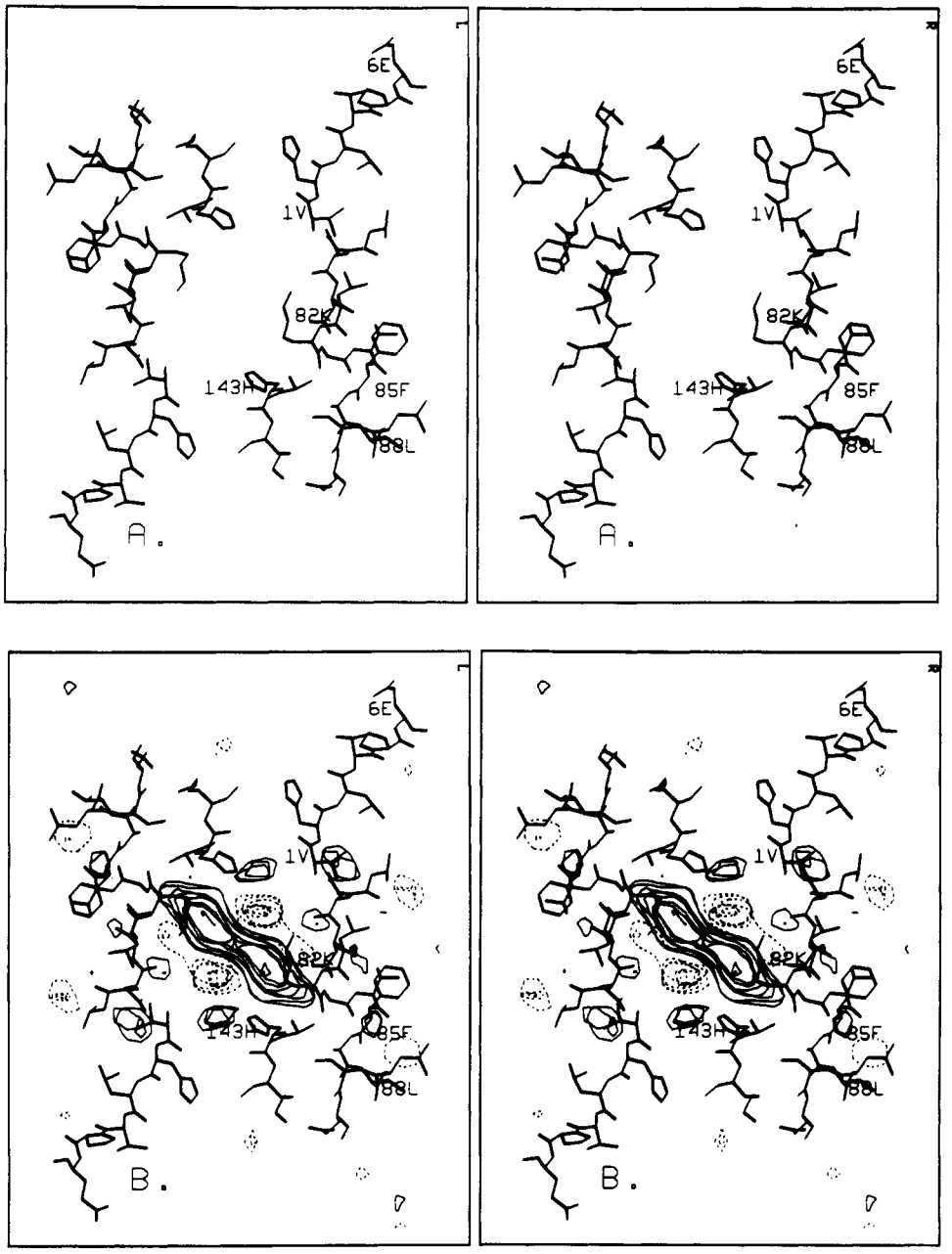


FIGURE 5: Difference electron density map of CO-β_4 cross-linked by bis(3,5-dibromosalicyl) fumarate – native CO-β_4. The same region of the atomic model is shown as in Figure 2A. The difference densities were again contoured in increments of $\pm 2\sigma$. (A) Stereo-line drawing of the atomic model. (B) Four contiguous sections of the difference electron density map centered about Lys-82β superimposed on the atomic model. The band of positive difference electron density is due to the cross-link bridge and, as in hemoglobin A, is joining the two Lys-82β residues. The series of negative and positive difference electron density peaks within the F′ and F helices in the deoxy structure (Figure 2) are not present in this difference map, showing that in the liganded quaternary state these regions of the molecule are not perturbed by the cross-link.

bridging group from four- to five- to six-carbon units progressively reduces the magnitude of the perturbation of the acceptor site. Correspondingly, the solubilities of the cross-linked derivatives decrease successively, approaching that of native deoxyhemoglobin S. The strict correlation between structure and function observed in this series also provides the first direct evidence from solution studies that Phe-85β and Leu-88β which form the contact site for Val-6 in the crystal structure (Wishner et al., 1975; Love et al., 1979) also participate in the fiber. Neither chemically modified derivatives nor mutant hemoglobins have been used previously to probe this important region of the molecule.

The strong dependence of the solubility on the length of the cross-link provides an important constraint on the design of new antisickling compounds. Alone the requirement for specificity of the reagent limits the span of the bridging group to between that of the succinate and glutarate derivatives, 6.8 and 8.1 Å, respectively. Outside of this range, the reactivity decreases markedly (Walder et al., 1980; Wood et al., 1981). However, even over this small interval, there is a very large difference in solubility of the cross-linked derivatives, particularly when considered in terms of the potential clinical benefit. Using the empirical relationship derived by Hofrichter et al. (1976) between the supersaturation ratio and the delay time for the nucleation of polymerization, one would predict that at the same hemoglobin concentration the delay time for the succinate derivative would be 1000-fold greater than that for the glutarate derivative. Consequently, in the design of new antisickling agents, it will be essential to maintain the length of the bridging group at about 7 Å. Pendant groups may be

added to the cross-link bridge to further enhance the specificity of the reagent or to make improvements in other desirable pharmacological properties. These changes may have secondary effects on the solubility, but such effects are likely to be relatively small compared to the large increase in the solubility due to the perturbation of the Val-6 acceptor site. Alternatively, new compounds may be derived by varying the nature of the leaving group (see Scheme I). Changes in this portion of the molecule obviously would have no effect on the functional properties of the modified hemoglobin.

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