Structural Basis for the Potent Antisickling Effect of a Novel Class of Five-Membered Heterocyclic Aldehydic Compounds

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Naturally occurring five-membered heterocyclic aldehydes, including 5-hydroxymethyl-2-furfural, increase the oxygen affinity of hemoglobin (Hb) and strongly inhibit the sickling of homozygous sickle red blood (SS) cells. X-ray studies of Hb complexed with these compounds indicate that they form Schiff base adducts in a symmetrical fashion with the N-terminal αVal1 nitrogens of Hb. Interestingly, two cocrystal types were isolated during crystallization experiments with deoxygenated Hb (deoxyHb): one crystal type was composed of the low-affinity or tense (T) state Hb quaternary structure; the other crystal type was composed of high-affinity or relaxed state Hb (with a R2 quaternary structure). The R2 crystal appears to be formed as a result of the aldehydes binding to fully or partially ligated Hb in the deoxyHb solution. Repeated attempts to crystallize the compounds with liganded Hb failed, except on rare occasions when very few R state crystals were obtained. Oxygen equilibrium, high performance liquid chromatography (HPLC), antisickling, and X-ray studies suggest that the examined heterocyclic aldehydes may be acting to prevent polymerization of sickle hemoglobin (HbS) by binding to and stabilizing liganded Hb in the form of R2 and/or various relaxed state Hbs, as well as binding to and destabilizing unliganded T state Hb. The proposed mechanism may provide a general model for the antisickling effects of aldehyde containing small molecules that bind to N-terminal αVal1 nitrogens of Hb. The examined compounds also represent a new class of potentially therapeutic agents for treating sickle cell disease (SCD).

Introduction

Sickle cell disease (SCD) is one of the most prevalent hematologic genetic disorders in the world[1,2] and results from a single point mutation of βGlu6 in Hb to βVal6 in HbS. Two classical quaternary structures of Hb are known: (1) the deoxy conformation (also referred to as the tense or T state) and (2) the oxygenated conformation (also referred to as the relaxed or R state). When the Hb allosteric equilibrium shifts toward the R state, a high-affinity Hb, which readily binds to and holds oxygen, is obtained; the converse is true for the T state. Perutz,[3] and Baldwin and Chothia[4] elucidated, at atomic level resolutions, the tetrameric structures of the T and R states of Hb. The Hb tetramer is composed of two αβ dimers that are arranged around a 2-fold axis of symmetry. This dimer arrangement results in a large central water cavity. The source of structural tension in the T state results from (1) interdimer salt bridges and hydrogen bonds and (2) the preferential binding of an indigenous allosteric effector, 2,3-diphosphoglycerate (2,3-DPG), which engages in salt bridges between the two β-subunits.[5] The Hb T→R transition occurs as a result of oxygen binding, which leads to the disruption of many of the T state intersubunit interactions, as well as the expulsion of 2,3-DPG. During the allosteric transition, the α1β1 dimer rotates by 12−15° with respect to the α2β2 dimer.[4] The R state structure possesses a smaller central water cavity, as well as fewer intersubunit salt bridges and hydrogen bonds, than the T state.

For a long period of time, the allosteric equilibrium of Hb (embodied in the two-state MWC model[6]) was believed to involve only the T↔R transition, with the R state structure thought to be the only relaxed conformer of this protein. However, recent studies have revealed the existence of multiple relaxed Hb conformers, including the R2, RR2, R3 states and others.[7−10] There is still controversy regarding the physiological importance of these relaxed state Hbs and how they might relate to one another with respect to Hb allostery. Silva et al.[7] have suggested that the R2 structure is an intermediate between the T and R structures. However, further analyses show that the R2 state is in itself another relaxed end-state structure.[11,12] Srinivasan and Rose[13] have further suggested that the R2 structure may be the physiologically relevant end-state, and that the R structure is an intermediate that is trapped between the R2 and T states (as a result of the high-salt crystallization conditions used to generate the R state structure).

Structurally, normal Hb and HbS have almost identical residue positions—even in the A helix of the β chains

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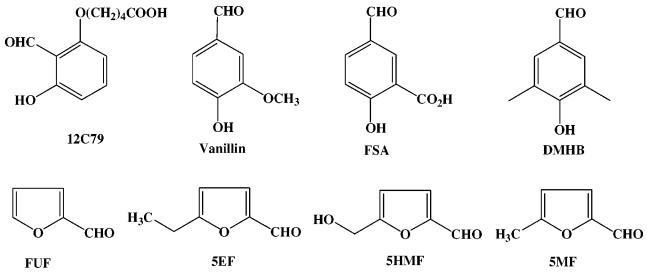

Figure 1. Structures of the furanic compounds, as well as other compounds mentioned in the text.

where the mutation occurs. In HbS, the presence of the βVal6 mutation results in the formation of new hydrophobic contacts between the mutation region of one HbS molecule and a region defined by βPhe85 and βLeu88 in the heme pocket of another HbS. Importantly, these hydrophobic interactions, which occur only in the deoxy or T state conformer of HbS, induce polymerization of the T state molecules into fibers. The formation of HbS polymers causes the normally flexible red blood cells (RBC) to adopt rigid, sicklelike shapes that block small capillaries, causing both local tissue damage and severe pain. Other studies on the gelation of deoxyHbS, and various other Hb variants, have also provided crucial information on other Hb−Hb contact points that are important for stabilizing the HbS polymer.[14,15]

Regulating the Hb allosteric equilibrium, in favor of the relaxed conformation, has been of interest in medicine, as oxygenated HbS (oxyHbS) does not form polymers. Hence, compounds that bind to HbS, and cause an allosteric shift to the high-affinity HbS, have been hypothesized to possess clinical value as agents to treat SCD. This hypothesis has proven to be viable, as two compounds—vanillin[16,17] and 12C79[18−20] (Figure 1) that shift the allosteric equilibrium to the high-affinity Hb—have shown antisickling properties. Both compounds are aldehydes and subsequently have been found to form Schiff base-adducts with Hb. Vanillin is a common food flavoring compound and, because it is relatively nontoxic, is a very attractive therapeutic agent for SCD. A low-resolution structure of T state complexed with vanillin showed that this compound binds to two different sites on the surface of Hb (near residues αHis103, αCys104 and βGln131 (site 1), and residues βHis116 and βHis117 (site 2).[16] The authors proposed that vanillin decreased HbS polymerization by destabilizing the low-affinity T state, which in turn caused a shift in the HbS allosteric equilibrium toward the high-affinity R state form. Interestingly, additional studies of several analogues of vanillin[21,22] showed that these compounds, unlike vanillin, bind to the N-terminal αVal1 of Hb T state, and surprisingly effect opposite allosteric shifts.

In the current studies, we combined the use of aldehydic covalent modifiers of Hb with our knowledge of the molecular regulation of the allosteric equilibrium to produce potent antisickling compounds that should be clinically safe. Specifically, we examined 5-hydoxymethyl-2-furfural (5HMF) and several of its analogues, including furfural (FUF), 5-methyl-2-furfural (5MF), and 5-ethyl-2-furfural (5EF) (Figure 1) for their antisickling potencies. These compounds significantly shift the allosteric equilibrium to the high-affinity Hb and also act as potent inhibitors of SS cells sickling. In fact, one of the compounds, 5HMF, was about 3.5 times more potent than vanillin in inhibiting the sickling of SS cells. Remarkably, the addition of these compounds to deoxy-Hb solution resulted in the formation of crystals that turn out to be R2 Hb quaternary structure. The binding of the compounds to the N-terminal αVal1 of Hb appears to be linked to the regulation of the allosteric equilibrium and may explain the observed structure−activity relationships.

Table 1. Oxygen Equilibrium Studies with Normal Whole Blood[a]

compound[b]	P_{50} (mmHg)[c]	ΔP_{50} (mmHg)[d]	n_{50}[e]
control (DMSO)[f]	25.8 ± 0.0	-	2.3 ± 0.2
5 mM vanillin	19.1 ± 2.0	−6.8	2.0 ± 0.2
5 mM FUF	14.5 ± 0.1	−11.4	2.3 ± 0.0
5 mM 5MF	9.7 ± 0.2	−16.2	1.7 ± 0.1
5mM 5EF	10.1 ± 0.8	−15.7	1.8 ± 0.1
1 mM 5HMF	19.2 ± 1.5	−6.6	2.1 ± 0.1
2 mM 5HMF	15.0 ± 0.5	−10.8	2.0 ± 0.1
5 mM 5HMF	8.3 ± 0.4	−17.5	1.9 ± 0.1

[a] The results are the means ± SE for two measurements. [b] The ratio of compound to Hb at 1 mM, 2mM, and 5 mM compound concentrations are 0.8, 1.6, and 4, respectively. [c] P_{50} is the oxygen pressure at which AA cells (40% hematocrit) in the absence or presence of compound is 50% saturated with oxygen. [d] ΔP_{50} is P_{50} of compound treated AA cells − P_{50} of control. [e] n_{50} is the Hill coefficient at 50% saturation with oxygen. [f] The final concentration of DMSO was about 1.2 mM in all samples, including control.

Results and Discussion

Vanillin is clinically tested for SCD therapy and was studied with the examined heterocyclic aldehydes, also referred to as furanic compounds. We have also previously published detailed functional and antisickling properties of vanillin.[16,17] Relatively high concentrations of compounds (1−5 mM) were used to react with Hb in the solution studies. This is necessary, as the concentration of Hb within RBCs is approximately 5 mmol/L, and at 25% hematocrit with a blood volume of 4 L, 10 mmol/L compound is needed to produce a 2:1 compound−Hb adduct. There are two identical binding sites in Hb since it possesses a 2-fold axis of symmetry. All compounds were solubilized in DMSO (1−2 mM) and used for the test. The corresponding control experiments (without test compound) also contain DMSO (1−2 mM).

Oxygen Equilibrium and HPLC Studies. The compounds were tested for their ability to left-shift the oxygen equilibrium curve (OEC) of both AA and SS cells and quantified by their ability to decrease in P_{50} (partial pressure of oxygen at 50% Hb saturation). Allosteric effectors that increase Hb oxygen affinity decrease the P_{50} (left-shift the OEC) relative to the control. This results in a negative ΔP_{50} value. Table 1 summarizes the effects of vanillin, FUF, 5MF, 5EF, and 5HMF on AA cells at 5 mM. The most potent compound is 5HMF ($\Delta P_{50} = -17.5$ mmHg), followed by 5MF ($\Delta P_{50} = -16.2$ mmHg) and 5EF ($\Delta P_{50} = -15.7$ mmHg), and FUF (−11.4 mmHg). The least left-shifting is vanillin (−6.8 mmHg). Table 1 also shows that 5HMF left-shifts the OEC in a dose-dependent manner. The Hill coefficients of the modified Hbs (1.7−2.1), with the exception of that of FUF (2.3), are smaller compared to that of control (2.3).

Table 2 (columns 2 and 3) also summarizes changes in P_{50} and ΔP_{50} for SS cells treated with vanillin, FUF, and 5HMF at 5 mM concentration. All compounds shift the OEC to the left, and as observed in the oxygen

Table 2. Oxygen Equilibrium and Adduct Formation Studies with Homozygous Sickle Red Blood Cells[a]

compound	SS cells P_{50} (mmHg)[b]	SS cells ΔP_{50} hemolysate (mmHg)[c]	hemolysate P_{50} (mmHg)[d]	hemolysate ΔP_{50} (mmHg)	HbS adduct (%)[e]
control (DMSO)[f]	31.2 ± 1.0	-	11.2 ± 0.2	-	-
vanillin	17.7 ± 2.2	−13.5	8.1 ± 1.1	−3.1	15 ± 3.6
FUF	15.4 ± 1.7	−15.8	5.3 ± 0.6	−5.9	24 ± 5.7
5HMF	6.0 ± 1.2	−25.2	1.8 ± 0.3	−9.4	70 ± 10.0

[a] The results are the means ± SE for two measurements. [b] P_{50} is the oxygen pressure at which SS cells (10% hematocrit) or hemolysate (in the absence or presence of 5 mM compound) is 50% saturated with oxygen. [c] ΔP_{50} is P_{50} of compound treated SS cells or hemolysate − P_{50} of control. [d] P_{50} values obtained from hemolysate after incubation of compounds with SS cells. [e] HbS adduct values obtained from HPLC elution patterns of hemolysate after incubation of compounds with SS cells. [f] The final concentration of DMSO was about 1.0 mM in all samples, including control.

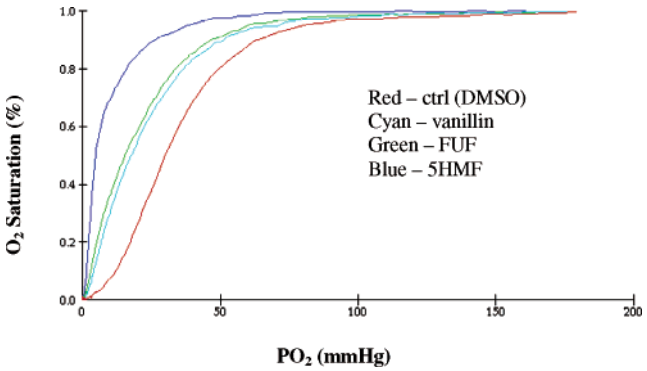

Figure 2. The effect of 0 mM compound (control, in red), 5 mM vanillin (cyan), FUF (green), and 5HMF (blue) on the OECs of intact SS cells. The curves are from right to left, respectively.

equilibrium studies with AA cells, 5HMF is the most potent compound (ΔP_{50} = −25.2 mmHg) followed by FUF (ΔP_{50} = −15.8 mmHg), and last vanillin (ΔP_{50} = −13.5 mmHg). In the absence of compound, the OEC of SS cells suspension showed a sigmoid curve, while the shape changed to hypoberlic with 5 mM incubation of the SS cells with the three compounds (Figure 2). Although not reported in the Table 2 or Figure 2, 5HMF also left-shifts the OEC in a dose dependent manner with ΔP_{50} of −5.8 mmHg and −13.6 mmHg at 1 mM and 2 mM concentrations, respectively. In addition, 5HMF has a major effect on the cooperativity, as the shape of the OEC changed from sigmoid to hyperbolic with increased 5HMF concentration. Similar effect on cooperativity has been reported for vanillin.[16]

The absorption spectra of Hb in red blood cells that were taken before and after measurement of the OEC showed no significant oxidation of Hb during the experiment.

To determine whether the left-shift of compound-treated SS cells is the result of direct interaction of the compound with HbS, the hemolysate of vanillin-, FUF-, and 5HMF-treated SS cells were subjected to both OEC and HPLC studies. The results are summarized in Table 2 (columns 4−6). Each of the tested compounds produces a new HbS modified peak that eluted before that of the parent HbS peak, indicating the formation of a reversible Schiff-base covalently modified HbS adduct (Figure 3). 5HMF-modified HbS by the greatest degree (70%), followed by FUF (24%), and last vanillin (15%). The compounds also shift the OEC of the hemolysate to the left. 5HMF causes the largest Hb left-shift (ΔP_{50} of −9.4 mmHg), followed by FUF (ΔP_{50} = −5.9 mmHg) and last vanillin (ΔP_{50} = −3.1 mmHg). These shifts follow the same trend observed during the whole blood studies. Since the compounds probably exist in unbound and

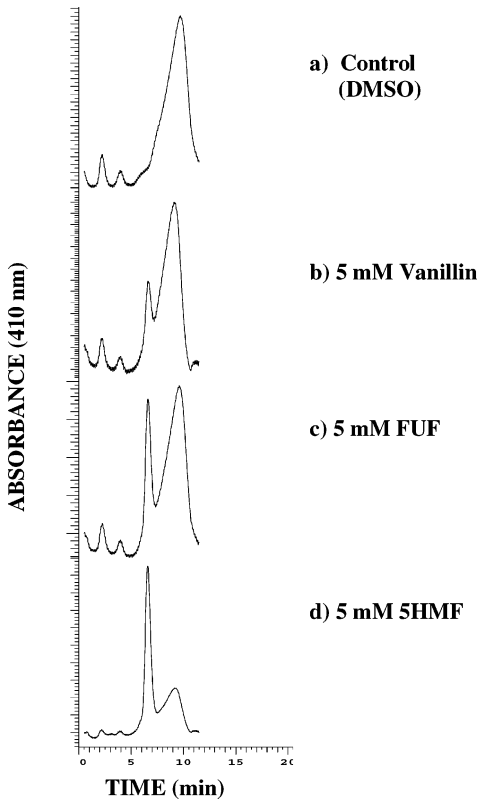

Figure 3. Cation-exchange HPLC patterns of hemoglobin prepared from SS cells that had been preincubated with 0 (control) and 5 mM of vanillin, FUF and 5HMF. (a) Hemolysate prepared from untreated SS cells. (b−d) Hemolysate prepared from suspensions of SS cells that had been preincubated in the presence of 5 mM vanillin, FUF, and 5HMF for 1 h. Note the new peaks at approximately 7 min, indicating the Schiff-base adduct for the test compounds. The area of the peak is greatest in 5HMF, followed by FUF and last vanillin.

bound forms in the solution studies, lysis of the cells may increase the unbound form because of dilution. This is consistent with the observed lower left-shift of the hemolysate compared to the SS cells. The values reported for HbS modification may also be an underestimation due to the same reason given above.

Results from these studies clearly indicate that the furanic compounds possess the ability to (1) pass through RBC membranes, (2) bind to and/or modifies HbS, and (3) shift the OEC to the high-affinity HbS, which does not form polymers. Furthermore, we have found that the change in the oxygen affinity of SS cells suspension caused by these compounds depends on the degree of binding to HbS; 5HMF showed the highest amount of compound−HbS adduct (modified HbS), and as expected, was the most potent OEC left-shifter. Also, the results clearly suggest that substitution, as well as

Table 3. Antisickling Studies with Homozygous Sickle Red Blood Cells[a−c]

compound	inhibition of sickling of SS cells (%)	MCV[d] (fl)
control (DMSO)	0	61.5
5 mM vanillin	20 ± 6.5	61.0
1 mM FUF	0	60.3
2 mM FUF	0	60.0
5 mM FUF	30 ± 7.0	62.8
1 mM 5HMF	13	62.5
2 mM 5HMF	42 ± 1.0	61.0
5 mM 5HMF	90 ± 5.0	61.4

[a] The results are the means ± SE for two measurements. [b] Antisickling studies with SS cells (10% hematocrit) under 4% oxygen. [c] The final concentration of DMSO was about 2.0 mM in all samples, including control. [d] MCV is the mean corpuscular volume.

substitution type at the 5-position of the central furan ring, is important to biological activities. 5HMF, which possesses an alkyl alcohol at the 5-position of the furan ring, is more potent than either 5MF or 5EF, both of which possess hydrophobic moieties at this position. FUF, without a substitution, is the least potent. This is consistent with the X-ray results (see below), which indicate that the hydroxyl moiety of 5HMF is intimately involved in interactions that stabilize the relaxed state Hb. The Hill's coefficients of the modified Hbs are relatively smaller compared to the unmodified Hb, suggesting a decrease in cooperativity. This is expected because of the apparent weakening of interdimer interactions by the binding of the compounds to the T state, leading to increased oxygen affinity, reduced cooperativity, and a shift toward the high-affinity Hb.

Antisickling Studies. Upon exposure of SS cells suspension to only 4% oxygen, in the absence of test compound, all cells underwent sickling. In the presence of vanillin, FUF, and 5HMF (at 5 mM concentration) the percentage of SS cells decreased by 20%, 30%, and 90%, respectively (Table 3, column 2) after 5 h. 5HMF inhibited sickling the most, followed by FUF and vanillin. These results follow the same trend observed in the left-shift of the OEC, as well as the compound−HbS adduct formation. Column 2 of Table 3 also shows that FUF did not inhibit cell sickling at lower concentrations (1 and 2 mM). In contrast, 5HMF decreased the formation of SS cells in a dose-dependent manner at all studied compound concentrations.

Under 100% nitrogen (and without 5HMF), about 5% of the SS cells were sickled after 40 min, which increased to 65% after 60 min. The cells were completely sickled after 80 min. However, for 5HMF incubated SS cells, 5% were sickled after 40 min, which increased to 10% after 60 min, and then to 20% after 80 min. Eventually all cells became sickled in 2 h. Thus, when 100% nitrogen is used for the testing, 5HMF does not prevent sickling, but may only prolong sickling for a few hours. This is in contrast to the experiments done with 4% oxygen that show 90% inhibition of sickling even after 5 h. The use of 4% oxygen in antisickling studies is justified, because under physiological condition, the circulating SS cells in patients are never exposed to a fully deoxygenated condition, and a significant proportion of the intracellular HbS molecules are in the nonpolymerizing oxy-form.

In general, the furanic compounds, especially 5HMF have very strong antisickling properties. At 5 mM concentration, 5HMF inhibited cell sickling by ∼3.5 and 2.5 times more than vanillin and FUF, respectively. Remarkably, even at 2 mM, 5HMF reduces cell sickling by 42%, twice as much as vanillin at 5 mM concentration. Thus, the antisickling action of these compounds seems to result from their ability to bind to, as well as modify, HbS and left-shift the OEC toward the high-affinity Hb. On the basis of structure−activity relationships, we predict that 5MF and 5MF will exhibit antisickling activities that lie between those of FUF and 5HMF.

The effect of compounds on SS cells size was also studied, and as shown in Table 3 (column 3), incubation of SS cells with 1, 2, and 5 mM of FUF or 5HMF, and only 5 mM of vanillin, did not dehydrate the cells. Since polymerization of HbS and sickling of SS cells are linked to intracellular concentration of HbS, any agent that causes dehydration of RBCs would increase the molar concentration of HbS and presumably increase polymer formation. Although not reported in the experiment, the compounds did not promote formation of metHbS or membrane-associated denatured HbS during the antisickling studies.

Crystallization Studies. X-ray crystallographic studies of the heterocyclic aldehydes were conducted to determine the binding sites of these compounds in T and R state Hbs with the hope of explaining their potent biological activities. Both T and R state crystallization experiments were conducted with FUF and 5HMF using high-salt as previously reported.[10,23] The compounds were first reacted with deoxyHb under nitrogen gas atmosphere and carbonmonoxy Hb (COHb) under carbon dioxide atmosphere, respectively. This was then followed by reduction of the Schiff base adduct (formed between the aldehyde and the N-terminal nitrogen of Val1) with sodium cyanoborohydride ($NaCNBH_4$) to the corresponding irreversible alkylamine covalent bond before the complex solution was crystallized. Reduction of the Schiff base is necessary to resolve the bound compound crystallographically,[21,22] because at the pH of ∼7.0 where both the solution and X-ray experiments were conducted, the Schiff base interaction is reversible and leads to poorly resolved compound density. Note that none of the solution studies use $NaCNBH_4$, and they perfectly show modification of Hb or HbS. Thus, the compounds may exist essentially in bound form in the X-ray studies, while they exist in unbound and bound forms in the solution studies, with the ratio dependent on pH, dilution, and compound concentration, among other factors. Unexpectedly, the T state crystallization experiments resulted in two different crystals—a rectangular crystal (space group $P2_1$), which is isomorphous to T state native crystal, and a trigonal crystal (space group $P3_221$), which was later determined to have a relaxed state conformation in the form of R2 quaternary structure. R2 state native crystals have previously been obtained under low-salt conditions,[7] but not under high-salt conditions.

It is believed that the predominantly deoxyHb solution used for crystallization experiments contained partially ligated Hb or even fully ligated Hb in R2 state form. The furanic compounds may then have reacted

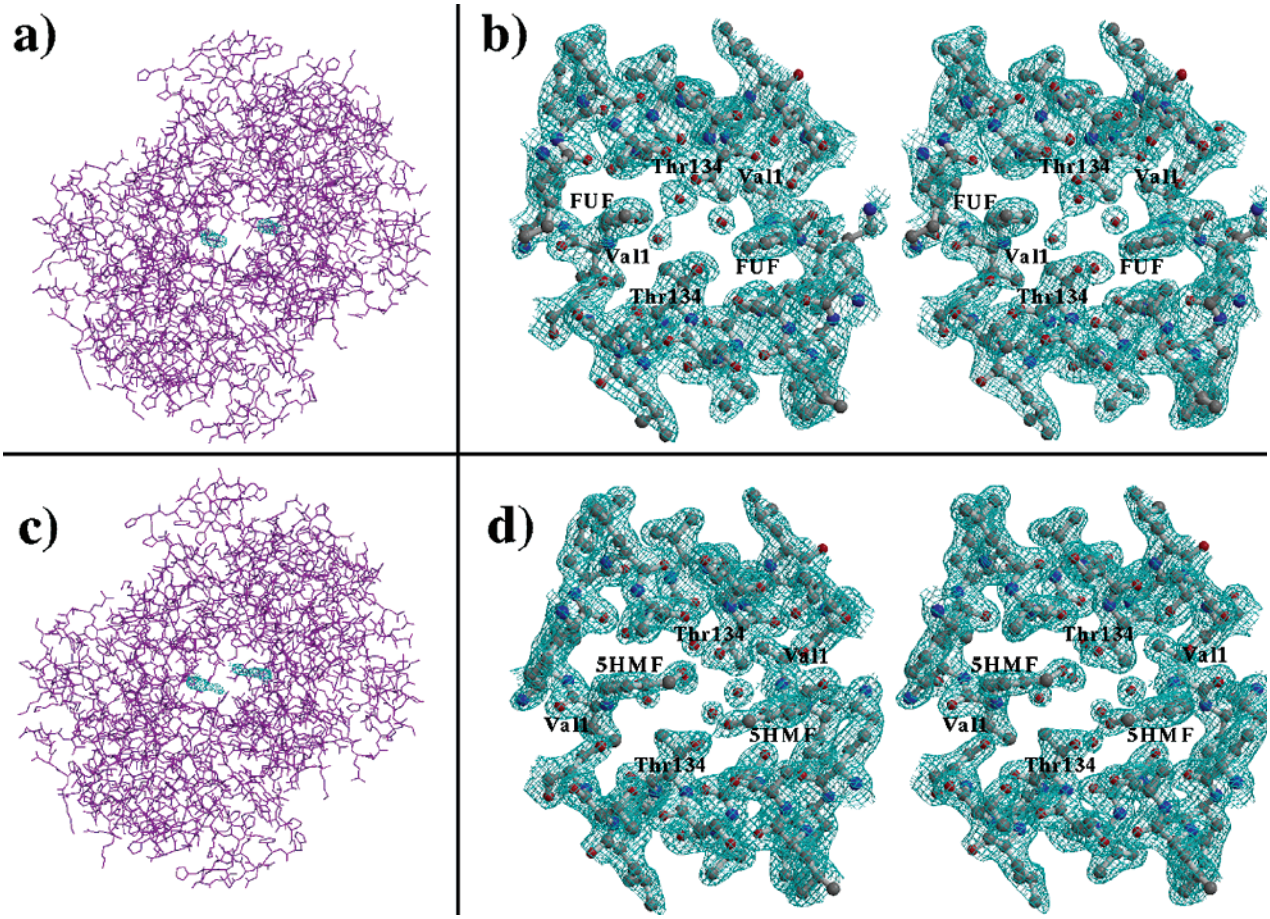

Figure 4. Final $2F_o - F_c$ electron density maps of the bound furanic compounds in the central water cavity (close to the mouth of the α-cleft) of the R2 state Hb complex structures. Two compounds bound in a symmetry-related fashion to the N-terminal αVal1. Maps were contoured at 1.0 σ level and superimposed with the refined models. The figures were drawn using BOBSCRIPT[39] and RASTER3D[40] and labeled using SHOWCASE (Silicon Graphics, Inc). (a) A global view of the bound FUF molecules looking down the two-fold axis of Hb. (b) Stereo figure of a close-up view of FUF binding site. (c) A global view of the bound 5HMF molecules. (d) Stereo figure of a close-up view of 5HMF binding site. The solvent densities found at the binding sites of FUF and 5HMF are also shown in c and d.

with the R2 state Hb and stabilize it to increase the R2 state Hb fraction, which subsequently crystallized out. Approximately $(3-5) \times 10^{-6}$ moles of Hb were used for each crystallization setup, and it would only take a minute quantity of air in the anaerobic chamber to ligate the Hb. Also, the ~3% CO and metHb in the starting Hb solution may still exist as ligated form in the deoxygenated Hb. When sodium dithionite is used to completely deoxygenate the Hb solution, we only observe T state crystals with no compound bound. This is probably due to the fact that the aldehyde is easily reduced to the inactive alcohol derivative.

Unlike the T (and R2) state experiment, repeated R state cocrystallization experiments yielded very few crystals and only on rare occasions when low concentrations of the compounds (≤4 molar excess to the Hb tetramer) were used, with most of the CO-liganded Hb complex still remaining in solution. Since the compounds are known to bind and modify liganded Hb as indicated by HPLC studies, we speculate that the liganded complex that failed to crystallize may be R2 state Hb or other relaxed state Hbs. And even though we did not observe R2 state or any other relaxed state crystals, it is likely that the high salt R state crystallization conditions used were not conducive for crystallizing out these relaxed state Hbs. This is consistent with the fact that R2 state crystals from fully ligated normal Hb have only been observed under low salt.[7] The few R state crystals that were obtained are probably unbound or weakly bound Hb that still exist as R state Hb at low compound concentration, as indicated by the almost nonexistent compound density at the binding site of the R state complex structures. We point out that in the absence of the aldehydes; we easily obtain R state crystals from liganded Hb using the same R state crystallization conditions.

Description of the R2 State Complex Structures. The structure of the FUF−Hb complex in the R2 state crystal was determined by molecular replacement method[24] using the α1β1-α2β2 R2 state native Hb structure (PDB code 1BBB) as a search model. We used the refined FUF−Hb structure as a starting model for the refinement of the 5HMF−Hb complex structure. In both complexes, strong and well-defined densities (with occupancies of approximately 100%) were identified for a pair of FUF or 5HMF molecules bound at the N-terminal αVal1 residues in a symmetry-related fashion (Figure 4a−d). The N-terminal αVal1 binding site is located in the central water cavity of Hb close to the mouth of the α-cleft.

The R2 state complexes and R2 state native have essentially the same Hb quaternary structures (rmsds

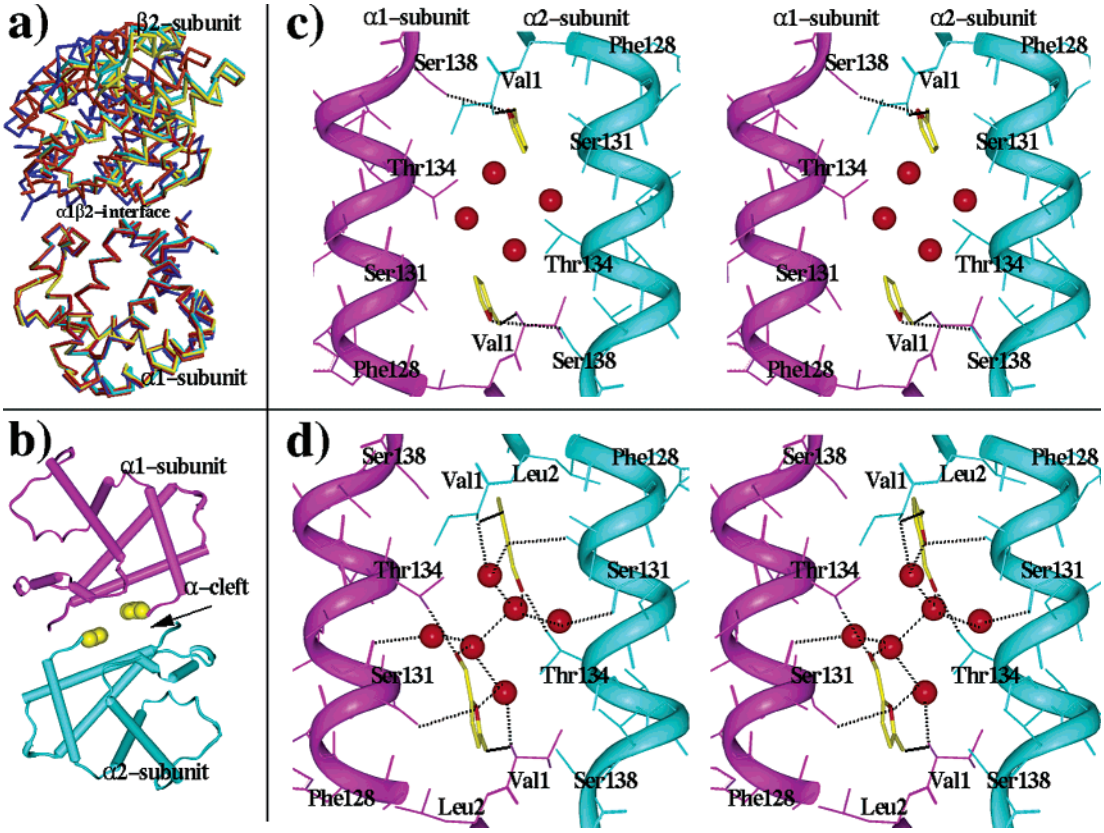

Figure 5. Crystal structures of different Hb states and the R2 state Hb complexes. The figures were generated with INSIGHTII (Molecular Simulations, Inc. 9685 Scranton Rd., San Diego, CA), MOLSCRIPT[41] and RASTER3D[40] and labeled with SHOWCASE. (a) Superposition of R (brown), R2 (red), and T (blue) native structures with those of the R2 FUF−Hb (yellow) and R2 5HMF−Hb (cyan) complex structures. The structures were superimposed as described in the test. For clarity, only the α1β2 dimers are shown. Note that the quaternary structures of the R2 native and the R2 complexes are identical but different from those of the R and T native structures. (b) A R2 complex structure depicting α1 (magenta) and α2 (cyan) subunits of Hb where FUF or 5HMF (yellow in CPK model) bind in a symmetry-related fashion at the α-cleft to the two N-terminal αVal1. (c) Stereo figure of a close-up view of FUF (yellow) binding site. Binding site residues are shown in sticks. Water molecules are in red sphere, while hydrogen bonds are represented by dashed lines. α1-subunit in magenta, while α2subunit in cyan. The covalent linkage between the aldehyde of the compound and the nitrogen of the protein is represented by a heavy line. (d) Stereo figure of a close-up view of 5HMF (yellow) binding site.

of ∼0.4 Å) when the two are superimposed using all Cα atoms with the exception of three residues from the N- and C-terminals of the four subunits in each structure. However, similar comparison of the R2 state complex structures with R (PDB code 1AJ9) and T (PDB code 2HHB) native Hb structures shows very significant quaternary structural differences, with rmsd of ∼1.8 Å and ∼3.3 Å, respectively. Figure 5 shows the α1β2 dimers of the R2 state complexes and the R, R2, and T native Hb structures after superposition of the α1β1 dimers (using all Cα atoms with the exception of three residues from the N- and C-terminals) with the R2 state complexes as a reference point. As previously analyzed and reported by Silva et al., for R, T, and R2 native Hb structures,[7] the allosteric transitions between the R2 complex structures and those of the R and T structures show extensive reorganization of the α1β2, α1α2, and β1β2 interfaces in the three Hb states.

Following are detailed descriptions of the interactions between the R2 state Hb and the compounds at the α-cleft of the α1Val1 binding site (Figure 5b). The other symmetry-related molecule engages in similar, but opposite, interactions at the α2Val1 binding site. A covalent interaction between the aldehyde and α1Val1 nitrogen directs the furan ring toward the central water cavity. In the FUF−Hb structure, the bound compound appears to have two alternate conformations that differ by almost 180°. This is clearly due to the fact that the small FUF molecule (no ring substituent) can easily rotate in the active site without any steric constraint. One conformer, with the ring oxygen facing the protein residues, is shown in Figure 5c. The aromatic oxygen engages in a very weak intersubunit hydrogen bond with α2Ser138 OG (3.6 Å), which serves to tie the two α-subunits together. Interestingly, if the compound is rotated to its alternate conformation, the oxygen faces the water cavity and engages in a weak intrasubunit hydrogen bond with α1Ser131 OG (3.5 Å). There are very few hydrophobic interactions (<3.8 Å) between the furan ring and the protein residues, involving only Lys127 and Ala130.

Unlike FUF, there is no evidence of compound rotation of the bound 5HMF molecule, and it assumes a conformation with the ring oxygen facing the water cavity. A rotation of 5HMF as observed with FUF would involve steric contact between the hydroxymethyl substituent and some of the binding site residues. The observed interaction between FUF and αSer138 OG is

therefore absent in the 5HMF−Hb complex structure (Figure 5d). Similar to FUF, the ring oxygen of 5HMF engages in an intrasubunit hydrogen bond with α1Ser131 OG (3.1 Å), albeit stronger. In addition, the 5-hydroxymethyl substituent of 5HMF also makes a strong intrasubunit hydrogen bond interaction with α1Thr134 OG1 (2.6 Å); this interaction is absent in the FUF−Hb complex structure. While FUF ties the two α-subunits together by making a weak intersubunit hydrogen bond with α2Ser138 OG, the two 5HMF molecules are joined together by a strong network of six water-mediated hydrogen bonds, through the hydroxyl and the ring oxygen moieties that tie the two α-subunits together (Figure 5d). Four of these water molecules are conserved in the FUF binding pocket, but they are mobile and do not engage in hydrogen bond contact with the FUF molecules. These water molecules do not interact with FUF, apparently due to the mobility of the compound, as well as the lack of hydroxymethyl moiety in FUF. Like the FUF−Hb structure, there are very few hydrophobic interactions (<3.8 Å) between 5HMF and Hb. The increased number of interactions between 5HMF and protein residues (versus FUF), as well as the strong water-mediated hydrogen bonds that tie the two α-subunits together in the 5HMF complex structure, may partly explain why 5HMF is biologically more potent than FUF.

Description of the T State Complex Structures. Since the T state cocrystals were isomorphous to that of T state native crystals, the α1β1-α2β2 T state native structure (PDB code 2HHB) was used as a starting model for the refinement of the T state FUF- and 5HMF−Hb structures. Unlike the R2 state complex structures, the T state complexes only show delocalized compound density and repeated model building to improve the density was not successful to allow for reliable compound fitting. The T state complexes and T state native have essentially the same Hb quaternary structures (rmsds of ∼0.4 Å) when all Cα atoms with the exception of three residues from the N- and C-terminals of the four subunits in each structure are superimposed. Superposition of the αVal1 binding sites also shows very few structural differences.

Description of the R State Complex Structures. The isomorphous α1β1 dimer R state native structure (1LJW) was used as a starting model to refine the FUF−Hb and 5HMF−Hb complex structures. The structures only show very sparse density at the N-terminal αVal1 binding sites for both FUF and 5HMF, significantly lower than observed for the T and R2 state complexes. No compound was fitted to the density. Even though the tetramer structures between the native R and the refined R complexes of FUF and 5HMF are indistinguishable (rmsds ∼0.4 Å, when all Cα atoms with the exception of three residues from the N- and C-terminals of the four subunits in each structure are superimposed), the C-terminal α-residues (Trp140 and Arg141) display significant positional differences. In the complex structures, these residues have rotated away at the αLys139, displacing αArg141 by almost 180° from its position in the native structure, while αTyr140 has oriented away by ∼2 Å. αTyr140 OH now engages in hydrogen bonds with αVal93 O and αPro95 N in the complex structures, versus diagnostic R state native hydrogen bonds with αVal93 N and O.

Molecular Docking Studies of the N-terminal αVal1 Binding Pockets of T, R, and R2 State Hb Structures. 3, 5-Dimethyl-4-hydroxybenzaldehyde (DMHB, Figure 1) that shifts the OEC slightly to the left and right-shifting effectors have been shown to bind to N-terminal αVal1 of T state Hb.[21,22] Therefore, we anticipated that the furanic compounds, when reacted to Hb, would produce T and R state cocrystals with the compounds bound to the N-terminal αVal1 nitrogen. Surprisingly, the T state crystallization studies led to the formation of both T and R2 state cocrystals, while the R state crystallization studies produced only few R state crystals with very weak compound density. To our knowledge, this is the first report of mixed crystal formations as a result of addition of an allosteric effector to deoxyHb. We decided to visually model the furanic compounds to the N-terminal αVal1 binding pockets of known T, R, and R2 native Hbs to help explain these unexpected results. Shown in Figure 6a is an overlay of the α-cleft of the three native Hbs, with 5HMF modeled to the binding site. The native structures were superimposed using the invariant α1β1 dimer (Cα residues) on the BGH frame as defined by Baldwin and Chothia.[4] The T state has the largest binding pocket, followed by the R2 state, and last by the R state. Docking studies show that 5HMF can easily fit the T and R2 state binding sites without steric interference. In contrast, the binding pocket of the R state Hb is sterically crowded due to the presence of the C-terminal residues of Tyr140 and Arg141. Thus, for 5HMF to be able to bind to the R state there must be rearrangements of the binding pocket residues. This is exactly what occurs in the R state complex structures, which show larger binding pockets compared to the native R structure (Figure 6b). It seems reasonable that the penalty for rearrangement of the binding site pocket residues in the R state should considerably decrease the affinity of the compound for the R state Hb, compared to those of the T and R2 states. This is consistent with the X-ray studies that show very sparse to almost nonexistent density at the R-state binding site.

Although the T state cocrystals only show delocalized compound density, a hypothetical fit of 5HMF to the binding site with the aid of the density shows the compound bound covalently to αVal1 and possibly engaging in intrasubunit interactions with αThr134 OG1 and αSer131 OG. The delocalized density is probably due to mobility of the bound effector, as the binding pocket of the T state Hb is very large compared to the compound size. In contrast, the R2 state binding pocket is smaller than that of the T state and shows relatively tighter packing around the compounds. We have observed similar delocalized densities in effectors that bind to the N-terminal Val1 binding pocket of T state Hb.[16,21,22]

Mechanism for the Antisickling Activities of the Furanic Compounds. Oxygen equilibrium, antisickling, HPLC, molecular docking, and X-ray studies indicate that the furanic compounds may be acting to inhibit SS cells sickling by covalently binding to and stabilizing the relaxed state HbS and/or binding to and destabilizing the T state HbS (with a concomitant shift

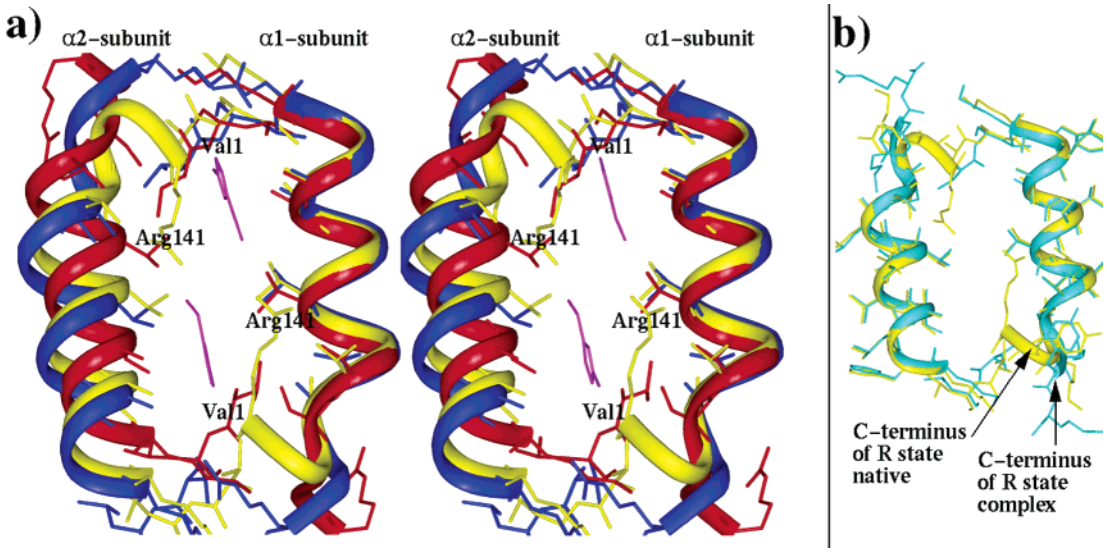

Figure 6. Structural differences at the N-terminal αVal1 binding pockets of the different Hb states. The structures were superimposed using the invariant α1β1 dimer on the BGH frame (see text). The figures were generated with INSIGHTII and labeled with SHOWCASE. (a) Stereo figure of the overlay of the N-terminal αVal1 binding pockets of T (blue), R (yellow), and R2 (red) native Hbs. 5HMF (magenta) is modeled into the binding pocket. Note how the C-terminus occupies the binding pocket of R state Hb. (b) Overlay of the N-terminal αVal1 binding pockets of the R native and FUF−Hb R complex structures.

of the allosteric equilibrium to the high affinity HbS). As pointed out by several investigators, both R and R2 state Hbs, as well as several other relaxed Hb states can potentially coexist in solution.[7−10] On the basis of the T state crystallization experiments, which resulted in the formation of R2 state cocrystals, as well as the modeling studies, it is compelling to suggest that the compounds may be interacting with and stabilizing R2 state Hb (that occurs as partially or fully ligated Hb in the crystallization solution in equilibrium with the predominant T state Hb), driving the allosteric equilibrium predominantly to the soluble relaxed state conformation, and that the compound-HbS adducts observed during the HPLC analyses of SS cells in air are mostly modified R2 state Hb. The T state crystallization experiments may mimic in vivo physiological conditions, where the circulating SS cells in patients are only partially deoxygenated with a significant proportion of the intracellular HbS molecules in the nonpolymerizing oxy-form. Thus, the compounds could similarly act on liganded Hb (probably in the form of R2 state Hb) in vivo to increase the solubility of the HbS. The mechanism is also consistent with the fact that increasing the oxygen affinity is known to prevent polymerization of HbS at partial deoxygenation.[25]

In the oxygen equilibrium studies, we observed significant decreases in the Hill's coefficients of the modified Hbs, as well as changes in the shape of the OECs from sigmoidal to hypoberlic with increasing concentration of the aldehydes, indicating an effect on cooperativity produced by compound binding. Thus, even though we make compelling arguments for direct compound binding to the relaxed state Hb, the X-ray, and in particular the oxygen equilibrium studies, suggest that the compounds could bind directly to deoxyHb and weaken the T state interdimer interactions, leading to a shift in the allosteric equilibrium to increase the concentration of the relaxed state conformation. It is likely that the relaxed state may be in the form of R, as suggested by previous studies, or it could be a mixture of the various relaxed states, including R2. The allosteric equilibrium is primarily regulated by stabilization or destabilization of the T state conformation, and this notion is consistent with landmark findings by Abraham's group,[21,22] in which the authors hypothesized that agents such as 5-formylsalicylic acid (FSA, Figure 1), that form Schiff base adducts with the N-terminal αVal1 nitrogen of the T state, as well as salt bridges and hydrogen bonding contacts with the opposite dimer (across the 2-fold axis), add more constraints to the T state and produce a low-affinity Hb. In contrast, agents such as DMHB (Figure 1), which bind to the T state in a similar fashion, but do not contain acid groups to engage in intersubunit salt bridges/hydrogen bonding interactions, left-shift the allosteric equilibrium and produce a high-affinity Hb. The latter effectors disrupt the T state salt or proposed water-mediated bridges between the two α-chains,[26] as well as inhibit nonspecific chloride ion binding.[27] The furanic compounds, like DMHB, also lack acid groups for intersubunit interactions and as a result bind to and destabilize the T state Hb and shift the allosteric equilibrium to the relaxed state.

On the basis of the structural studies, it is hypothesized that the differences in the biological activities of the furanic compounds are due to their modes of binding to both the relaxed and T state Hbs. In the relaxed state Hbs, such as observed in the R2 state structures, 5HMF possesses the ability to stabilize the relaxed conformation to a greater degree than FUF, and it is equally possible that 5HMF destabilizes the T state more than FUF. Both 5MF and 5EF are more potent than FUF, but less so than 5HMF. The substituent at the 5-position of 5MF and 5EF should translate into additional hydrophobic interactions, as well as prevent or reduce compound rotation when bound to Hb, compared to FUF. However, the lack of the hydroxymethyl moiety in 5MF and 5EF is expected to reduce the affinity of these compounds for Hb, compared to 5HMF.

Studies with vanillin[16] and pyridoxal[28] have suggested that the antisickling activities of these compounds are partially due to the shifting of the allosteric equilibrium toward the more soluble R state HbS with binding to T state HbS. However, results from our studies suggest that R2 state HbS or possibly a combination of the various relaxed state HbS maybe involved in left-shifting the allosteric equilibrium to the high-affinity HbS. Consistent with this observation is the fact that, vanillin also binds to deoxyHb and forms R2 state crystals, similar to the furanic compounds (Safo, unreported results). The vanillin studies also attributed the antisickling effects to stereospecific inhibition of T state HbS polymer formation due to an apparent binding of the effectors at the surface of Hb molecule.[16] The latter suggestion has also been made for a number of other compounds that prevent gelling of HbS, because X-ray studies also show the compounds bound at the surface of Hb molecule.[29−31] We cannot discount a possible contribution to the antisickling activities by direct inhibition of T state HbS polymerization, even though we do not observe binding of the furanic compounds to the surface of the Hb. Nevertheless, the lack of SS cells sickling inhibition by 5HMF at 100% nitrogen argues against such a contribution.

Summary and Conclusions. The above studies indicate that the furanic compounds readily pass through serum albumin and RBC membrane, form a Schiff-base adduct with intracellular oxy- and deoxyHbS, and shift the oxygen equilibrium to the left, thereby increasing the fraction of soluble relaxed state HbS. We also show for the first time that, the soluble relaxed state Hb may not exist exclusively in the R state conformation, but possibly a mixture of the various relaxed state Hbs, including the R2 state. In fact, the conditions under which the R2 crystals were formed may mimic physiological conditions, where partially deoxygenated HbS may bind with the furanic compounds in vivo to increase soluble R2 state HbS.

Due to the chronic nature of SCD, as well as the large amount of Hb in the body, large and frequent doses of drugs are needed to effectively treat the disease. Therefore, any antisickling agent that works by modifying HbS must possess little, if any, toxicity, and should be very potent. 5HMF has a very low toxicity profile, as the acute oral dose (LD_{50}) has been reported to be 2.5 g/kg for male rats and 2.5−5.0 g/kg for females rats (US EPA, 1992). In comparison, vanillin, which is considered nontoxic, has a LD_{50} of 1.58 g/kg in rats.[16] We also point to the fact that certain foods that are consumed on a daily basis, such as coffee and caramel products, possess concentrations of 5HMF that sometimes exceed 6 g/kg.[32] The other furanic compounds also occur in nature, and with the exception of FUF, there are no reports about possible adverse effects. Antisickling agents, such as vanillin and 12C79, also bind covalently to Hb, and both have been shown to be clinically nontoxic.[16,19,20] Remarkably, 5HMF is about 3.5 times more potent than vanillin, which is currently under clinical studies for the treatment of SCD. The low molecular weight, low toxicity, and high potency of the furanic compounds make them viable drug molecules to treat SCD. The results from these studies also present a coherent picture of the antisickling potencies and atomic-level mechanisms of new antisickling agents. With this information, it will be possible to perform structure−activity studies that will result in the development of analogues with enhanced potency.

Experimental Section

Materials and General Procedures. The compounds, vanillin, FUF, 5MF, 5EF, and 5HMF (see Figure 1), were purchased from Aldrich Chemical Co. All compounds were solubilized in DMSO (1−2 mM) and used for the test. The corresponding control experiments (without test compound) also contain DMSO (1−2 mM). Normal red blood (AA) cells were collected from adult donors. SS cells were obtained from patients with SCD. Purified human adult Hb in 50 mM Bis Tris buffer, pH 6.8, was prepared from discarded human blood as previously described[10] and stored at −80 °C until ready for crystallization.

Oxygen Equilibrium Studies with Normal Whole Blood. Normal blood samples (hematocrit 40%) in the presence of 5 mM vanillin, FUF, 5MF, 5EF, and 5HMF (solubilized in DMSO) were equilibrated at 37 °C for 1 h. The samples were then incubated in IL 237 tonometers (Instrumentation Laboratories, Inc. Lexington, MA) for approximately 10 min at 37 °C and allowed to equilibrate at oxygen tensions 7, 20, and 60 mmHg. The samples were aspirated into an IL 1420 Automated Blood Gas Analyzer and an IL 482 or IL 682 Co-oximeter (Instrumentation Laboratories) to determine the pH, pCO_2, pO_2, and Hb oxygen saturation values (sO_2). The measured values of pO_2 and sO_2 at each oxygen saturation level were then subjected to a nonlinear regression analysis using the program Scientist (Micromath, Salt Lake City, UT) to calculate the P_{50} and Hill coefficient values (n_{50}). A dose−response study with 5HMF was performed at final compound concentrations of 1 and 2 mM.

Oxygen Equilibrium Studies with Homozygous Sickle Red Blood Cells. SS cells were suspended in PBS to a final hematocrit of 10%. Vanillin, FUF, and 5HMF (solubilized in DMSO) were added to this suspension at final concentrations of 5 mM and incubated at 37 °C for 1 h. A 40 μL aliquot of this suspension was added to 4 mL Hemox buffer and subjected to oxygen equilibrium analysis (37 °C) using a Hemox-Analyzer (TCS Scientific Corp., Southampton, PA).[33]

Transport through Homozygous Sickle Red Blood Cells Membrane and Reaction with HbS. The compound-treated SS cells obtained in the preceding experiment (oxygen equilibrium studies with SS cells) were hemolyzed by adding 5 volumes of 5 mM potassium phosphate buffer, pH 7.4, containing 0.5 mM EDTA. After centrifugation, the hemolysate was subjected to both oxygen equilibrium analysis using Hemox-Analyzer and cation-exchange HPLC analysis using a Hitachi HPLC apparatus (Model D-7000 Series) and a Swift WCX column (Swift WCX-PEEK: 50 mm × 4.6 mm, Isco, Inc., Lincoln, NE). The column was developed using a linear gradient of phase B from 25% to 90% at 410 nm (Mobile Phase A: 40 mM Bis-Tris, 5 mM EDTA, pH 6.5; Phase B: 40 mM Bis-Tris, 5 mM EDTA, 0.2 M sodium chloride, pH 6.5). The HbS adduct formation (modification of HbS) values are expressed in percentages, using the following formula:

$$\text{Mod HbS (\%)} = \frac{\text{peak area of modified Hb}}{(\text{peak area of modified Hb} + \text{peak area of unmodified Hb})} \times 100$$

Antisickling Studies with Homozygous Sickle Red Blood Cells. The effects of vanillin, FUF, and 5HMF on the inhibition of SS cells sickling were evaluated using a mixture of 4% oxygen and 96% nitrogen as previously described.[34] Briefly, SS cells suspended in buffered saline solution, pH 7.4 (hematocrit of 10%), were incubated at 37 °C with 4% oxygen in the presence of 5 mM compound solubilized in DMSO. Aliquots (10 μL) of the suspensions were obtained after 5 h and fixed with 2% glutaraldehyde solution without exposure to air. Morphological analysis and percentage of SS cells that

Table 4. Crystal Information, Data Collection, and Refinement Parameters for the Hb Complex Structures[a]

	FUF (R2 state)	5HMF (R2 state)	5HMF (T state)	FUF (R state)
		Data Collection Statistics		
space group	$P3_221$	$P3_221$	$P2_1$	$P4_12_12$
cell dimensions (Å)	91.40 91.40 142.00	91.86 91.86 143.53	62.61 82.47 53.46 99.52	53.46 53.46 192.88
molecules/asymmetric unit	1 tetramer	1 tetramer	1 tetramer	1 dimer
resolution (Å)	69.14−2.25 (2.30−2.25)	69.57−1.85 (1.90−1.85)	82.47−1.86 (1.90−1.86)	84.0−2.00 (2.10−2.00)
no. of measurements	124853 (9905)	286747 (6589)	108495 (4373)	105005 (2596)
unique reflections	32647 (1991)	56802 (2940)	41917 (1934)	18357 (1526)
$I/\sigma I$	7.0 (1.8)	12.5 (1.8)	15.8 (5.5)	13.2 (2.8)
completeness (%)	97.0 (94.1)	93.1 (64.2)	90.7 (68.5)	92.0 (57.4)
R_{merge} (%)[b]	7.5 (24.9)	6.9 (31.8)	6.5 (18.6)	6.9 (32.7)
		Structure Refinement		
resolution limit (Å)	69.14−2.25 (2.37−2.25)	69.57−1.85 (1.97−1.85)	52.72−1.86 (1.98−1.86)	51.5−2.00 (2.13−2.00)
σ cutoff (F)	0.0	0.0	0.0	0.0
no. of reflections	32645 (4983)	56780 (698)	41895 (5756)	18316 (1880)
R_{factor} (%)	21.7 (38.6)	18.4 (29.3)	16.3 (22.6)	21.3 (29.0)
R_{free} (%)[c]	27.4 (39.7)	22.3 (31.9)	20.7 (29.0)	26.3 (34.1)
rmsd standard geometry				
bond lengths (Å)	0.011	0.013	0.015	0.012
bond angles (deg)	1.87	1.89	1.72	1.90
dihedral angles				
most favored regions	91.4	92.8	93.6	92.8
additional allowed regions	8.6	7.2	6.4	7.2
Luzzatti est. coordinate error				
R_{factor}	0.30	0.20	0.17	0.25
R_{free}	0.38	0.25	0.23	0.33

[a] Numbers in parentheses refer to the outermost resolution bin. [b] $R_{merge} = \Sigma(I - I)/\Sigma I$. [c] 5% of the reflection which were used for the calculation of R_{free} were excluded from the refinement.

were not sickled were conducted using a computer-assisted image analysis system as described elsewhere.[35] Dose−response studies of FUF and 5HMF at compound concentrations of 1 and 2 mM were also performed. A control experiment using 100% nitrogen was also performed with and without 5HMF.

The Effect of Compounds on Homozygous Sickle Red Blood Cells Size. To study the effect of the compounds on the degree of hydration/dehydration of SS cells, the compound-treated SS cells obtained in the preceding experiment (anti-sickling studies with SS cells) were evaluated with a Hemavet Cell Analyzer to determine the mean corpuscular volume (MCV).

Crystallization Experiments. Crystallization experiments to obtain T and R state crystals were conducted with two of the compounds, FUF and 5HMF. The experiment involved 4−25 molar excess of the compounds to Hb (tetramer). For T state crystallization experiments, Hb solution was evacuated for at least 1 h, and the compounds solubilized in DMSO were incubated with the deoxyHb (60 mg/mL protein) for at least 1 h to form the Schiff base adduct. Sodium cyanoborohydride ($NaCNBH_4$) in 4−25 molar excess of Hb was added to reduce the reversible Schiff base adduct to the corresponding irreversible alkylamine covalent bond. Subsequent crystallization of the compound−deoxyHb complex solutions in 10 mL test tubes using 3.2−3.6 M sulfate/phosphate precipitant (pH 6.5) was performed in a glovebox under nitrogen atmosphere as previously described[10,23] for obtaining high-salt T state crystals.

The compound−Hb complexes were also crystallized in the R state form using COHb, following a previously described procedure.[10,23] Hb solution was evacuated for about 10 min, and the resulting deoxyHb solution was fully saturated with CO to generate COHb. The compounds solubilized in DMSO were then reacted with the COHb, followed by addition of $NaCNBH_4$ to reduce the Schiff base adduct. Crystallization was carried out with a solution of 30−50 mg/mL protein, 3.2−3.4 M Na^+/K^+ phosphate, pH 6.4, and two drops of toluene in 10 mL test tubes. The experimental procedures were done under aerobic conditions. Most of the experiments did not result in crystal formation, except on few occasions where cocrystals isomorphous to R state native crystals (space group $P4_12_12$) were isolated (4−30 days).

Data Collection, Processing, and Structure Refinement. X-ray diffraction data sets for the R, R2, and T state cocrystals were collected at 100 K using a Molecular Structure Corporation (MSC) X-Stream Cryogenic Cooler System (MSC, The Woodlands, TX), an R-Axis II image plate detector equipped with OSMIC mirrors, and a Rigaku RU-200 generator operating at 50 kV and 100 mA. Prior to use in diffraction, the crystals were first washed in a cryoprotectant solution containing 50 μL mother liquor and 10−12 μL glycerol. The data sets were processed with MSC BIOTEX software program. All structure refinements and omit maps were performed with the CNS program.[36] Model building and correction were carried out using the graphic program TOM.[37] The X-ray data for all complexes are summarized in Table 4. The program LSQKAB as implemented in the CCP4 program suite[38] was used for structure comparison.

Structure Determinations and Refinements of the R2 State Complex Structures. The structure of the FUF−Hb complex in the R2 state crystal was the first to be determined by molecular replacement method[24] using the $\alpha 1\beta 1$-$\alpha 2\beta 2$ R2 state native Hb structure (PDB code 1BBB) as a search model. The translation function using the space group $P3_221$ gave a solution of a tetramer in the asymmetric unit with a final correlation coefficient of 69.2 and R_{factor} of 35.5% for data between 8 and 4.0 Å. The molecular replacement model was subjected to a rigid body refinement, followed by conjugate gradient minimization and simulated annealing. Strong and clear densities were identified for two FUF molecules bound at the N-terminal αVal1 residues in a symmetry-related fashion (Figure 4a and 4b). The N-terminal αVal1 binding site is located in the central water cavity of Hb close to the mouth of the α-cleft. The electron density from the bound compound overlapped that of the αVal1 nitrogen, suggesting an alkylamine covalent interaction between FUF and the nitrogen (Figure 4b). The electron density map also showed ligation of the four heme atoms, and water ligands were fitted to the density. Although the omit map indicated significant oxygen bound to the heme, fitting of O_2 produced distorted geometry of the Fe−O−O bonds and angle. The inability to refine the ligand as oxygen is probably due to the low resolution of the data. Several alternate rounds of conjugate gradient minimization, simulated annealing, individual B factor refinements, and the addition of 7 sulfate anions and 297 water molecules, with manual model corrections, brought the final R_{factor} to 21.7% and R_{free} to 27.4% at 2.25 Å resolution.

The starting model for the refinement of the 5HMF−Hb complex structure was the FUF−Hb structure, after the

deletion of FUF, water molecules, and sulfate anions. A round of rigid body, conjugate gradient minimization, and simulated annealing refinements also showed two 5HMF bound at the two symmetry-related N-terminal αVal1 nitrogens (Figure 4c and 4d). In contrast to the FUF−Hb structure, O_2 molecules were ligated to the heme atoms. Several alternate rounds of conjugate gradient minimization simulated annealing, individual *B* factor refinements, and the addition of 7 sulfate anions and 538 water molecules, with intermittent manual model corrections, brought the final R_{factor} to 18.4% and R_{free} to 22.3% at 1.85 Å resolution. The atomic coordinates and structure factors have been deposited in the RCSB Protein Data Bank with accession codes 1QXD and 1QXE for the FUF−Hb and 5HMF−Hb structures, respectively.

Structure Determinations and Refinements of the T state Complex Structures. The starting model for the refinement of the T state 5HMF−Hb structure was the isomorphous α1β1-α2β2 T state native structure (PDB code 2HHB). After rigid body refinement, and subsequent gradient minimization and simulated annealing, the electron density maps for the structure, unlike those of the R2 state complex structures, revealed only delocalized and uninterpretable densities at the N-terminal αVal1 binding sites. Repeated cycles of refinements, addition of water molecules, and model building did not show improved density at the binding site to successfully model 5HMF. There were no other apparent binding sites. The final Rfactor and Rfree for the 5HMF−Hb structures are 16.3% and 20.7% at 1.86 Å resolution, respectively. Other statistics for the crystal are reported in Table 4.

The 5HMF−Hb structure, without water and ligands, was used as a starting model for the refinement of the FUF−Hb structure. Similar to the 5HMF−Hb structure, refinements of the FUF−Hb structure did not result in any interpretable density at the binding pocket. The structure was not refined to completion, and detailed statistics for the crystal are not reported in the Table 4.

Structure Determinations and Refinements of the R State Complex Structures. The isomorphous α1β1 dimer R state structure (1LJW), after deletion of water molecules and phosphate anions, was used as the starting model to refine the FUF−Hb structure. Similar to the T state complexes, repeated refinements of the FUF−Hb R structure with model building showed only sparsely and uninterpretable density at the N-terminal αVal1 binding pocket. The final R_{factor} and R_{free} are 21.3 and 26.3 at 2.0 Å resolution, and detailed statistics for the crystal are reported in Table 4.

The FUF−Hb structure, without water and ligands, was used as a starting model for the refinement of the 5HMF−Hb structure. Similar to that of the FUF crystal, refinements also showed sparsely and uninterpretable density at the binding pocket, and the refinement was aborted. No detailed statistics for the crystal are reported in the Table 4.

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