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Physiological and x-ray studies of potential antisickling agents

(hemoglobin/binding sites/oxygen equilibria)

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ABSTRACT **Several aromatic compounds have been found to inhibit the gelling of sickle cell hemoglobin. We have tried to correlate the antigelling activity of such compounds with the stereochemistry of their binding sites in the hemoglobin molecule. This approach led to the discovery that two known antilipoproteinemia drugs, clofibrate and gemfibrozil, have antigelling activity. X-ray analysis showed that three pairs of molecules of clofibric acid, the active metabolite of clofibrate, bound to the walls of the internal cavity of deoxyhemoglobin A; only one pair bound to a quite different site, between helices A, E, and H of the α chains of carbon monoxide hemoglobin A. Unlike other antigelling agents, clofibric acid and related compounds decrease rather than increase the oxygen affinity of hemoglobin.**

Many chemical compounds have been studied as possible inhibitors of the gelation of sickle cell deoxyhemoglobin (Hb S), but no agent has proved sufficiently effective and innocuous to be approved for therapeutic treatment (for review, see ref. 1). We wondered whether x-ray diffraction studies of the binding modes of antigelling compounds to hemoglobin crystals might help us to improve their design. By what appeared to be logical arguments, we found that two known drugs—clofibric acid (CFA) and gemfibrozil (GFZ), used for treatment of patients with hyperlipoproteinemia—have antigelling activity.

The rationale for our study was as follows. Many known antigelling agents, such as phenylalanine, contain aromatic rings (2–5). Because toluene, a simple aromatic hydrocarbon, is required for the crystallization of human carbon monoxide hemoglobin A (HbCO) (6), and four to nine molecules are bound by HbCO (7, 8), we argued that the toluene-binding sites might be related to the phenyl-binding sites of antigelling agents to Hb S. Because toluene is not observed in the HbCO electron density map (9), two heavy atom analogues, methylphenylmercury (MePhHg) and iodobenzene, were used to try to locate the toluene site; to our surprise, MePhHg bound to two buried cysteines,‡ making it more suitable as a heavy atom reagent for protein crystallography than as a marker for the aromatic binding site. However, C_6H_5I binds to HbCO near Trp-14α(A12), between helices A and E, not far from a known intermolecular contact in Hb S fibers (10) and at the binding site to deoxyhemoglobin A (Hb) of dichloromethane, which is both an anesthetic and an antisickling agent (11). C_6H_5I also has weak antigelling activity.‡ However, a low-resolution crystal structure of Hb crystals grown in the presence of C_6H_5I showed no evidence of binding. X-ray analysis of crystals with antigelling aromatic amino acids gave a blank for phenylalanine with Hb and L-lysyl-L-tyrosine·HOAc with HbCO, and a multitude of small structural changes for both phenylalanine with HbCO and L-arginyl-L-tyrosine·HOAc (Arg-Tyr) with Hb but no evidence for a single binding site.

Encouraged by the discovery of C_6H_5I in what looked like a binding site for aromatic antigelling agents, we next turned to a similar halogenated phenyl compound, *p*-bromobenzylalcohol (*p*-BrBzlOH), which has been reported (12) to have antigelling properties. This agent also binds to HbCO, close to the site where C_6H_5I had been located. At this point it was suggested to us (D. Witiak, Ohio State University; personal communication) that the antilipoproteinemia drug clofibrate, ethyl-2-(*p*-chlorophenoxy)-2-methylpropionate, was similar in structure and should be tested. We found the active metabolite of clofibrate, CFA [2-(*p*-chlorophenoxy)-2-methylpropionic acid], to have strong antigelling activity. Crystals of HbCO grown in the presence of CFA indicated binding in the same area near Trp-14α(12A), but the map was not interpretable, possibly because of low occupancy. To our surprise, crystals of Hb with CFA showed no binding near Trp-14α(12A). Instead, CFA was bound with high occupancy at two pairs of sites in the internal cavity that runs along the molecular symmetry axis between the α subunits and was bound with lower occupancy at another pair of sites deeper down in the cavity near the β subunits. This binding in the cleft produces small distortions of the tertiary structure, which may account for the antigelling activity. At this stage C. Seymour suggested to us that a newer antilipoproteinemia drug with lesser side-effects also may have antigelling activity. This was GFZ [dimethyl-5-(2,5-xylyloxy)valeric acid]. Her suggestion proved correct, but its binding site has not yet been determined. The *p*-bromobenzyl alcohol-related compound *p*-bromobenzyloxyacetic acid shows higher antigelling activity but binds to Hb in a different site in the α subunit.

Unlike other classes of antigelling agents that raise the oxygen affinity, CFA lowers it. It also makes Hb S less soluble in concentrated ammonium sulfate or sodium potassium phosphate and more heat labile. Binding studies of CFA showed up to 10 mol (HbCO) or 12 mol (Hb) of drug per tetramer at high concentrations of drug.

MATERIALS AND METHODS

CFA from Sigma was used directly, and another batch from Aldrich was recrystallized from water. GFZ was isolated from capsules made by Parke, Davis and given to us by C. Seymour. The solid material was boiled in water, filtered, and dissolved in chloroform. Removal of the solvent gave pure GFZ with the reported melting point. Arg-Tyr from Vega Biochemicals (Tucson, AZ) was given to us by A. N. Schechter.

Hb crystals were grown as described (6) with HbCO at 0.155

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Abbreviations: Hb S, sickle cell deoxyhemoglobin; Hb, deoxyhemoglobin A; HbCO, carbon monoxide hemoglobin A; MePhHg, methylphenylmercury; Arg-Tyr, L-arginyl-L-tyrosine·HOAc; *p*-BrBzlOH, *p*-bromobenzylalcohol; CFA, clofibric acid; GFZ, gemfibrozil; P_{50}, oxygen pressure at which hemoglobin is half saturated.

‡ Abraham, D. J. & Phillips, S. E. V. (1980) International Symposium on Abnormal Hemoglobins: Genetics, Populations and Diseases, Sept. 6–11, 1981, Jerusalem, Israel.

mM (1 gm/100 ml) and Hb A at about 0.165 mM (1.07 gm/100 ml). C_6H_5I and *p*-BrBzlOH were added to the crystallization tubes in large excess (without toluene) for crystallization of HbCO. HbCO crystals with CFA (12 mM) grew from a precipitate (without toluene) in 2.05–2.25 M phosphate buffer. Higher concentrations of CFA (20 mM and up) greatly reduce HbCO solubility without giving crystals. Similarly, Hb with 50 mM CFA precipitated and crystallized at ≈1.8 M buffered $(NH_4)_2SO_4$, compared to about 2.3 M for pure Hb. HbCO crystals with phenylalanine (39 mM) and Arg-Tyr (1.2 mM) required the addition of toluene for growth. Hb crystals also were grown with 15 mM phenylalanine and 6.2 mM Arg-Tyr.

Antigelling effects were measured as described (13), except that the EPR tubes were opened after centrifugation, the supernatant was removed, and Hb was assayed spectrophotometrically as the cyanide–Hb complex at 540 nm. The tubes contained Hb concentrations of 24–26 gm/100 ml. Phenylalanine also was tested for comparison with the other compounds reported here and at 40 mM, with each run as a second control. The control solubility of Hb S (i.e., no additive) averaged 17.0 gm/100 ml for the CFA, GFZ, and phenylalanine assays (10 runs). Oxygen equilibrium curves were determined in 250-ml tonometers by using 15 μM heme in 0.05 M [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane, pH 7.4/0.1 M NaCl (14). Hb was prepared by the same procedure as that used for crystallizations (6). The binding of CFA to HbCO and Hb was determined spectrophotometrically by a modified version of the method reported for 2,3-bisphosphoglycerate (15).

The stability of hemoglobin solutions with CFA was tested by using the methods described in ref. 16. In the heat stability test, 150 μM (tetramer) solutions of HbO_2 and HbO_2 S in 0.15 M phosphate (pH 7.4), containing 0, 5, 10, 20, and 50 mM drug were heated for 1 hr at 50°C. In the other test, the concentration of Hb and drug were the same, but the medium was 15.3% isopropanol/0.1 M Tris·HCl, pH 7.4. The solutions were heated to 37°C for 10 min.

X-ray diffraction data were collected on a Nonius CAD4 4-circle diffractometer for Friedel pairs to a resolution of up to 2.6 Å. Crystals used ranged from 0.5 to 1.0 mm in their longest dimension. For each derivative, data from various crystals were merged; corrections for radiation damage, absorption, Lorentz and polarization factors were applied; and symmetry equivalent observations were averaged to form a unique set of intensities. Table 1 shows maximum resolution and statistics for each derivative.

Difference electron density maps were calculated by using the refined coordinates of native Hb (17) and HbCO (9), with coefficients $|F_d| - |F_n|$ and phases α_c, where F_d and F_n are observed structure amplitudes for derivative and native crystals and α_c is the phase calculated from the atomic model. For Hb derivatives, the maps were averaged over the two crystallographically independent $\alpha\beta$ dimers if the initial map showed evidence of symmetry about the molecular dyad. The observed data for native HbCO had been collected by photographic methods (9).

Table 1. X-ray data for Hb and HbCO derivatives

Derivative	Resolution, Å	R[d − n],* %
HbCO-Phe	3.2	17.2
Hb-Phe	3.2	4.5
Hb-Arg-Tyr	3.6	5.4
HbCO-C_6H_5I	2.8	18.6
Hb-C_6H_5I	4.0	2.9
HbCO-*p*-BrBzlOH	2.6	18.2
HbCO-CFA	2.8	26.7
Hb-CFA	2.8	10.7
Hb-*p*-BrBzl-OAcOH	3.1	9.5

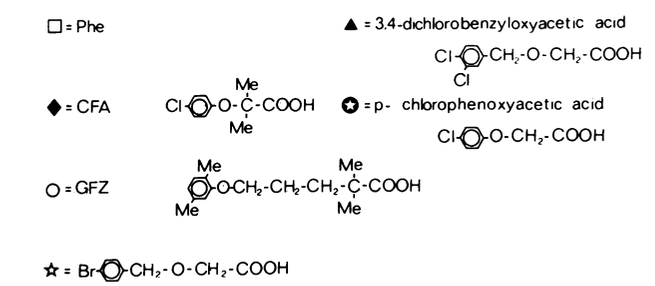
Crystal forms: HbCO = $P4_12_12$; $Z = 4$; $a = b = 53.7$, $c = 193.0$ Å; $\alpha = \beta = \gamma = 90°$ and Hb = $P2_1$; $Z = 2$; $a = 63.4$, $b = 83.6$, $c = 53.9$ Å; $\beta = 99.3$, $\alpha = \gamma = 90°$. *p*-BrBzlOAcOH, *p*-bromobenzyloxyacetic acid.

* R[d − n] = $\Sigma_{hkl}|[F_d - F_n]|/\Sigma_{hkl}|F_d|$ where F_d, F_n are derivative and native observed structure amplitudes for each reflection.

RESULTS AND DISCUSSION

Fig. 1 shows the results of our antigelling assays with CFA, GFZ, and phenylalanine and some more potent halogenated benzyloxy and phenoxy acid derivatives reported recently (18). At 5 mM, GFZ and phenylalanine were about equivalent and more active than CFA. *p*-Bromobenzyloxyacetic acid and 3,4-dichlorobenzyloxyacetic acid were much better antigelling agents at this low concentration and at 10, 20, and 40 mM. The trend in relative activity for CFA and GFZ altered slightly at 10 mM, with CFA about equal to phenylalanine and GFZ somewhat more active. At both 20 and 40 mM, CFA was more potent than both GFZ and phenylalanine.

Because clofibrate (ethyl ester of CFA) and GFZ are clinically approved agents for treatment of hyperlipoproteinemia, it is of interest to compare our antigelling ratios with those reported as necessary for a clinically less severe condition of sickle cell anemia. Sunshine *et al.* (19) have suggested that, in the double heterozygous conditions of S/β^+-thalassemia, ratios of solubility of Hb S (drug treatment)/Hb S (control) should range from



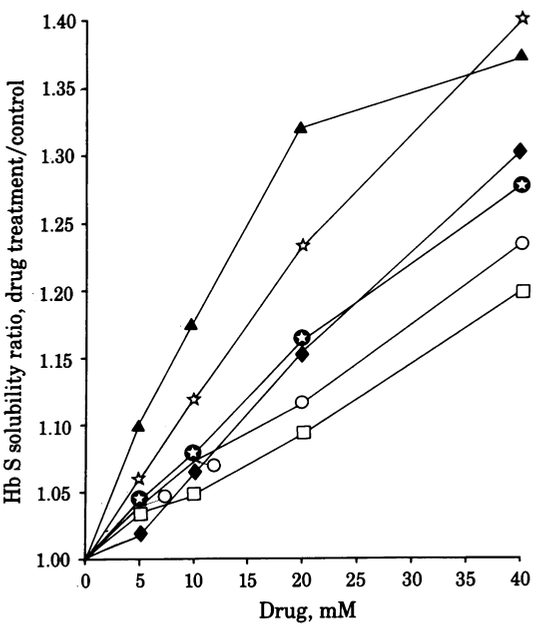


FIG. 1. Increase in solubility of Hb S as a function of concentration of various antigelling compounds.

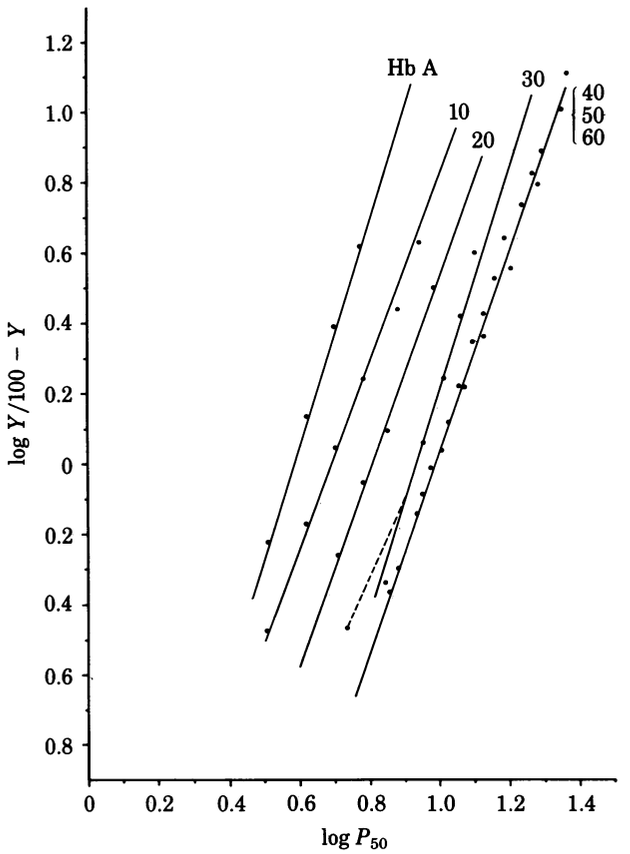


FIG. 2. Influence of CFA on the oxygen equilibrium of human Hb A. Numbers labeling lines through the data points indicate the millimolar CFA concentration. *Y*, fractional saturation of Hb with O_2.

1.06 to 1.17. For conditions that are much less severe, such as sickle/hereditary persistance of fetal hemoglobin, solubility ratios range from 1.19 to 1.26. At present, clofibrate is recommended at a dose of 2 gm per day for adults, producing a plasma level of about 1 mM CFA (20). The concentration of Hb S in homozygous patients is about 1.5 mM, which gives a ratio of 1 mM drug/1.5 mM Hb S. Because our gelling experiments averaged 3.8 mM in Hb S, it would be necessary for CFA to show a suitable solubility ratio at 2.5 mM to produce a clinical effect, but in fact the solubility ratio at 2.5 mM CFA was only 1.01. The only compound that approached the necessary solubility ratio was 3,4-dichlorobenzyloxyacetic acid. Another complicating factor is that 95% of the total CFA is bound to plasma proteins (20, 21). Treatment with other acidic substances might displace CFA from these proteins, but the prospects for effective treatment with CFA alone appear unpromising at present dosages. Our stability studies with CFA indicate that it precipitates HbO_2 S at concentrations above 20 mM, but no precipitate was observed in our antigelling assays. Such high concentrations, with production of Heinz bodies and lysing of the cells, are not likely to be reached in erythrocytes.

CFA decreased the oxygen affinity of Hb at concentrations as low as 10 mM (Fig. 2). At 40 mM and above, the oxygen affinity shifted to a value (log P_{50} = 0.98, where P_{50} is the oxygen pressure at which hemoglobin is half saturated) comparable to that produced by 2 mM 2,3-bisphosphoglycerate (log P_{50} = 1.12) at the same strength, pH, and temperature (22). Other antigelling agents studied to date increase the oxygen affinity.

Because CFA decreases the oxygen affinity of Hb, it must bind preferentially to Hb, but our binding studies showed a high affinity for both HbCO and Hb, and an increased affinity for the latter became evident only at high drug concentrations. Our studies indicated the binding of as many as 9 or 10 mol of drug at very high concentrations (90–100 mM) for HbCO and 10–12 mol for Hb (60–80 mM). Conceivably, precipitation of Hb at the bottom of the ultracentrifuge tubes can lead to increased binding of the drug, in which case the results of our binding studies may not be representative of the binding in solution. The upturn of the Hb curve at high drug concentrations may be due to decreased solubility of Hb and increased binding of the drug by the precipitate. We also tried to measure the binding of the drug in solution by equilibrium dialysis but found our results vitiated by its binding to the dialysis membrane.

Difference electron density maps of HbCO with C_6H_5I and *p*-BrBzlOH showed similar difference peaks in the cleft between helices A and E, near the site occupied by Trp-14α(A12). When models of the compounds were fitted to the peaks, it became apparent that there was not enough room in the cleft for them and the indole side chain of Trp-14α(A12). The peaks can be explained only if these compounds displaced the indole ring (Fig. 3). There was a positive peak, which was much stronger in the *p*-BrBzlOH derivative than in the C_6H_5I derivative, corresponding to a new position occupied by the indole ring in both maps. All contacts between C_6H_5I or *p*-BrBzlOH

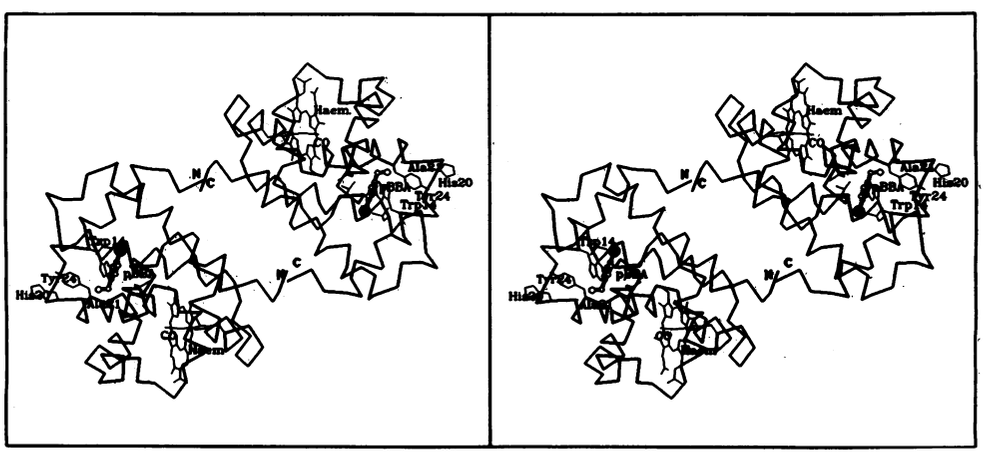


FIG. 3. Stereo view of the $\alpha_1\alpha_2$ dimer down the molecular dyad, shown as the C_α backbone with heme groups and selected side chains. *p*-BrBzlOH (indicated as pBBA) is shown in its site near Trp-14α(A12). His-20α(B1) is involved in an intermolecular contact in the Hb S polymer. Br is shown as a filled circle, and other atoms of *p*-BrBzlOH are shown as open ones. C_6H_5I binds in the same site as does *p*-BrBzlOH but with its aromatic ring reversed, with iodine pointing towards Ala-21α(B2) (not shown).

FIG. 4. Stereo diagram of the difference electron density map of Hb with CFA, viewed down the molecular dyad, showing the four major sites between the α subunits. Solid bonds show α subunits and open ones, β subunits, with the CFA molecules 1 and 2 fitted to the peaks. Contours are at intervals of ± 0.15, commencing at ± 0.1 $e^-/Å^3$. The two minor CFA sites between the β subunits are not shown.

and the protein were nonpolar, except for a hydrogen bond from the hydroxyl of *p*-BrBzlOH to the main chain carbonyl of Ala-21α(B2). This residue, together with Trp-14α(A12), was close to His-20α(B1), which makes a hydrogen bond with Glu-22β(B4) of a neighboring molecule along a single fiber of Hb S (23). Therefore, distortion in this region may affect the stability of the fiber. However, because no binding has yet been established in this region for the Hb structures, we cannot offer this as a mechanism of action. Other peaks in both maps showed similar widespread small changes in the protein, some of which appeared also in the HbCO-phenylalanine map, even though it showed no peaks in the site occupied by *p*-BrBzlOH or C_6H_5I.

The difference map for CFA with HbCO also showed electron density in the site near Trp-14α(A12), but it was not readily interpretable. Changes indicated elsewhere differed from those for C_6H_5I and *p*-BrBzlOH and were more widespread.

Fig. 4 shows a projection of the electron density of Hb with CFA around the 2-fold symmetry axis running between the α subunits. The map shows four distinct regions of positive difference density related in pairs. To each of these a molecule of CFA can be fitted. Fig. 5 shows their attachment to the protein chains. Molecules designated CFA2 each have one carboxyl oxygen pointing to the dyad axis, as if they were binding a cation (NH_4^+) between them, and the other oxygen forming a hydrogen bond to the side-chain hydroxyl of Thr-134α(H17). Their hydrophobic skeletons hug the groove between helices G_α and H_α. Molecules designated CFA1 are attached to the interface between helices G_α and G_β by nonpolar interactions only, and their carboxyls point into the solvent in the internal cavity.

Clusters of positive and negative difference density in several regions of the α subunits showed movements of helix F and of the C terminus of helix H to be associated with CFA binding. There were no peaks, however, near Trp-14α(A12), nor were there features in the HbCO-CFA maps in the sites observed for CFA1 or CFA2 in Hb-CFA.

The electron density difference map for *p*-bromobenzyloxyacetic acid with Hb showed only one major peak, indicating binding at yet another site inside the CD corner of one α subunit. The noncrystallographic symmetry-related α subunit showed no binding in this region.

CONCLUSIONS

There can be a variety of approaches to the problem of preventing sickle cell formation. One consists in making derivatives

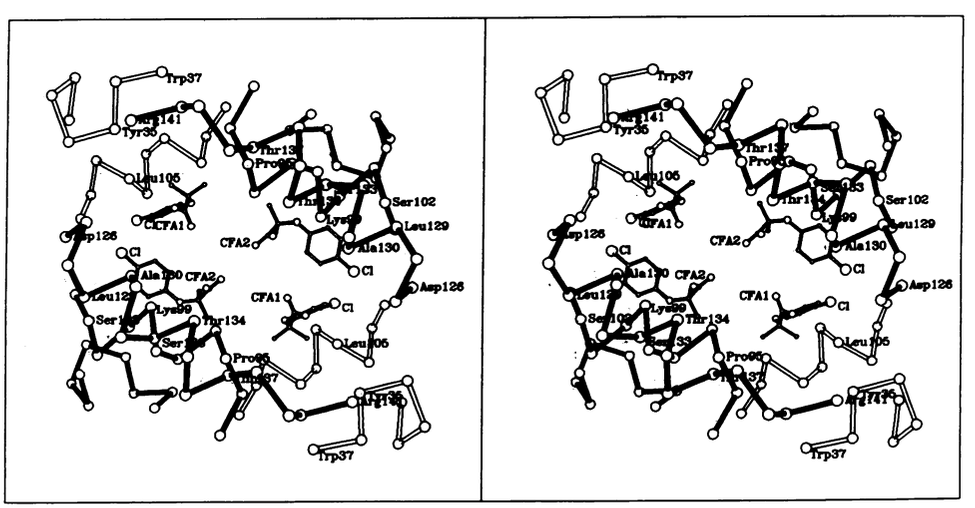


FIG. 5. Stereo diagram of the four CFA molecules bound to Hb in the same orientation as in Fig. 4. C_α atoms only are shown for α (solid bonds) and β (open bonds) subunits. Labeled residues are those with contacts to bound CFA molecules. All interactions are nonpolar, except for the hydrogen bond from Thr-134α(H17) to CFA2.

that shift the allosteric equilibrium towards the more soluble oxy form. Another, performed here, is to modify the deoxy form in some way that decreases the stability of the Hb S polymer. The initial results of this work, from the HbCO structures, seemed to encourage the design of antigelling agents on the basis of our x-ray results. The binding of the antigelling compounds C_6H_5I, *p*-BrBzlOH, and, apparently, CFA to the same region in HbCO as the antigelling agent dichloromethane binds to in Hb presented a coherent picture. The finding that Hb does not bind CFA molecules there but in the cleft between the α subunits raises doubts about the relevance of the binding site in the relaxed *R* structure (HbCO) to the antisickling activity. Alternatively, the linear aggregation of Hb that is common to crystallization and gelation may prevent drug binding at this position. Yet, on the other hand, dichloromethane does bind to the site near Trp-14α(A12) in the tight *T* structure (Hb). Even more puzzling are our observations (*i*) that phenylalanine and Arg-Tyr, which both have antigelling activity, have little effect on the x-ray diffraction pattern of Hb and (*ii*) that phenylalanine has a strong effect on the pattern for HbCO without showing any specific binding site, whereas one would expect antigelling compounds to bind to Hb rather than HbCO (i.e., the *T* rather than the *R* structure) and to bind at a specific site. At present, our intention to proceed by stereochemical logic has been thwarted by the unpredictability of nature, but we have opened up some avenues to the development of potential antisickling agents. The results of the x-ray studies, on the other hand, are consistent with the oxygen equilibrium measurements, for they support the binding of less than two molecules of CFA to HbCO and up to six molecules of CFA to Hb.

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