Allosteric Modifiers of Hemoglobin: 2-[4-[[(3,5-Disubstituted anilino)carbonyl]methyl]phenoxy]-2-methylpropionic Acid Derivatives That Lower the Oxygen Affinity of Hemoglobin in Red Cell Suspensions, in Whole Blood, and in Vivo in Rats†

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ABSTRACT: Two new potent allosteric effectors of hemoglobin, RSR-4 [2-[4-[[(3,5-dichloroanilino)carbonyl]-methyl]phenoxy]-2-methylpropionic acid] and RSR-13 [2-[4-[[(3,5-dimethylanilino)carbonyl]methyl]-phenoxy]-2-methylpropionic acid], are compared to the previously reported compounds L3,5 and L3,4,5 [Lalezari, I., Lalezari, P., Poyart, C., Marden, M., Kister, J., Bohn, B., Fermi, G., & Perutz, M. F. (1990) *Biochemistry 29*, 1515]. Unlike L3,5 and L3,4,5, RSR-4 and RSR-13 are less impeded by physiological concentrations of serum albumin. RSR-4 has also been shown to be more effective than L3,5 in shifting the allosteric equilibrium of bovine Hb toward the low-affinity T-state. X-ray crystal studies show that both RSR-4 and RSR-13 bind to only one pair of symmetry-related sites in the Hb central water cavity whereas previous studies on L3,5 and L3,4,5 demonstrated a second pair of symmetry-related binding sites near Arg 104β. Three major interactions between these allosteric effectors and Hb include the acid group with the guanidinium group of C-terminal Arg 141α, the effector's amide oxygen with the ammonium ion of Lys 99α, and the π electrons of the halogenated or methylated aromatic ring and Asn 108β. No explanation has been found for the difference in number of binding sites observed for RSR-4 and RSR-13 (two sites) compared to L3,5 and L3,4,5 (four sites); also no correlation has been made between the number of binding sites and degree of allosteric shift in the oxygen equilibrium curve. We also show that the new allosteric effectors (1) readily cross the red cell membrane in the presence of serum albumin solutions, (2) restore to normal the oxygen equilibrium curves of outdated blood, (3) are not inhibited from entering erythrocytes in the presence of an anion-channel blocking agent (DIDS), and (4) shift the oxygen dissociation curve to the right in vivo in rats. The possible use of these effectors as therapeutic agents is discussed.

During our search for a drug to treat sickle cell anemia, we discovered that the antilipidemic drug clofibric acid (Figure 1) lowered the oxygen affinity of hemoglobin solutions (Abraham et al., 1983, 1982). Later that year, Perutz and Poyart (1983) reported that bezafibrate, another antilipidemic drug, was much more effective in lowering the oxygen affinity of hemoglobin solutions and of suspensions of fresh intact red cells. X-ray crystallographic studies showed that bezafibrate binds to specific sites in the central water cavity of deoxyhemoglobin (Perutz et al., 1986); i.e., one bezafibrate molecule spanned the sites occupied by two clofibric acid molecules (Abraham et al., 1983, 1984; Mehanna & Abraham, 1990). Bezafibrate and clofibric acid act by stabilizing the deoxy structure, shifting the allosteric equilibrium toward the low-affinity deoxy form. Bezafibrate and clofibric acid do not bind in a specific manner to oxy- or carbonmonoxyhemoglobin (Perutz et al., 1986; Mehanna & Abraham, 1990). After these initial reports, both clofibric acid and bezafibrate were evaluated as radiosensitizing agents that deliver oxygen to ischemic tumors (Hirst et al., 1987). Perutz and Poyart (1983) expressed the hope that potent allosteric effectors of hemoglobin that pass freely in and out of the red blood cell may also have other clinical applications (i.e., extension of the shelf life of blood, use in treatment of ischemia, stroke, and polycythemia, etc.).

Lalezari et al. (1988, 1990) next reported a series of urea derivatives [2-[4-[[(arylamino)carbonyl]amino]phenoxy]-2-methylpropionic acids] with greater allosteric potency than bezafibrate, but disappointingly the effects of bezafibrate and the urea derivatives were significantly inhibited by serum albumin (Lalezari et al., 1990).

Our goal has been (1) to design and synthesize new potent allosteric effectors of hemoglobin, (2) to synthesize allosteric effectors that are resistant to serum albumin binding, and (3) to determine the binding sites of the allosteric effectors in order to explain the stereospecific reasons for differences in

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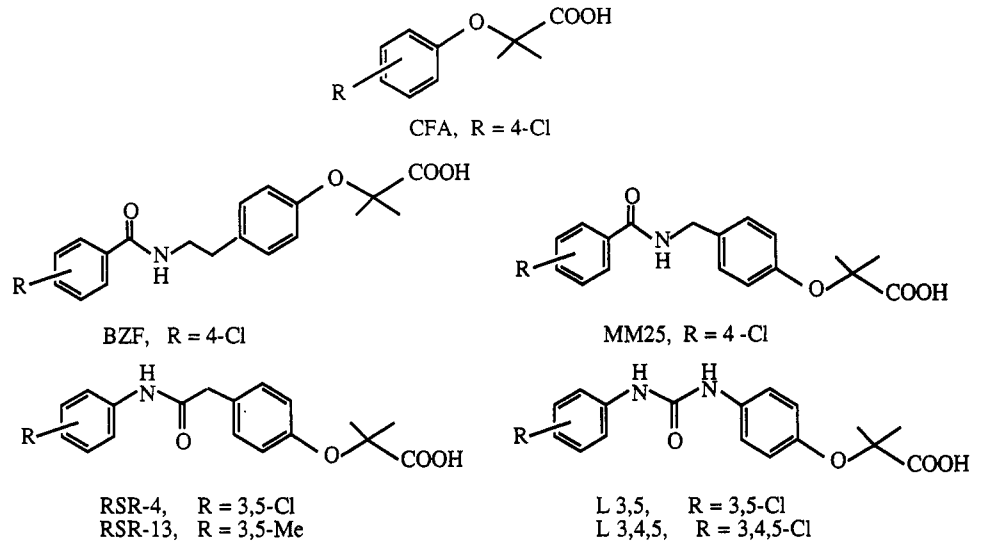


FIGURE 1: Structures of the allosteric effectors discussed in this paper. CFA is clofibric acid; BZF is bezafibrate.

allosteric potency (Randad et al., 1991; Wireko et al., 1991). Therefore, we varied the three-atom linkage between the two aromatic rings in L3,5 (Lalezari et al., 1988, 1990) and discovered that RSR-4 and RSR-13 had ideal structural features for allosteric activity and were also active in whole blood, indicating minimal inhibition by serum albumin (Randad et al., 1991). We also determined, crystallographically, the binding site for six of the new allosteric effectors including RSR-4 (Wireko et al., 1991).

Since the allosteric effectors described above bind at a different site than the naturally occurring red cell allosteric effector (2,3-diphosphoglyceric acid, DPG), they produce an additive right shift in the oxygen equilibrium curve in the presence of DPG. DPG binds in the cleft between the two β-subunits in deoxy-Hb (Arnone, 1972), whereas the synthetic allosteric effectors modeled after bezafibrate bind near the top of the α-subunits and extend deep into the central water cavity to the α,β-subunit interfaces.

We now report detailed studies concerning the oxygen-releasing properties of the new effectors (RSR-4 and RSR-13) and compare them to previously reported molecules (L3,5 and L3,4,5) in normal adult hemoglobin (HbA) and bovine hemoglobin. The oxygen dissociation curves at physiological concentrations of serum albumin with hemoglobin solutions or whole blood were carefully evaluated, and efforts were made to determine the membrane transport mechanisms for these molecules. The positive effect of the allosteric effectors RSR-4 and/or RSR-13 on Hb with outdated blood and their marked activity in vivo demonstrate their potential clinical usefulness.

MATERIALS AND METHODS

Human blood was collected on heparin from healthy nonsmoking donors. Bovine blood was provided by Pr Delpech, INA (Paris-Grignon). Red blood cells were washed three times in isotonic buffer, kept at 4 °C, and used within 24 h after sampling. Hemoglobin Yakima, a high-oxygen-affinity variant [$\alpha_2\beta_2$ 99(G1) Asp $\gg$ His], was obtained from a known heterozygous carrier. Fresh Yakima red cells were processed in the same way as described above.

L3,5 and L3,4,5 were provided by Dr. Lalezari (Montefiore Hospital, New York); RSR-4 [2-[4-[[(3,5-dichloroanilino)-carbonyl]methyl]phenoxy]-2-methylpropionic acid] and RSR-13 [2-4-[[(3,5-dimethylanilino)carbonyl]methyl]phenoxy]-2-methylpropionic acid] have been synthesized following the procedure described in Randad et al. (1991). Human serum albumin (HSA), bovine serum albumin (BSA), and 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS) were purchased from Sigma (St. Louis, MO). Due to their low solubility in aqueous buffer, 10 mM stock solutions of the compounds were prepared in a slight excess of sodium bicarbonate and back-titrated to pH 7.4. For example, RSR-4 (Randad et al., 1991) or RSR-13 was dissolved at 60 °C in a 140 mM NaCl and 50 mM bis-Tris buffer at room temperature upon progressive addition of $NaHCO_3$ to obtain 10 mM stock solutions. The pH was carefully adjusted to 7.4 after complete solubilization and before use of the solutions. Fresh solutions were prepared each week. Stock solutions of DIDS (1 mM) were prepared, in the dark, on the day of the experiments in a pH 7.4 buffer. Human serum albumin (defatted HSA) and bovine serum albumin (BSA) were prepared freshly as 1 mM stock solutions, pH 7.4.

Equilibrium Measurements. The allosteric modulation of the effectors on freshly prepared solutions of hemoglobin or red cell suspensions was measured by the change in p_{50}, the partial pressure of oxygen for half-saturation. Oxygen equilibrium curves (OEC) were carried out with the Hemox analyzer (TCS, Southampton, PA) as described previously (Perutz et al., 1986; Lalezari et al., 1990; Perutz & Poyart, 1983) under the following conditions: pH 7.4, 140 mM NaCl and 50 mM bis-Tris buffer at 37 °C. In all experiments, the concentration of hemoglobin in the red cell suspensions was 20–25 μM hemoglobin tetramer (Hb_4) as measured by Drabkin's method (cyanomethemoglobin) at the end of each recording. The final concentration of the allosteric effector in the cuvette was 0.5 mM, resulting in an effector/Hb_4 ratio of 20 in the absence of albumin. OEC were also made in the same buffer containing 12.5, 25, 50, and 100 μM HSA (for human red cell suspensions) or BSA (for bovine cell suspensions). The serum albumin experiments were carried out with traces of an antifoaming agent. This agent has no deleterious effect on the red cell suspensions. The molar ratio of albumin to tetrameric hemoglobin was varied from 0.5 (at 12.5 μM HSA) to 4 (at 100 μM HSA); in whole blood this ratio is approximately 0.3 (0.7 mM albumin plus plasma proteins/2.25 mM Hb_4). Therefore, the concentration of albumin used in the present studies was always larger relative to tetrameric hemoglobin than that found in whole blood.

Equilibrium oxygen binding curves were also recorded for red cell suspensions (10% hematocrit) that had been reacted for 30 min in the dark at 37 °C with 20 μM DIDS, a specific inhibitor of the red cell membrane anion channel.

Kinetic Experiments. The kinetics of the interaction between the effectors and intracellular hemoglobin were measured in the absence and presence of varying concentrations of HSA. The red cell suspensions were equilibrated under room air (p_{O_2} = 140 mmHg) at 37 °C in the optical cuvette of the Hemox analyzer, which provides a constant stirring of the suspensions. At time 0, the drug was added to the stirred suspensions, which resulted in a rapid increase in absorbance at 560 nm until a plateau was reached, usually within 10 min. Changes in absorbance were recorded and stored on tape for further analyses.

An increase in absorbance at 560 nm (ϵ_{max} for deoxy-Hb in the visible spectrum) under room air indicates the formation of partially deoxygenated Hb in the red cells and a decreased oxygen affinity of Hb, provided that the pH is held constant. We found that the change in absorbance at 560 nm (ΔA_{560}) upon addition of the effectors was linearly related to the p_{50} values of these cells:

$$p_{50}\ (\text{mmHg}) = 220\Delta A_{560} + 27.0\ (r = 0.994)$$

Examples of these kinetic recordings are illustrated in Figure 4. Two indices were calculated from the experimental values: ΔA_{560} is the difference in optical density from the base line to the plateau and $t_{1/2}$ is half the time for ΔA_{560}. Any inhibitory effect by albumin was therefore demonstrated by a decrease in either the p_{50} shift or ΔA_{560} relative to the control values obtained in the absence of albumin.

X-ray Crystallographic Studies. Hemoglobin for the crystal studies was prepared from human blood, and T-state deoxy crystals were obtained from ammonium sulfate solutions according to the procedure of Perutz (1968). RSR-4 or RSR-13 (2 effectors/1 Hb) was dissolved in a slight excess of sodium bicarbonate to facilitate dissolution and was added to a 6 g % solution of deoxy-Hb in the glove box. A 1 g % Hb RSR-4 or RSR-13 solution was added to each crystallization tube. Crystals grew in approximately 1 week. X-ray data were collected with Friedel pairs using ω scans on a Rigaku AFC5R diffractometer equipped with a rotating anode and a 60-cm-long evacuated beam tunnel. The crystals with allosteric effectors were isomorphous with native deoxy-Hb: RSR-4, 63.2, 83.7, and 53.8 Å, 99.4°; RSR-13, 63.2, 83.6, and 53.7 Å, 99.5°. Data collection (RSR-4 to 2.4-Å and RSR-13 to 2.8-Å resolution) was controlled by TEXAN software from Molecular Structure Corp. (The Woodlands, Texas). There was little radiation damage to crystals, and new crystals were mounted when standard reference reflection intensities fell by 10%. All data were corrected for radiation damage when appropriate. The *R*-factors for the Friedel pairs and derivatives vs native were as follows: RSR-4 (2.4 Å), 14.6% and 18.9%, and RSR-13 (2.8 Å), 7.2% and 12.5%, respectively. The difference electron density maps were obtained using the Cambridge computer programs (CCP4). The highest peaks in the difference electron density maps were observed to be at 7–8σ, and difference density maps were contoured at 3σ and above. The allosteric effector molecules were well resolved and easily fit to the difference density.

Outdated Blood Studies. For the outdated blood studies, 40-day-old packed red cells (80% hematocrit) were obtained from the blood bank at the Medical College of Virginia, Richmond, VA. The cells had been stored in Adsol formulation at 1–4 °C, pH 6.84. Fresh red cells obtained from volunteers were used for the control studies in Table III. The fresh red blood cells were centrifuged and washed with saline, pH 6.8. The oxygen dissociation curves were recorded on an Aminco Hem-O-Scan (Travenol Laboratories).

Table I: Oxygen Binding Measurements for Fresh Human Red Blood Cells on Addition of the Effectors L3,5, RSR-4, and RSR-13[a]

effector (0.5 mM)	HSA (μM)	p_{50}[b] (mmHg)	ΔA_{560}	$t_{1/2}$ (s)
none	none	27		
L3,5	none[c]	60	0.181	33
	12.5		0.124	92.5
	50.0	32	0.058	110
	100.0	28	0.029	115
+DIDS[d]	none	54	nd	nd
RSR-4	none[c]	52.5	0.152	60
	12.5	48.5	0.107	92
	50.0	44.0	0.086	76
	100.0	36	0.042	87
+DIDS[d]	none	49	nd	nd
RSR-13	none[c]	37.8	0.085	139
	50.0	34.0	0.082	138
	100.0	32.6	0.067	125

[a] Conditions of the measurements were pH 7.4, 140 mM NaCl and 50 mM bis-Tris buffer, at 37 °C. p_{50} is the partial pressure of oxygen for half-oxygen saturation; HSA is human serum albumin (defatted); the hemoglobin concentration was 20–25 μM on a tetramer basis; the HSA/Hb_4 molar ratio was varied from 0.5 to 4. ΔA_{560} is the maximum absorbance change at 560 nm under room air on addition of the effector; $t_{1/2}$ is the time(s) for half-ΔA_{560}. [b] Mean of at least two measurements. [c] The effector to tetrameric Hb molar ratio was 20 in the absence of HSA. [d] Red cells were incubated for 30 min at 37 °C at pH 7.4 in the presence of 20 mM 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS) prior to the recording of the oxygen binding curves.

In Vivo Studies. The in vivo effect of RSR-4 and RSR-13 was assessed in conscious rats that received (through a chronic femoral vein catheter) an initial 85.7 ± 3.7 mg/kg dose of RSR-4 or 84.0 ± 4.0 mg/kg dose of RSR-13, followed by 85.7 ± 3.7 mg/(kg h) RSR-4 or 84.0 ± 4.0 mg/(kg h) RSR-13, respectively, for 2 h. The oxygen dissociation curve was measured before infusion with a Hemox analyzer and, after the 2-h infusion, was manually measured with a tonometer and visible spectroscopy to avoid a dilution step (used with the Hemox analyzer) that would disrupt the equilibrium between the drug and erythrocytes. Tonometry was performed at a constant p_{CO_2} of 40 mmHg at 37 °C. The pH of the blood was 7.443 ± 0.012 (n = 5) for the RSR-13 experiment and 7.412 ± 0.098 (n = 7) for the RSR-4 experiment. Control rats received an infusion of buffer solution with $NaHCO_3$. A slight molar excess of $NaHCO_3$ was used to better solubilize RSR-4.

RESULTS

Oxygen Equilibrium Studies. Table I demonstrates that, in the absence of human serum albumin, RSR-4 and L3,5 are almost equally potent effectors in raising the p_{50} of hemoglobin in fresh human red cells containing normal concentrations of 2,3-diphosphoglycerate. However, the allosteric effect produced by RSR-4 is much less inhibited by 50 or 100 μM albumin than observed with L3,5. While RSR-13 is the weakest in reducing the oxygen affinity of Hb, relative to L3,5 or RSR-4, it is much less affected by serum albumin, and its effect persists even in the presence of 100 μM HSA. Figure 2 shows the oxygen equilibrium curves for a fresh red blood cell suspension upon addition of RSR-4 with and without HSA. Figure 3 summarizes the differences in allosteric effector activity for L3,4,5, L3,5, RSR-4, and RSR-13 in the presence of serum albumin. RSR-13 is by far the least affected

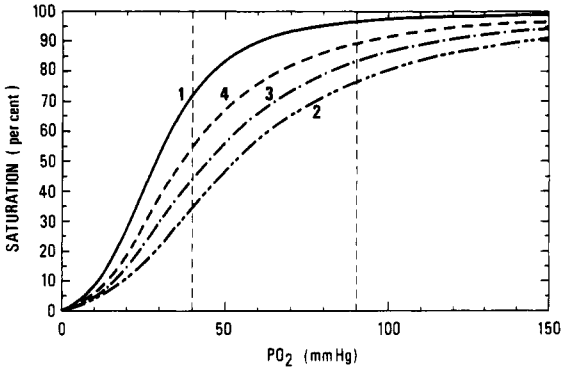


FIGURE 2: Equilibrium oxygen binding curves for human red cell suspensions: (1) control curve; (2) curve 1 + 0.5 mM RSR-4; (3) curve 2 + 50 μM HSA; (4) curve 2 + 100 μM HSA. The concentration ratios of albumin/Hb in this experiment are above those found in vivo. For example, in curve 3 serum albumin is about 6 times and curve 4 about 12 times in excess above the concentration ratio of albumin/Hb found in vivo (see Materials and Methods for details). The vertical lines indicate the physiological p_{O_2} values in the arterial blood (90 mmHg) and in the mixed venous blood (40 mmHg). The change in oxygen saturation between these two lines allows an estimation of the capability of the red blood cells to deliver oxygen to the tissues [ΔY (%); see Table IV].

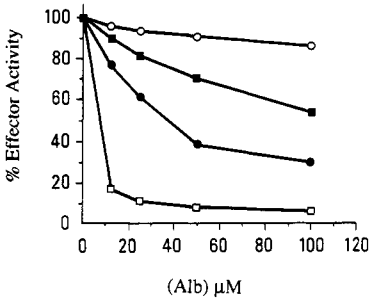


FIGURE 3: Percent of RSR-13 (○), RSR-4 (■), L3,5 (●), and L3,4,5 (□) allosteric effector activity in the presence of albumin (HSA) in buffered red cell suspensions. Effector concentration was 0.5 mM except for L3,4,5, which was 0.25 mM. Hemoglobin concentration was 20–25 μM on a tetramer basis.

by serum albumin inhibition and exhibits greater than 90% allosteric effector activity even in the presence of 50 μM albumin, which is ≈7 times the albumin/Hb ratio present in vivo. RSR-4 effector activity is also greater than L3,5 and L3,4,5 at all serum albumin/Hb ratios studied.

Kinetic Studies on the Transport of Allosteric Effectors across Red Cell Membranes. The results from the kinetic studies are also presented in Table I. In the absence of albumin, $t_{1/2}$ is 33 s for L3,5, 1 min for RSR-4, and over 2 min for RSR-13. The differences in $t_{1/2}$ are probably due to the structural changes (the 3,5-dichloro atoms in L3,5 and RSR-4 are substituted by methyl groups in RSR-13; see structures in Figure 1). In the presence of albumin, the ΔA_{560} is decreased and the $t_{1/2}$ values are increased. However, the $t_{1/2}$ values are independent of the concentration of albumin, whereas ΔA is not. This may indicate that, at a concentration of 12.5 μM, HSA is saturated with the effector. No change in $t_{1/2}$ was observed for RSR-13 upon addition of albumin. A typical example of the kinetic results is illustrated in Figure 4.

The presence of DIDS, a specific covalently bound inhibitor of the membrane anion channel, does not inhibit the effects of the compounds (Table I). This was also true for L3,4,5 (not shown) and indicates that the mechanism by which these compounds penetrate into the red cells is likely to be a diffusion process, probably driven by the affinity of the huge concentration of Hb in the cells.

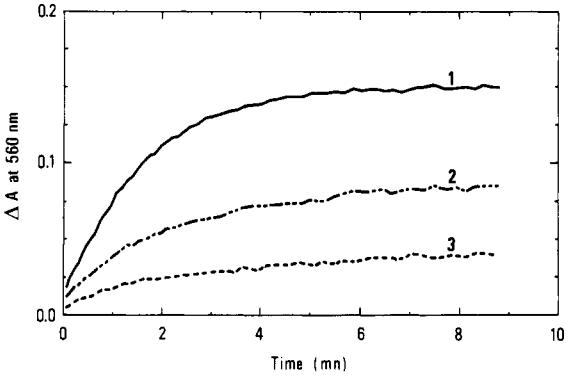


FIGURE 4: Kinetic recordings of the changes in absorbance (A_{560}) versus time after addition of 0.5 mM RSR-4 at increasing concentrations of defatted human serum albumin: (1) none; (2) +50 μM; (3) +100 μM. Experimental conditions were pH 7.4, 140 mM NaCl, and 37 °C. Red blood cell suspensions were equilibrated under room air. These traces correspond to conditions for curves 2–4 in Figure 2.

Table II: Oxygen Binding Measurements for Fresh Bovine Red Blood Cells and Isolated Hb Solutions on Addition of the Effectors L3,5, RSR-4, and RSR-13[a]

	p_{50}		
effector	RBC	HbA	Hb bovine
control	27	5	14.5
L3,5[b]	35	47	37
RSR-13	31	nd	nd
RSR-4	54.0	35.5	59
RSR-4 + BSA (50 μM)[c]	45.0	nd	nd

[a] Experimental conditions for bovine RBC's were as described in the legend of Table I for human RBC. p_{50} (mmHg) is the p_{O_2} for 50% saturation; BSA is bovine serum albumin. OEC for Hb solutions were made at pH 7.2, 100 mM NaCl and 50 mM bis-Tris buffer at 25 °C. Catalase (20 μg/mL) and 50 μM EDTA were added to oppose oxidation of the hemes. The concentration of the effectors was 0.5 mM in all conditions. [b] See footnote *c*, Table I. [c] See footnote *b*, Table I.

Bovine Red Cell Studies. Table II compares the p_{50} shifts induced by the effectors for bovine red cell suspensions and isolated bovine Hb solutions to those observed for human Hb. It is known that bovine red cells, which do not contain DPG, exhibit a p_{50} value similar to that of fresh human red cells (Table II). By contrast, the p_{50} value of isolated bovine Hb is about 3 times higher than human HbA. It has also been demonstrated that bovine Hb does not bind exogenous organophosphates in the presence of 100 mM chloride but does bind these effectors in the absence of chloride (Fronticelli et al., 1988). We were interested in knowing whether bovine hemoglobin was able to bind our effectors in the presence or absence of chloride anions. The results given in Table II show that the three effectors, L3,5, RSR-4, and RSR-13, bind to bovine Hb in red cells. Interestingly, while L3,5 and RSR-13 induce a small p_{50} shift (30% and 15%, respectively), the effect of RSR-4 results in the doubling of the control p_{50} and persists even in the presence of 50 μM BSA.

Differences in the p_{50} shifts between human and bovine red cells were also observed: the order of potency in lowering the oxygen affinity was L3,5 ≈ RSR-4 > RSR-13 in human (Table I) and RSR-4 ≫ L3,5 > RSR 13 in bovine (Table II). A similar potency was observed in Hb solution studies (Table II) with the RSR-4 effect being significantly larger than that of L3,5.

Since these effectors bind in the Hb central water cavity (Perutz et al., 1986; Lalezari et al., 1990, 1988; Abraham et al., 1983; Wireko et al., 1991) at sites which have also been

Table III: p_{50} (mmHg) and ΔY (%) [Y(90%) − Y(40%)] for Outdated Red Blood Cell Suspensions (40 Days) in the Presence or Absence of RSR-13

origin	effector	p_{50} (mmHg)	ΔY (%)
fresh human RBC's	none	38	38
stored human RBC's	none	32	31
stored human RBC's	RSR-13 (1 mM)	39	37
stored human RBC's	RSR-13 (2 mM)	45	40

implicated for chloride binding (O'Donnell et al., 1979), we have also investigated the role of increasing chloride concentration (0–400 mM) with bovine and human Hb solutions in the presence of RSR-4 and L3,5. Surprisingly, the p_{50} shifts induced by the two compounds (0.5 mM) were identical at all chloride concentrations, indicating that RSR-4 and L3,5 antagonize chloride binding in both human and bovine hemoglobin solutions. Conversely, these results demonstrate that the differences in the effects of RSR-4 and L3,5 in the two hemoglobin solutions cannot be ascribed to differences in chloride reactivity. The reason for the apparent difference in RSR-4 binding to bovine Hb relative to the closely related L3,5 compound requires further investigation. The key residues in human Hb that bind with the allosteric effectors are Lys 99α, Arg 141α, and Asn 108β (Wireko et al., 1991), and these residues are also present in bovine hemoglobin. A stronger interaction by RSR-4 with these same residues or with other sequence differences in bovine Hb must probably account for the increased allosteric effect of RSR-4 vs L3,5 and RSR-13.

Outdated Blood Studies. We have evaluated the capability of RSR-13 to restore the oxygen affinity of stored blood. The procedure involves measurement of the oxygen equilibrium curves on red cell suspensions, which were stored in Adsol formulations at 4 °C, in either the absence (controls) or presence of RSR-13. The results are summarized, as p_{50} values, in Table III. The oxygen dissociation curves demonstrate that 1 mM RSR-13 is able to restore, to near normal, the oxygen transport capabilities (p_{50} and ΔY) of 40-day-old red blood cells. Untreated samples (controls) were found left shifted to 32 mmHg. This increase in the oxygen affinity of stored blood is attributed to the decreased concentration of DPG. However, the oxygen dissociation curves of samples treated with 1 mM RSR-13 showed a p_{50} of 39 mmHg, comparable with the p_{50} of fresh red cells. The oxygen dissociation curves of samples treated with 2 mM RSR-13 were right shifted further (P_{50} of 45 mmHg). Similar concentrations of RSR-13 were added to 50-, 60-, or 70-day-old red cells with similar results. The control oxygen dissociation curves of 60- and 70-day-old red cells were right shifted compared to the 40-day-old samples, possibly due to a decrease in pH (Bohr effect). Similarly, it was observed that the pH of the RBC solution treated with RSR-13 (4 mM) was higher than that of untreated samples, suggesting a favorable decrease in the rate of glycolytic metabolism (less pyruvic and lactic acid). A similar effect has been observed with another allosteric effector (Hyde et al., 1984). Addition of RSR-13, to red cells prior to measurements or during storage, also preserved the oxygen delivery capabilities of red cell hemoglobin. There was no indication of hemolysis at the end of these experiments.

In Vivo Estimates for Percent of Oxygen Delivery. Table IV contains the calculated amounts of increased oxygen transport [ΔY (%)] using these agents. Extrapolation to in vivo values can only be estimated since the Hb concentration in these studies (50 μM) is much lower than that found in whole blood (about 2 mM). The ratio of drug to hemoglobin required in dilute Hb solutions is much greater than that necessary to obtain the same degree of binding at the higher levels of Hb found in vivo. Table IV also clearly shows that, even in the presence of HSA, RSR-4 greatly improves oxygen delivery (+60% relative to the control value). This would indicate a potential clinical benefit for patients suffering from hypoxic problems.

Table IV: Calculated Oxygen Delivery Index for Red Blood Cell Suspensions in the Presence or Absence of 0.5 mM L3,5 or RSR-4[a]

origin	effector	HSA (μM)	ΔY (%)
human	control	none	24.5
	L3,5	none	42
	+HSA	50	30
		100	25
human	RSR-4	none	41.5
	+HSA	50	39
		100	34
human	none	none	5
HbA/Yakima	RSR-4	none	22
	+HSA	50	19

[a] Experimental conditions were as described in the legend of Table I. The oxygen delivery index was calculated from the experimental oxygen binding curves as the difference in hemoglobin oxygen saturation (percent) corresponding to the p_{O_2} in the arterial blood (90 mmHg) and that in the mixed venous blood (40 mmHg). HbA/Yakima red blood cells are from a heterozygous carrier of the high-affinity Hb variant.

Studies with Hemoglobin Yakima, a High-Affinity Mutant. We have also measured oxygen binding to red cells containing 47% of Hb Yakima ($\alpha_2\beta_2$ D99H), a high-oxygen-affinity, noncooperative Hb variant. Table IV shows that, by reducing the oxygen affinity of the functional HbA (53%) in these red cells, RSR-4 is able to restore an almost normal oxygen delivery to the RBC from this patient.

Crystal Structures of RSR-4 and RSR-13 Bound to Deoxyhemoglobin and Allosteric Effector Structure–Activity Relationships. The X-ray-determined binding sites for L3,5 (Lalezari et al., 1990) and RSR-4 (Wireko et al., 1991) have been reported previously. In this study we determined the RSR-13 crystal structure binding site to hemoglobin and compared it with RSR-4, L3,5, bezafibrate, and a lesser active allosteric effector, MM-25 (Randad et al., 1991; Wireko et al., 1991).

RSR-13 binds in the central water cavity in a conformation identical to that found for RSR-4 (Wireko et al., 1991). The only difference between the two bound structures results from the difference in bond lengths due to the substitution of methyl for chlorine at the 3,5 positions on the terminal aromatic ring. RSR-13 difference maps exhibited a symmetry-related pair of densities around the molecular 2-fold axis that bisects the hemoglobin tetramer (Figure 5). The electron density clearly defined the position of the effector at the same locale as that found for bezafibrate (Perutz et al., 1986). The reason(s) for the differences in allosteric shifts observed for these effectors is (are) not immediately apparent. All of the bis-aromatic effectors shown in Figure 1 are closely related to bezafibrate, yet MM-25 is somewhat stronger than bezafibrate in its ability to right shift the oxygen equilibrium curve (Randad et al., 1991), while the other three molecules are much stronger allosteric effectors with L3,5 $\approx$ RSR-4 > RSR-13 as shown above.

The allosteric effector structure–activity differences can be summarized as follows: (1) The weakest effector, bezafibrate, has a four-atom linkage between the two aromatic rings, while the other effectors have a three-atom bridge. A four-atom bridge may make the effector too long to maximize

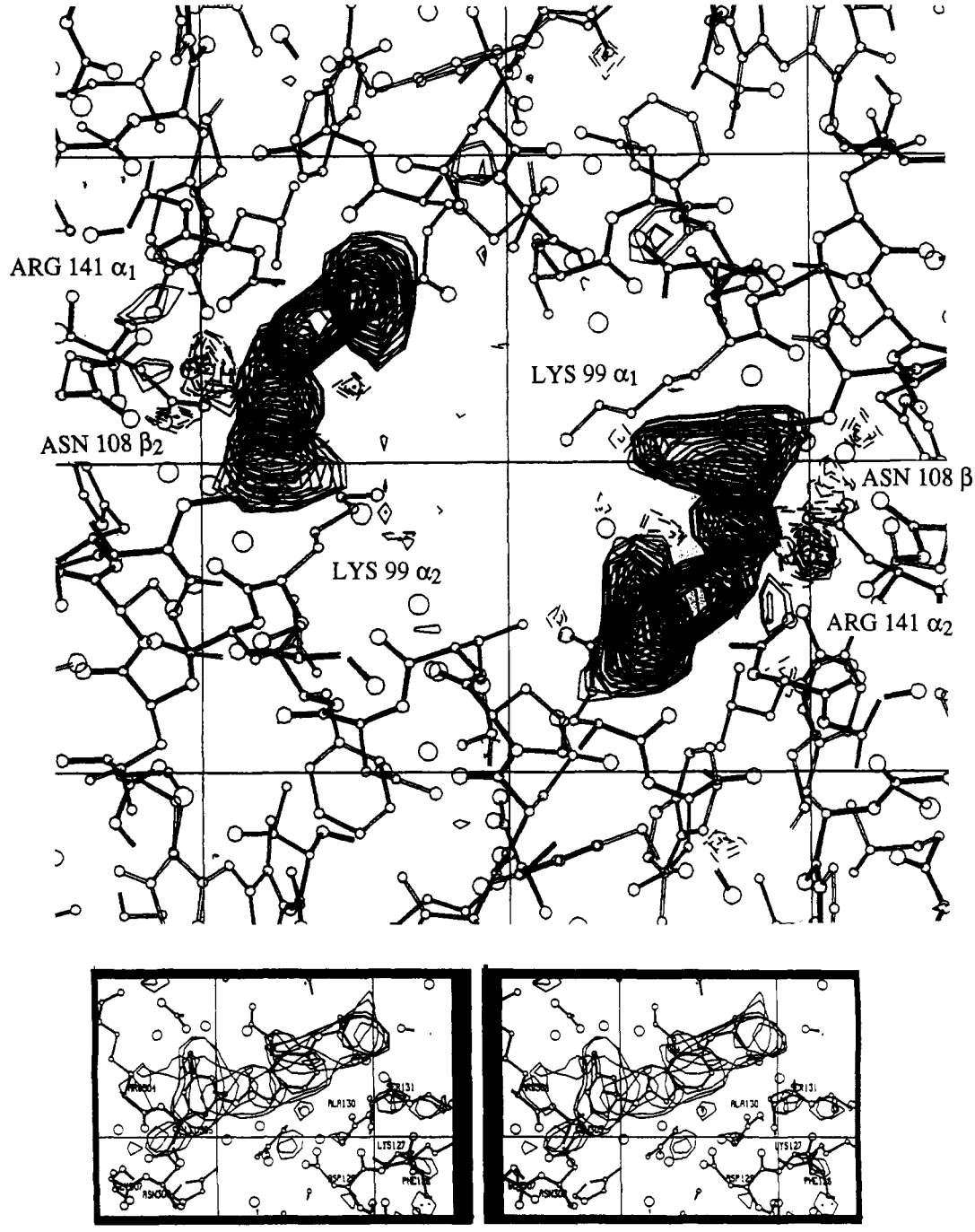


FIGURE 5: (a, top) A view looking down the molecular 2-fold axis that bisects the hemoglobin central water cavity. A symmetrically related pair of difference electron density peaks (dark contours) clearly indicates the positions of RSR-13 molecules. Lys 99α, which points into the central water cavity, helps define the primary binding cavity for these effectors (see also Figure 6d). (b, bottom) Stereoview of RSR-13 fit into the difference electron density (a single contour level at 3σ).

key interactions. It was previously pointed out that the halogenated ring of bezafibrate is quite close to the aromatic ring of Phe 36α (Perutz et al., 1986). (2) The L series has a urea linkage between the aromatic rings, while the RSR and MM series have an amide and methylene bridge between the aromatic rings (Randad et al., 1991; Wireko et al., 1991). (3) The stronger acting RSR series differs from the much weaker MM series by simple reversal of the amide bond. (4) Both weaker acting effectors, MM-25 and bezafibrate, have the amide carbonyl oxygen of the amide linkage next to the halogenated aromatic ring (Figure 1).

A quantitative analysis of protein–allosteric effector interactions was reported previously using a new software package (HINT) developed in our laboratory (Wireko et al., 1991; Kellogg et al., 1992). HINT evaluates and visualizes the degree of hydropathic interactions between a protein and ligand and demonstrated that the major interactions between effectors such as RSR-4 with the protein include the salt bridge between the protonated nitrogen atoms of the Arg 141α guanidinium group with the acid oxygens of the allosteric effector, the interaction of the Lys 99α side-chain ammonium group with the carbonyl oxygen of the effector amide group, and the Asn 108β side-chain NH interaction with the π electrons of the halogenated or methylated aromatic rings of the allosteric effectors.

HINT also calculated a significant difference in interaction constants between the RSR and MM series due to the inversion of the amide bond that directs the carbonyl atom of the amide linkage toward the Lys 99α ammonium ion in the RSR (and LR) series but positions the amide oxygen away from the Lys

99α ammonium ion in the MM series (Wireko et al., 1991). The difference in amide oxygen direction between RSR-4 and MM-25 is shown with the overlapped bound allosteric effectors in Figure 6a. This single difference in effector amide bond direction appears to be an important component in the degree of allosteric effector activity observed. Solution binding measurements with these and other allosteric effectors (Randad et al., 1991) are being conducted (Richmond) to determine whether differences in binding energies are correlated with the number of binding sites observed in the crystal and the degree of allosteric shift exhibited by each agent.

The reason(s) why a few of these effectors, L3,5, L3,4,5 (Lalezari et al., 1990), and MM-25 (Wireko et al., 1991), have a second symmetry-related pair of binding sites near Arg 104β (Figure 6b) still remain(s) a puzzle. We have determined the crystal binding sites for 15 allosteric effectors with different three-atom arrangements, excluding the L series with the urea linkage. Only four of the effectors exhibited a second binding site (F. C. Wireko and D. J. Abraham unpublished results). The secondary site is not sterically well-defined by the protein and permits a more flexible environment for binding ligands, as can be seen by the three different orientations for L3,5, L3,4,5, and MM25 (Figure 6b,c). This secondary site is located in the central water cavity directly below the better defined primarily BZF site (Figure 6c). The primary binding site orients the effectors almost vertically along the cavity wall with little variation in binding modes (Figure 6d). Lys 99α appears to be a key residue in the formation of a cavitylike pocket at the primary binding site by wrapping around the middle of the effectors (Figures 5a and 6a,d). There does not appear to be a relationship between the degree of allosteric shift versus the number of sites bound, and there is no obvious reason to us why some effectors have a second binding site and others do not.

In Vivo Studies with the Allosteric Effectors. The above data suggest that RSR-4 and RSR-13 may be promising compounds for lowering whole blood oxygen affinity in vivo. This was confirmed in seven rats in which RSR-4, 85.7 ± 3.7 mg/kg, iv, followed by 85.7 ± 3.7 mg/(kg h) for 2 h, increased p_{50} from an average control value of 36.4 ± 1.6 to 60.7 ± 8.1 mmHg. The Hill coefficient decreased from 2.66 ± 0.13 to 1.74 ± 0.08. In eight control rats the p_{50} decreased from 36.7 ± 1.0 to 33.2 ± 1.4 mmHg. In five rats receiving RSR-13 at 84 mg/kg, followed by 84 mg/(kg h) for 2 h, p_{50} increased from 36.0 ± 2.7 to 61.4 ± 5.8 mmHg. The Hill coefficient decreased from 2.62 ± 0.10 to 1.73 ± 0.08. The animals did not show any sign of distress during infusion of either compound. Significant hemolysis was observed with RSR-4 with rat erythrocytes, but not with RSR-13.[1] No hemolysis is observed with RSR-4 or RSR-13 with human or bovine red cells under the in vitro conditions used in this paper.

DISCUSSION AND CONCLUSIONS

There has been considerable interest in medicine, the military health services, and the pharmaceutical industry in finding methods to increase oxygen delivery in vivo for ischemic insults, stroke, and trauma; to increase blood storage life; to discover radiosensitization agents; and to develop new blood substitutes. In all these instances, the availability of either autologous blood or recombinant Hb solutions is of major interest, provided the oxygen affinity can be decreased to enhance oxygen delivery to the tissues.

The RSR molecules described in this paper are the first strongly acting allosteric effectors that are able to shift the OEC of red blood cells to a large extent without being grossly affected by serum albumin. In order to exemplify their potential use in vivo, we have calculated the index of oxygen delivery to the tissues, assuming normal arterial and mixed venous blood p_{O_2} values. Table IV shows that, even in the presence of HSA, RSR-4 improves to a large extent the oxygen delivery to tissues (+60% relative to the control values). Since RSR-4 is less inhibited by albumin than the closely related compound L3,5, RSR-4 exhibits a greater effect in increasing oxygen release to tissues. Even though the p_{50} of RSR-13 is less than that produced by the other effectors, it is large enough to show a significant right shift in vivo. In this regard, it is worth recalling that allosteric modifiers that produce too great a shift of the OEC could inhibit oxygen uptake by red blood cells in the lungs and thereby hinder oxygen delivery in vivo at physiological mixed venous p_{O_2} values. Also, because of its lower affinity for serum albumin (Figure 3), RSR-13 may be the preferred allosteric effector to study clinically.

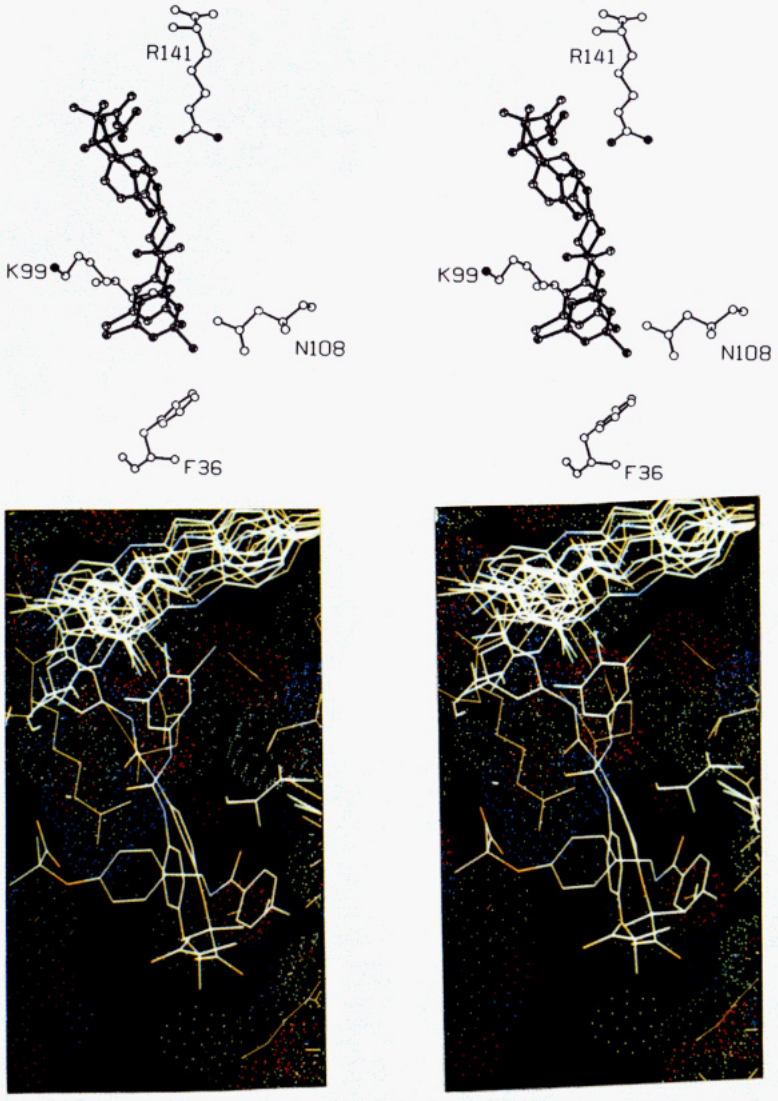
Another example of the beneficial effect of RSR-4 was demonstrated in studies of oxygen binding curves with red cells containing 47% of Hb Yakima, a high-oxygen-affinity, noncooperative Hb variant (Novy et al., 1967). Table IV shows that addition of RSR-4 to these red cell suspensions results in an almost normal oxygen delivery index. In this case the p_{50} shift is due to the binding of RSR-4 to the fraction of HbA present in the cells, as purified Hb Yakima remains locked in the high-affinity R-state in the presence of L3,4,5, L3,5, RSR-4, or DPG (Poyart et al., unpublished results). More direct information was obtained from the in vivo rat studies. These preliminary experiments demonstrated that the p_{50} shifts measured in rat blood upon infusion of RSR-13 were consistent with our in vitro studies.

In the absence of albumin, the p_{50} shifts that arise from the addition of RSR-4 or L3,5 to human RBC suspensions or HbA solutions are comparable. In the presence of albumin (Table I), a large difference was observed between the two compounds with a much lower inhibition of RSR-4 by serum albumin than that observed with L3,5, while RSR-13 is practically unaffected by serum albumin (Figure 3). The reason for this great difference between the serum albumin inhibitory effect (binding) of RSR-4, RSR-13, and L3,5 to serum albumin is puzzling since the major structural differences between the three compounds are small. L3,5 and L3,4,5 contain a urea bridge between the aromatic rings while RSR-4 and RSR-13 have a methylene group substituted for one of the amide nitrogens in the L series. Since the RSR series is less polar than the L series, it appears that small changes in hydrophobicity and/or structure can play a significant role in changing the affinity to ubiquitous binding proteins such as serum albumin.

The similarity in binding at the primary site for RSR-4, RSR-13, L3,5, and all other allosteric effectors illustrates the basic mechanism by which these molecules act. They keep the T-state Hb molecule from shifting to the R-state oxygenated structure by not permitting the central water cavity to narrow. Each of the symmetry-related effector molecules at the primary site interacts with three different subunits in the central water cavity tying the two halves of the Hb tetramer together.[2] The natural allosteric effector, 2,3-diphosphoglyceric acid, binds in a similar fashion but at the other end

[1] The concentration of RSR-4 or RSR-13 in plasma was not measured and cannot be estimated in the absence of data on the volume of distribution and clearance of these drugs.

[2] One effector molecule interacts with α_1, α_2, and β_1 residues, and the symmetry-related effector interacts with the corresponding symmetry-related residues on the α_2-, α_1-, and β_2-subunits.



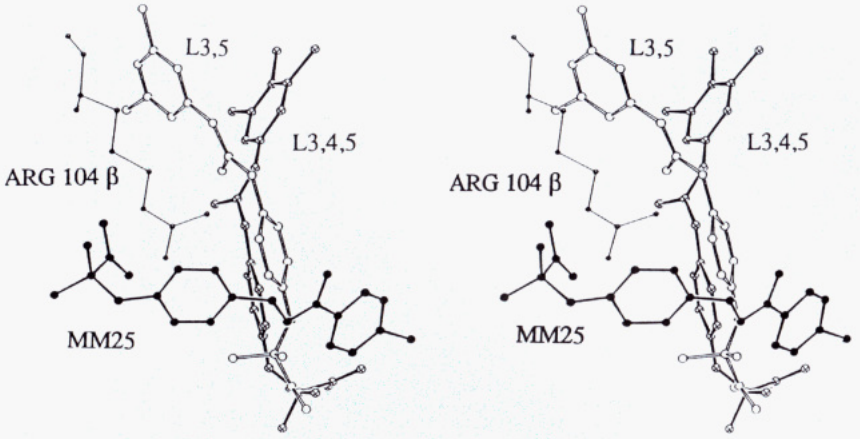


FIGURE 6: (a, top left) Stereoview of the overlap of the binding of RSR-4 (dark ellipsoids) and MM-25 (clear ellipsoids) at the primary binding site observed for bezafibrate (Perutz et al., 1986). Each effector molecule binds to three different subunits (two α and one β). Since RSR-4 and RSR-13 occupy the same positions except for the difference in bond lengths for the 3,5 atoms (Cl vs Me), only RSR-4 is shown. Note that MM-25 has the carbonyl oxygen of the amide bond pointed in a different direction away from the Lys 99α_1 (K99) ammonium ion. The other interacting residues illustrated are Arg 141β_2 (R141), Asn 108β_1 (N108), and Phe 36α_1 (F36). (b, top right) Stereoview of the secondary binding site for L3,5 (open circles), L3,4,5 (crosses in circles), and MM-25 (dark circles). The guanidinium side chain of Arg 104β (thin lines) influences the binding in this area. The different orientations of the three effectors illustrate the flexibility in binding modes at this site. (c, bottom left) Stereoview [with the same orientation as in (b)] of the secondary binding site for L3,5, L3,4,5, and MM-25. The primary site binding for the allosteric effectors (see overlapped structures, top center) is directly above the secondary site (bottom center). The protein residues are depicted with van der Waals spheres. (d, bottom right) Stereo pair of the primary BZF binding site for RSR-4, RSR-13, L3,5, and several other overlapped allosteric effectors (Wireko et al., 1991). Protein residues are again depicted with van der Waals spheres. The primary binding site is located vertically along the central water cavity wall (Figure 5a). The side chain of Lys 99α (from 9 o'clock pointing toward 12 o'clock) encircles the effectors and is key in defining the binding pocket.

of the tetramer, bridging the β-subunits. Since DPG binds at a different site, it acts in an additive manner when combined with these allosteric effectors.

In conclusion, the two new potent allosteric effectors RSR-4 and RSR-13 have been shown to possess properties that may make them attractive clinically. These effectors may also play a role in helping to elucidate the stereochemical details that trigger the allosteric transition. We recently discovered that RSR-13 and an analog (RSR-56) that replaces the 3,5 dimethyl groups in RSR-13 with methoxy groups stabilize tetraligated T-state deoxyhemoglobin crystals in high salt (Abraham et al., 1992). Difference density peaks of the partially tetraligated Hb confirm past ideas on the movement of key residues during the allosteric transition and also implicate the out of plane β-heme vinyl group in the process.

Several questions still remain to be answered: Why does the RSR series have less affinity than the L series for serum albumin? Why does RSR-4 have a much stronger allosteric effect on bovine hemoglobin than RSR-13 or L3,5? Why do RSR-4 and RSR-13 have only one primary binding site whereas L3,5, L3,4,5, and MM-25 have a second binding site. Further structural and biochemical experiments will be needed to answer these questions.

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SUPPLEMENTARY MATERIAL AVAILABLE

One table giving coordinates for hemoglobin allosteric effectors RSR-13 and RSR-4 (2 pages). Ordering information is given on any current masthead page.

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