New Effectors of Human Hemoglobin: Structure and Function†

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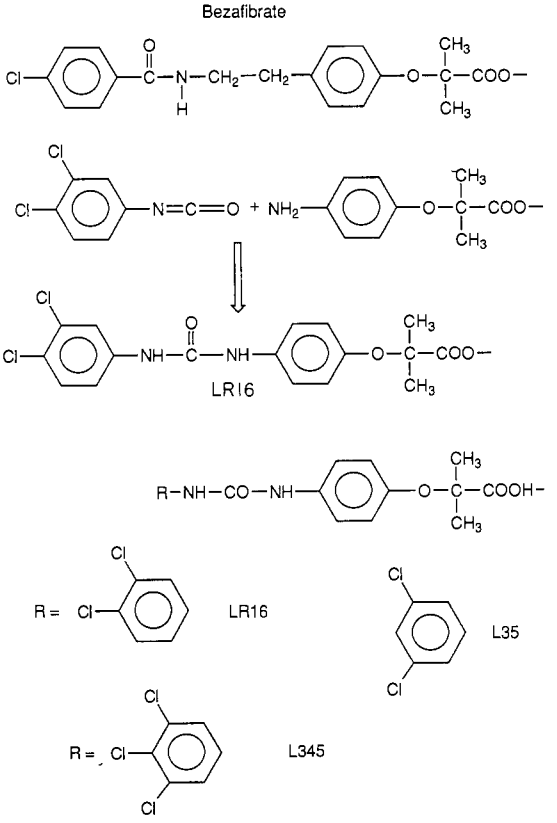
ABSTRACT: We describe the actions of two new allosteric effectors of hemoglobin, 2-[4-(3,5-dichlorophenylureido)phenoxy]-2-methylpropionic acid (L35) and 2-[4-(3,4,5-trichlorophenylureido)phenoxy]-2-methylpropionic acid (L345). Each of them binds to two pairs of symmetry-related sites in the central cavity of human deoxyhemoglobin. One pair of sites overlaps with that occupied by bezafibrate [Perutz et al. (1986) *J. Am. Chem. Soc. 108*, 1064–1078]. The other sites are new, and the pair occupied by L35 is different from that occupied by L345. All the sites are at least 20 Å from the site where organic phosphates are bound. L345 is by far the most potent allosteric effector of hemoglobin ever described. At a concentration of 0.1 mM, it raises the P_{50} of a suspension of red cells by 50%; at 0.2 mM it raises the P_{50} 2.5-fold. At acid pH, it reduces Hill's coefficient to near unity and prevents complete oxygen saturation even under 1 atm of pure oxygen. In azidemethemoglobin at pH 6, it induces a transition to higher spin. These properties are reminiscent of those of teleost fish hemoglobins that exhibit a Root effect. The influence of L35 and L345 and that of organic phosphates on the oxygen affinity are additive, but they compete with chloride. L35 acts more weakly than L345, but can be made to induce the same effects as L345 alone by adding inositol hexaphosphate. Both compounds increase the alkaline and acid Bohr effects. They alter the bimolecular kinetics of CO recombination after a flash by increasing the slowly reacting fraction of hemoglobin in the T state at the expense of the fast-reacting fraction in the R state. When [L345] > 1 mM, the slow fraction decreases again due to dissociation into $\alpha\beta$ dimers. Physiological concentrations of human or bovine serum albumin inhibit the actions of the effectors, even when palmitic acid or tripalmitin is added as decoy.

We recently described the structure and function of human hemoglobin complexes with bezafibrate and LR16, two novel allosteric effectors derived from clofibrate (Scheme I) (Perutz et al., 1986; Lalezari et al., 1988). They bind reversibly to hemoglobin in the red cells without damaging the cell membrane; they decrease the oxygen affinity of red cell suspensions and of hemoglobin A solutions through their preferential binding to deoxyhemoglobin, and they act synergistically with the natural effector 2,3-diphosphoglycerate. Their binding sites lie in the central cavity mainly between the α-chains and are distant from the organophosphate binding sites. Lalezari and Lalezari (1989) have synthesized several new compounds that have higher affinities for hemoglobin than bezafibrate and LR16. In this paper we describe the effects of their two most active compounds, L35 and L345, on the functions and structure of hemoglobin.

Scheme I



Material and Methods

2-[4-(3,5-Dichlorophenylureido)phenoxy]-2-methylpropionic acid (L35) and 2-[4-(3,4,5-trichlorophenylureido)phenoxy]-2-methylpropionic acid (L345) were synthesized as described by Lalezari and Lalezari (1989) (Scheme I).

Oxygen Equilibrium Studies. Experiments were performed with human red blood cells and purified HbA solutions. Blood was collected on heparin from a healthy nonsmoking donor at the blood bank of the hospital. It was centrifuged in the cold to remove the plasma and buffy coat and then washed twice in isotonic saline at pH 7.4. Pure HbA was prepared from a hemolysate by DEAE-Sephadex chromatography. The

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purity of the preparation was checked by isoelectric focusing. Buffers were made with 0.1 M NaCl and 50 mM Bis-Tris or Tris-HCl for pH values below or above 7.5, respectively. The compounds under study were dissolved in water by addition of 1 M $NaHCO_3$. The stock solutions were 20 mM for each compound. Inositol hexaphosphate (IHP) or 2,3-diphosphoglycerate (DPG) was used as sodium salt at the concentrations indicated in the tables. The pH values of the working solutions were adjusted to the desired values just before the experiments.

Oxygen dissociation curves were recorded with a continuous technique (Hemox-Analyzer, TCS, Southampton, PA) at 37 °C for red cell suspensions and at 25 °C for HbA solutions (Kister et al., 1987). For these experiments RBC suspensions (10% hematocrit) were first equilibrated in isotonic Bis-Tris buffer at 37 °C for 60 min at pH 7.4 in the presence of a given concentration of the compound. No hemolysis was observed after the addition of the compounds. For studies of HbA solutions, the compounds were added to the buffer just before the recordings. RBC suspensions or HbA solutions were first equilibrated under 1 atm of oxygen for 5 min and then slowly deoxygenated by having nitrogen bubbled through them. The duration of the recordings was 40–50 min to ensure full equilibrium.

Due to the potent effects of these compounds on the oxygen affinity of hemoglobin at pH values below 7.5, the solutions were not fully saturated under 1 atm of oxygen. The oxygen saturation of the hemoglobin solutions under pure oxygen was measured spectrophotometrically. At acid pH a significant amount of methemoglobin was formed during the recordings. This was estimated from the optical spectra of the carbonmonoxyhemoglobin solutions at three wavelengths according to the methods of Van Assendelft and Zijlstra (1973) and Benesch et al. (1973). Up to 10% methemoglobin was formed at pH 6, but negligible amounts (less than 5%) were formed at neutral or alkaline pH values. The reversal of the binding of the compounds to red cell hemoglobin was studied by recording the oxygen equilibrium curves of cell suspensions first equilibrated with the compound under study and then washed in a drug-free buffer and incubated with it for an additional 60 min at 37 °C. Experiments were also performed in solutions of HbA containing varying amounts of bovine serum albumin (BSA) or defatted human serum albumin (HSA), both from Sigma (St. Louis, MO). Their oxygen equilibrium curves were measured manually with the discontinuous method after step increments of oxygen to the fully deoxy solutions in a sealed glass tonometer (Poyart et al., 1978). This was necessary to avoid the foaming that occurs when nitrogen is bubbled through solutions containing albumin (Horbett, 1988) or hydrophobic compounds. In some experiments palmitic acid or tripalmitin (Sigma) saturated HSA was used. Fresh stock solutions (0.1 mM) of the reagents (alone or mixed) were prepared in 50 mM Bis-Tris buffer, pH 7, on the day of the experiment.

P_{50} and n_{50}, the oxygen partial pressure and Hill coefficient at half-saturation, respectively, were calculated from the Hill equation by linear regression analysis of data points between 40 and 60% oxygen saturation. $\bar{Y}$ was obtained from the total absorbance change between the oxy and the deoxy spectra and corrected when necessary for the amount of unsaturation under 1 atm of oxygen. Due to the uncertainty in estimating the K_R values in these experiments, no attempt was made to calculate the allosteric constants of the two-state model. Parameters of the Bohr effect were obtained from log P_{50} at varying pH by nonlinear regression analysis according to the Wyman equation (Wyman, 1948).

Table I: Oxygen Binding Parameters for Fresh Normal Red Cell Suspensions at 37 °C[a]

effector (mM)	P_{50} (mmHg)	n_{50}	P_{50}eff/P_{50}control	slope
none (control)	23.4	2.34		
Bzf				
1.0	28.6	2.0	1.2	0.2
5.0	37.6	2.3	1.6	
LR16				
0.2	28.3	2.0	1.2	
0.5	48.6	2.2	2.0	0.57
1.0	69.5	2.1	3.0	
L35				
0.1	28.8	2.0	1.2	
0.2	32.6	2.2	1.82	0.63
0.5	69.6	2.1	3.0	
1.0	123.0[b]	2.0	5.3	
L345				
0.1	33.5	2.0	1.4	
0.2	58.0	1.8	2.15	0.98
0.5	160.0[b]	1.6	11.0	

[a] Conditions: 0.14 M NaCl, 50 mM Bis-Tris (pH <7.5) or Tris-HCl buffer (pH >7.5), 37 °C. P_{50}eff/P_{50}control is the ratio of P_{50} with the effector to the control without it. The slopes were calculated as the difference in log P_{50} for a given variation of log (effector) concentration in molar. [b] These curves were recorded at pH 7.8 to ensure full oxygenation of the red cells under 1 atm of oxygen. P_{50} values were corrected to pH 7.4 by using a Bohr factor of 0.60.

X-ray Analyses. Human deoxyhemoglobin was allowed to react with varying concentrations of L35 and L345 and was then crystallized according to the method of Perutz (1968). Good crystals were obtained at final concentrations of 600 μM heme, 750 μM L35, and 300 μM L345. X-ray data were collected on a Hilger four circle diffractometer, corrected by the usual factors, and difference electron density maps were calculated.

Kinetic Studies. Ligand recombination kinetics were measured after photodissociation by a 30-ns laser pulse at 532 nm (YG585, Quantel, France). Observation was at 436 nm using cuvettes of 1 mm optical path length. Data were collected with a digital oscilloscope (Lecroy 9400) and transferred to an IBM microcomputer for further analysis (Marden et al., 1988).

Results

Oxygen Equilibria of Red Cell Suspensions. Table I gives the oxygen affinities (P_{50}) of fresh normal RBC suspensions at increasing concentrations of L35 and L345, compared to bezafibrate (Bzf) and LR16 (Lalezari et al., 1988). At high concentrations of the compounds L35 and L345 the oxygen equilibrium curves were recorded at pH 7.8 to ensure nearly complete oxygenation of the cell suspensions under 1 atm of oxygen. The P_{50} values were then normalized to pH 7.4 by using a Bohr factor of −0.6. Both compounds are potent effectors, showing that they permeate the red cell membrane. No hemolysis was noticed. After the cells previously incubated in the presence of 1 mM L35 or 0.2 mM L345 were washed, the normal oxygen affinity was recovered (not shown).

The potency in order of decreasing oxygen affinity is L345 > L35 > LR16 > Bzf (column 4 in Table I). L345 is by far the most potent compound of this series and of all the effectors of hemoglobin in the red cell ever described. Note also the low values of the cooperativity at half oxygen saturation (n_{50}). At the highest concentrations of L35 and L345 the oxygen binding curves became asymmetric, with n_{max} values at high oxygen saturations. Oxygen binding curves were also recorded for suspensions of 2,3-DPG-depleted red cells in the presence of L35 and L345. The cells had been depleted of DPG by incubation in an isotonic buffer for 18 h at 37 °C. The dif-

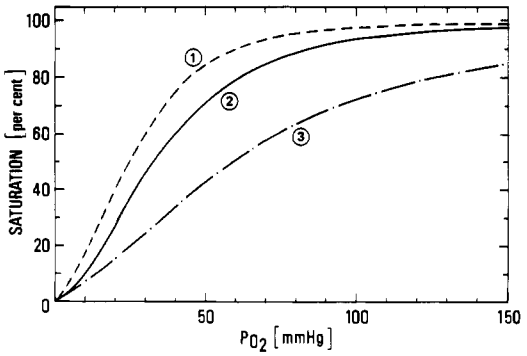

FIGURE 1: Oxygen equilibrium curves of red cell suspensions: (1) control curve; (2) red cells were incubated for 60 min at 37 °C in the presence of 0.2 mM L35; (3) as in (2) with 0.2 mM L345. All were in 0.14 M NaCl and 50 mM Bis-Tris buffer, pH 7.45, 37 °C.

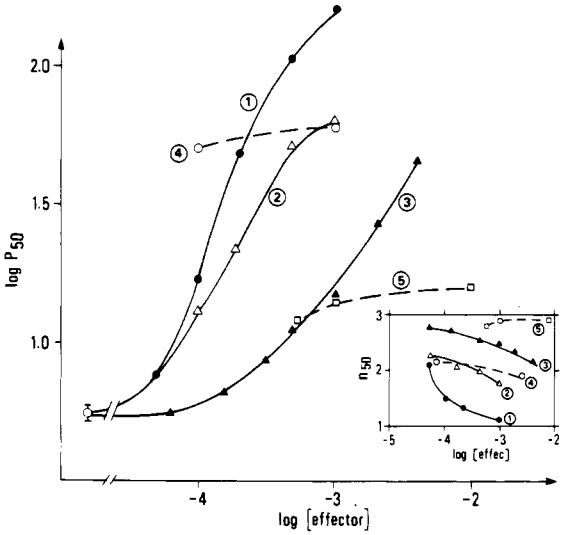

FIGURE 2: Titration curves of HbA solutions with the various effectors. Log P_{50} (mmHg) is the partial pressure of oxygen at half-saturation; n_{50} is the Hill coefficient at P_{50}. Log (eff) is the log 10 molar concentration of the effectors: (1) L345; (2) L35; (3) LR16; (4) IHP; (5) DPG. The slopes of these curves were calculated by linear regression analysis from the data points; the values (millimolar oxygen-linked effector bound per mole of heme) are L345 = 1.2, L35 = 0.7, and LR16 = 0.6. The insert illustrates the variations of n_{50} with increasing concentrations of the effectors. Conditions: 0.1 M NaCl, 50 mM Bis-Tris buffer, pH 7.2, 50 μM EDTA, 20 μg/mL catalase, 25 °C.

ferences in log P_{50} ± effector were similar to those for fresh red cells, indicating that, as for Bzf and LR16, the compounds L35 and L345 bind at sites different from those of the natural effector DPG. At constant intracellular pH, the effect of DPG on P_{50} levels off beyond a concentration of 1 mol/mol of hemoglobin tetramer (Duhm, 1976), but the effect of the present compounds does not level off within the range of concentrations used here, indicating that more than 1 mol of effector binds per mole of hemoglobin tetramer. The last column in Table I gives the slopes of the red cell titration curves with the three compounds which indicate that at least 2, 2.5, and 4 mol of LR16, L35, and L345, respectively, may be bound per hemoglobin tetramer. Figure 1 illustrates the profound effect of the new effectors on the oxygen affinity of red blood cells.

Oxygen Equilibria of HbA Solutions. Figure 2 summarizes the effect of varying concentrations of the compounds on log P_{50} of dilute solutions of hemoglobin. Values of log P_{50} at three concentrations of DPG and two concentrations of IHP are shown for comparison. The curves relating log P_{50} to log concentrations of LR16, L35, and L345 resemble those ob-

Table II: Parameters of the Bohr Effect in HbA and in HbA Reacted with L35 or L345

	ΔH^+ max	pH max	pK'_1	pK''_1	pK'_2	pK''_2
HbA	0.578	7.2	7.8	6.6	4.9	5.2
L35 (0.5 mM)	0.72	7.5	8.3	6.7	4.6	5.6
L345 (0.2 mM)	0.77	7.5	8.4	6.5	4.5	5.7

[a] $-\Delta H^+$ max is the maximum proton release per heme upon oxygenation. pH max is the pH at which $-\Delta H^+$ max is attained. pK'_1 and pK''_1 are pK values of a hypothetical alkaline Bohr group in the deoxy and oxy conformation, respectively. pK'_2 and pK''_2 have the same meaning for an acid Bohr group.

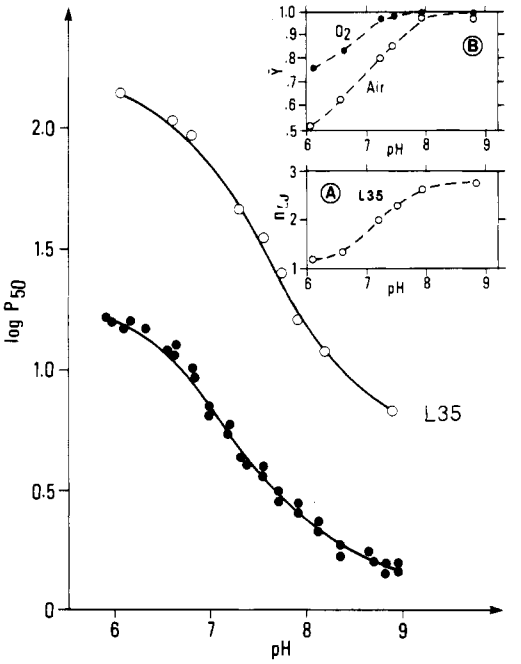

FIGURE 3: pH dependence of log P_{50} (mmHg) for solutions of HbA reacted with 0.5 mM L35 compared to the control Bohr curve for HbA. Insert A shows the pH dependence of the Hill coefficient in the presence of the effectors. Insert B shows the variation of the fractional oxygen saturation Y with pH in solutions of HbA reacted with L35 and equilibrated under room air or under 1 atm of oxygen.

tained with red blood cells suspensions (Table I). Again, no plateau of P_{50} values was reached at the concentrations used; 1 mM L35 lowered the oxygen affinity as much as 1 mM IHP (12-fold), while 1 mM L345 decreased it 30-fold. The insert in Figure 2 shows the variations of the Hill coefficient with effector concentration. High concentrations of LR16 lower n_{50} and decrease the oxygen affinity as much as IHP. L35 lowers n_{50} even at low concentration, and 1 mM L345 is sufficient to lower n_{50} to nearly unity. These results indicate that the compounds shift the allosteric equilibrium toward the low-affinity deoxy structure.

Influence of L35 and L345 on the Bohr Effect. Assuming only two Bohr groups, one for the alkaline and one for the acid pH range (Wyman 1948), L35 and L345 increase the alkaline and the acid Bohr effect as indicated by increases of ΔpK_1 and of ΔpK_2 (Table II). The pH of maximum proton release is also raised. Similar results were obtained in the presence of DPG and IHP (Kilmartin, 1974). All the effectors raise the pK values of groups with which they interact in the T state. Figure 3 (insert A) shows the large decrease in cooperativity with pH in the presence of L35, and insert B of Figure 3 gives the percent oxygen saturation of the L35–Hb complex at 1 atm of oxygen and in room air. The data indicate that at acid pH the L35–Hb complex is in an almost pure T state. Similar results were obtained with L345. This behavior is reminiscent

Table III: Effect of Anions on the Oxygen Binding Parameters of HbA Reacted with L35[a]

effector (mM)					
DPG	IHP	L35	P_{50} (mmHg)	n_{50}	P_{50}eff/ P_{50}control
0	0	0	1.9[b]	2.5	1.0
0	0	0.1	13.0[b]	2.2	6.8
0	0	0	5.4	2.9	1.0
0	0	0.1	15.4	2.3	2.9
1	0	0	16.3	2.8	2.9
1	0	0.1	43.0	2.3	8.0
0	1	0	69.5	2.0	13.0
0	1	0.5	172.0	1.5	32.0
0[c]	0.5	0.5	244.0	1.2	45.0

[a] Conditions: pH 7.2, 100 mM NaCl, 50 mM Bis-Tis, 50 μM EDTA, 20 μg/mL catalase, 25 °C. Heme concentration: 60–80 μM. [b] 10 mM Hepes buffer without chloride, pH 7.4, 25 °C. [c] 50 mM Bis-Tris HCl buffer, 100 mM NaCl, pH 6.1.

Table IV: Oxygen Binding Parameters of HbA Reacted with L35 or L345 in the Presence of Albumin

effector[a]	P_{50} (mmHg)	n_{50}
none +		
0.5 mM BSA	9.5	2.7
0.5 mM L35	60.7	2.1
0.5 mM L35 + 0.5 mM BSA	9.2	2.85
none +		
0.3 mM HSA	6.8	2.8
0.1 mM L345	17.0	1.9
0.1 mM L345 + 0.3 mM HSA	9.2	2.8

[a] Conditions: (first group) 0.1 M NaCl, 50 mM Bis-Tris buffer, pH 6.8, 25 °C, 200 μM heme; (second group) as in first group except pH 7. BSA, bovine serum albumin; HSA, human serum albumin (defatted).

of certain fish hemoglobins that exhibit a Root effect.

Effect of Chloride and Organophosphates on HbA Reacted with L35 and L345. In the absence of chloride, addition of 0.1 mM L35 decreases the oxygen affinity 7-fold. (Table III). In the presence of 0.1 M NaCl, L35 lowers the oxygen affinity only 3-fold, showing that chloride and the effectors compete for some of the same binding sites. By contrast, in the presence of L35 addition of DPG or IHP raises the P_{50} values beyond those for either effector alone, which indicates that the effects are additive, as found in our studies of red cell suspensions. Similar results were obtained for L345–Hb complexes. At acid pH, addition of L35 plus IHP (line 9 in Table III) results in extremely low oxygen affinity (P_{50} = 244 mmHg) and very low cooperativity, indicative of partially oxygenated Hb tetramers constrained in the T state.

Effect of Albumin on L35 and L345 Complexes. Table IV shows that in the presence of 0.3–0.5 mM of either bovine or human serum albumin (BSA or HSA) L35 has no effect and L345 only a slight effect on the oxygen affinity. When human serum albumin was saturated with either palmitic acid or tripalmitin, that inhibition was only slightly reduced. Kinetic experiments confirmed these equilibrium measurements.

Kinetic Studies. The bimolecular kinetics show the biphasic recombination typical of the allosteric Hb tetramer. The rapid and slow phases correspond to the fraction of sites rebinding CO to the high-affinity R and low-affinity T state, respectively. Figure 4 shows that L345 increases the slow phase at the fast phase's expense and that bovine serum albumin inhibits that effect. We tried to maximize the slow phase by raising the concentration of L345 as well as adding 1 mM IHP, but found that the slow phase *fell* when [L345] > 1 mM. Suspecting this to be due to dissociation into dimers, we then followed the kinetics in 0.5 mM L345 plus 1 mM IHP as a function of hemoglobin concentration. Figure 5 shows that the slow

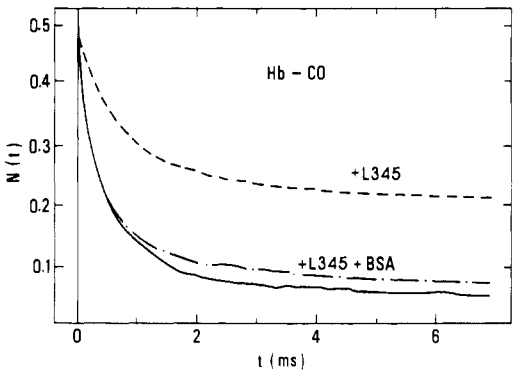

FIGURE 4: Bimolecular recombination kinetics of CO (0.1 atm) to Hb in the presence of 0.1 mM L345, 25 °C, pH 7. N(t) is the fraction deoxy. L345 increases the slow fraction, characteristic of the T state. The presence of BSA (0.5 mM) largely inhibits this shift. Similar results were obtained with HSA or HSA saturated with palmitic acid. The plain curve at the bottom is for Hb without effectors.

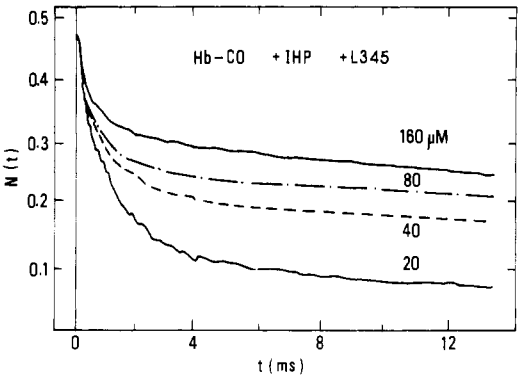

FIGURE 5: Bimolecular recombination kinetics of CO (0.1 atm) to Hb with both IHP (1 mM) and L345 (0.5 mM) at varying heme concentrations, 25 °C, pH 7. The combined effectors greatly enhance the slow fraction (T state) of the tetrameric form; however, the dependence on the total heme concentration indicates an increase in the fraction of dimers relative to Hb without effectors or with IHP alone.

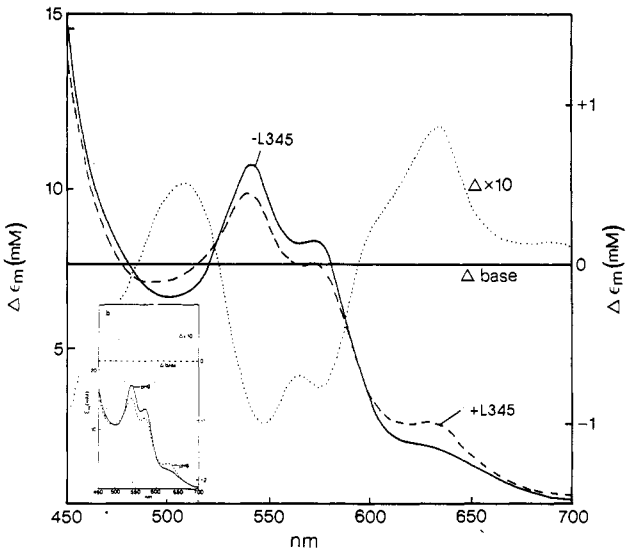

FIGURE 6: Visible absorption spectra of 65 μM (heme) human azide methemoglobin ± 250 μM L345 at pH 6.0. The inset shows the absorption spectra of trout IV azide methemoglobin at pH 9.0 and at pH 6.0 plus IHP.

phase diminishes with decreasing hemoglobin concentration, consistent with dissociation into dimers.

Influence of Effectors on the State of the Heme. In certain ferric hemoglobins the iron atoms are in a thermal equilibrium

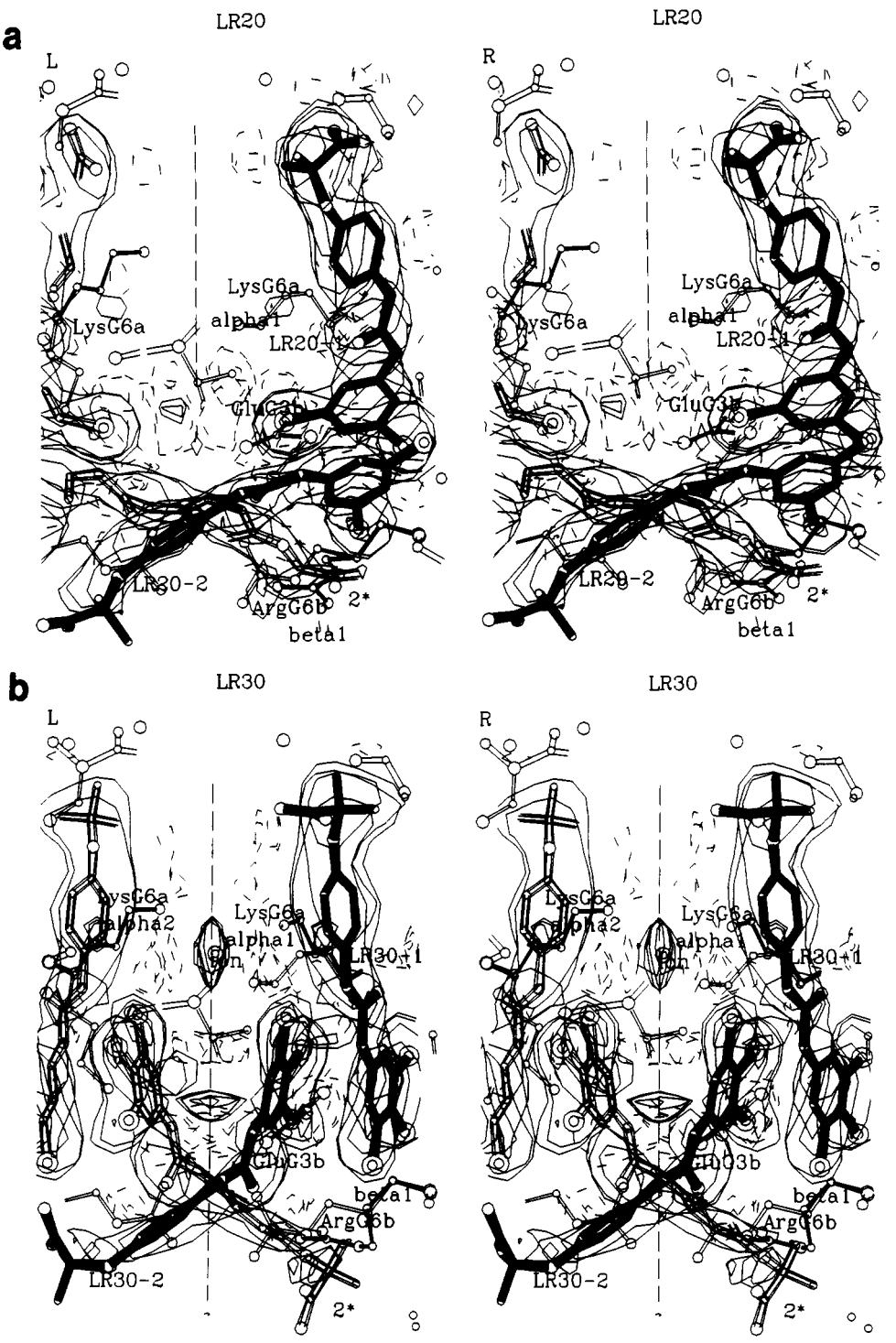

FIGURE 7: Stereoviews of the symmetry-averaged difference maps dxHb plus drug minus native dxHb for (a) L35 and (b) L345, with models of the drugs fitted to the difference maps. Both maps are contoured +0.1 e/Å^3 (solid contours) and −0.1 e/Å^3 (broken contours), or about 3 times the root-mean-square density. Only one site of each symmetric pair is shown in full. Thick bonds represent the drug; filled bonds are used at the site shown in full, open bonds at the other site of each symmetric pair. Thin open bonds represent the native dxHb model, and thin filled bonds represent protein residues whose positions were altered in accord with the difference map. The molecular dyad, about which the maps are averaged, is represented by the vertical broken line. In these pictures L35 is labeled LR20 and L345 is labeled LR30. X-ray data were collected from crystals grown from dxHb solutions containing the drugs, as previously described (Lalezari et al., 1988). Some details of the procedure and results are given below. Approximate average occupancy of drug sites estimated from electron count is in the range 20–40% for either drug.

	L35	L345
drug concentration in crystallization tubes (mM)	0.75	0.30
outer resolution limit (Å)	2.5	2.8
R factor between Friedel pairs (on intensity)	0.077	0.063
R factor with native data (on amplitude)	0.110	0.103
number of unique reflections in reduced data set	19101	12378
density in difference map (e/Å^3):		
root-mean-square over cell, before averaging	0.035	0.031
peak height, before averaging	0.53	0.40
peak height, after averaging	0.47	0.33

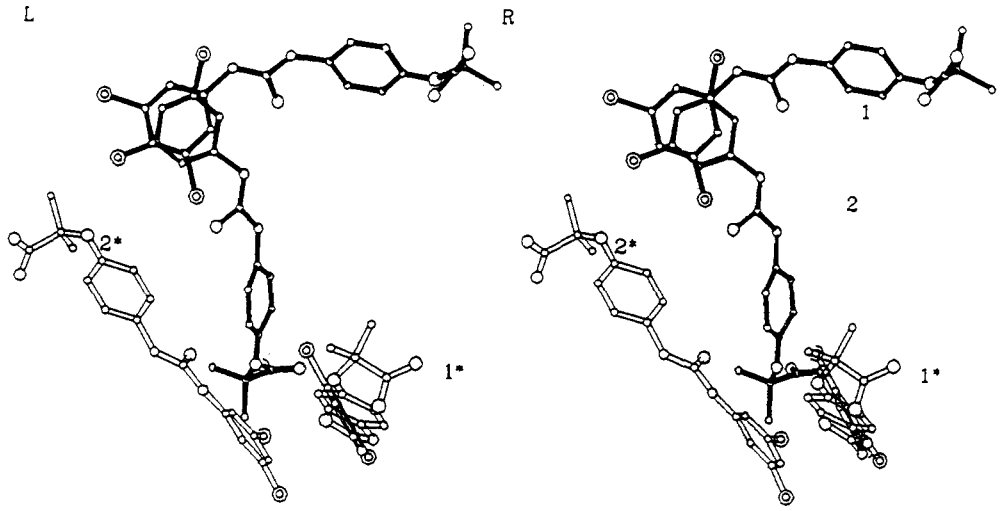

FIGURE 8: Stereoview of the sites of L35 in the central cavity of hemoglobin. Molecules 1 and 1* occupy the same sites as bezafibrate, and their interactions with the protein are similar to those shown in Figures 9–11 of Perutz et al. (1986).

between a low- and a high-spin state ($S = {}^1/_2$ and ${}^5/_2$). Fe–N bond distances are longer at high than at low spin. Tension at the heme iron in the T structure therefore biases that equilibrium toward high spin; in fish hemoglobins that exhibit a Root effect, this tension manifests itself by an increase in paramagnetic susceptibility, accompanied by a drop in the intensity of the low-spin and a rise in the intensity of the high-spin optical absorption bands when the pH is lowered from 9 to 7 and IHP is added. The effect is most marked in azide ($Hb^+N_3^-$) and nitric oxide ($Hb^+NO_2^-$) methemoglobins. In $Hb^+N_3^-$ of carp or trout IV the rise in paramagnetic susceptibility is equivalent to a change in free energy of about 1 kcal/mol of Fe (Perutz et al., 1978; Messana et al., 1978; Perutz, 1979). An even larger rise was found in carp $Hb^+NO_2^-$. We wondered whether similar tensions can be made to manifest themselves in human hemoglobin.

At acid pH, IHP converts the high-spin human Hb^+F^- and Hb^+H_2O from the R to the T structure, but has little effect on other human ferric hemoglobins. Hemoglobin Kansas [Asn G4(102)β→Thr] is an abnormal human hemoglobin with an allosteric equilibrium biased strongly to the T structure. Philo and Dreyer (1985) measured the effect of the R → T transition on the paramagnetic susceptibilities of several ferric mixed-spin derivatives of hemoglobin Kansas, but observed no significant rise in $Hb^+N_3^-$ and only very small rises in several other derivatives. They did not test $Hb^+NO_2^-$. Perutz and Cowan (1987) confirmed Philo and Dreyer's results with $Hb^+N_3^-$ Kansas, but found a 45% rise in the paramagnetic susceptibility of $Hb^+NO_2^-$ Kansas, much the same as that found in carp, showing that human hemoglobin can respond to the R → T transition by a rise in tension at the heme.

After Perutz and Poyart (1983) reported the additive effects of bezafibrate and organic phosphates on the allosteric equilibrium, Noble et al. (1989) measured the influence of IHP and bezafibrate, either separately or in combination, on the paramagnetic susceptibilities of several normal human ferric hemoglobins. They found the same large rise in $Hb^+NO_2^-$ that Perutz and Cowan had detected in $Hb^+NO_2^-$ Kansas (equivalent to 800 cal/mol of heme) and smaller rises in Hb^+ONC^- and Hb^+SCN^-, but still no significant rise in $Hb^+N_3^-$.

We have now found that L345 alone induces the same large changes in the visible absorption spectrum of normal human $Hb^+N_3^-$ as the change from pH 9 to pH 6 combined with IHP did in trout IV hemoglobin. Figure 6 shows that the rise in the high-spin bands at 500 and 630 nm and the fall in the low-spin bands at 540 and 570 nm are the same in the two hemoglobins. Since changes in the intensities of these bands are proportional to changes in paramagnetic susceptibility, these must also be the same. This is clear evidence that L345 induces a T state in human hemoglobin with a tension at the heme equivalent to that found in teleost fish hemoglobins at low pH.

Does this tension manifest itself only in the liganded T state or also in the deoxy T state? To answer this question, Professor T. Kitagawa kindly measured the Fe–N_ϵ stretching band in the resonance Raman spectrum of human deoxyhemoglobin with and without L345. This band is known to decrease in frequency from 222 cm^{-1} in human deoxyhemoglobin in the R state to 213 cm^{-1} in the T state (Nagai & Kitagawa, 1980). Addition of L345 to human deoxyhemoglobin in the T state at pH 7.4 reduced the Fe–N_ϵ stretching frequency from 213 to 211 cm^{-1} and that of the photodissociated transient from 222 to 220 cm^{-1}. These results show that combination with L345 makes itself felt at the hemes only weakly in deoxyhemoglobin in the T state, while the magnetic changes demonstrated that it makes itself felt strongly in the liganded T state.

Determination of Binding Sites by X-ray Analysis. Difference electron density maps of deoxyhemoglobin with bezafibrate or LR16 had shown one pair of symmetry-related binding sites in the central cavity, with the effectors adhering mainly to the α-chains and aligned roughly parallel to the molecular symmetry axis (sites 1 and 1*). The difference maps of L35 and L345 each showed an additional pair of binding sites (2 and 2*) near the midpoint of the hemoglobin molecule where the effectors enclose large angles with the symmetry axis (Figure 7). The dichlorobenzene rings of L35 stack in parallel pairs with staggered chlorines, site 1 with site 2 and site 1* with site 2* (Figure 8). The four trichlorobenzene rings of L345 form a single, close-packed, parallel stack in which five of the pairs of neighboring chlorines are eclipsed (Figure 9). Figure 10 shows L35 and L345 superimposed. Molecules in the bezafibrate sites 1 and 1* overlap; the other pair of sites occupied by L35 is different from that occupied by L345.

Figure 11 shows the contacts between the effectors and the protein. They are a mixture of polar and nonpolar interactions. Arg HC3α interacts with the carboxylates of the effectors in sites 1 and 1*, while the isobutyl groups in sites 2 and 2* are immersed in water. The amino groups of Asn G10β and Lys G6α and an imino group of His G10α donate hydrogen bonds

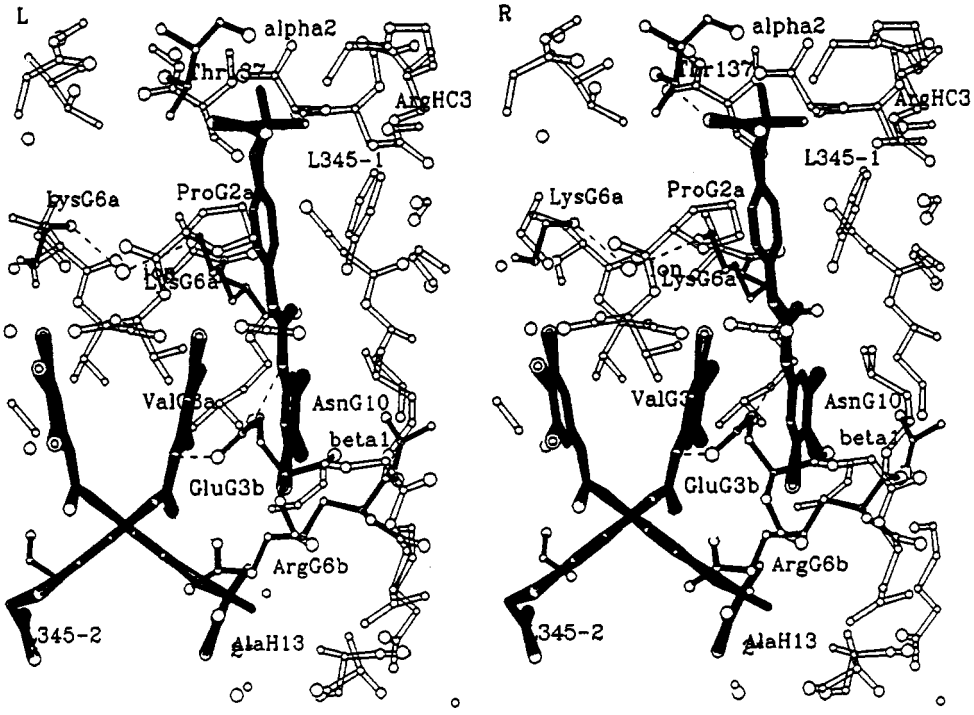

FIGURE 9: View of L345 perpendicular to the molecular symmetry axis. The molecule on the right occupies site 1, the bezafibrate site. The other symmetrically related pair occupies sites 2 and 2*. Note the hydrogen bonds from Glu G3β linking the urea moieties of the molecules in sites 2 and 2*.

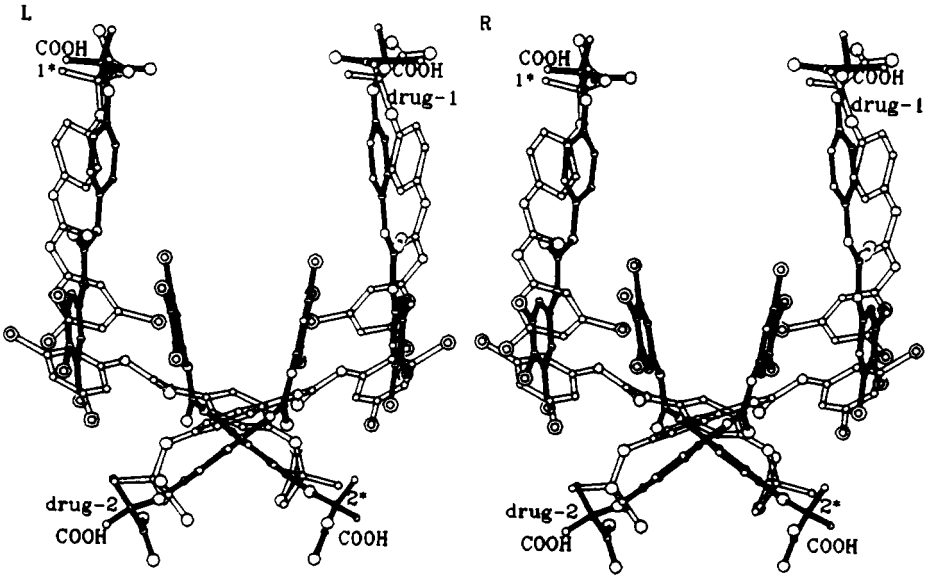

FIGURE 10: Superposition of L35 and L345. The vertical molecules occupy the bezafibrate sites.

to chlorines. The carboxylates of Glu G3β and Asp H9α accept hydrogen bonds from the urea moieties (Figures 9 and 12). Pro G3α and Thr H20α form van der Waals interactions with the isobutyl groups in sites 1 and 1*. The side chains of Leu G7α and Phe C1β and several of the methylenes of Lys G6α and Arg G6β are in contact with chlorines.

After the structure of deoxyhemoglobin with L35 had been solved, we detected faint positive peaks in sites 2 and 2* in the difference electron density map of LR16, showing weak occupancy of these same sites by LR16. Occupation of sites 1 and 2 by L35 and L345 is about equal, only about one site in three being occupied. We could not avoid this low occupation, as good crystals grew only at low drug concentrations, but it has prevented us from refining the structures and determining interatomic distances between drug and protein, because our electron densities are superpositions of the drug-free and drug-occupied protein.

Discussion

We have now examined the crystal structures of hemoglobin complexed with four related drugs: bezafibrate, LR16, L35, and L345. Bezafibrate has $CONHCH_2CH_2$ as a linker between the two benzene rings, while the other three compounds have NHCONH instead. Bezafibrate has one chlorine in position 4 (parallel to the linker), LR16 and L35 have two chlorines in positions 3 and 4 and 3 and 5, respectively. L345 has three chlorines in positions 3, 4, and 5 (Scheme I). All four compounds bind to the same pair of sites between the α-chains as bezafibrate which binds only at these sites. LR16 binds very weakly and L35 strongly at a second pair of sites; L345 binds strongly to a different pair of second sites. If we regard hemoglobin as a drug receptor, then our results show

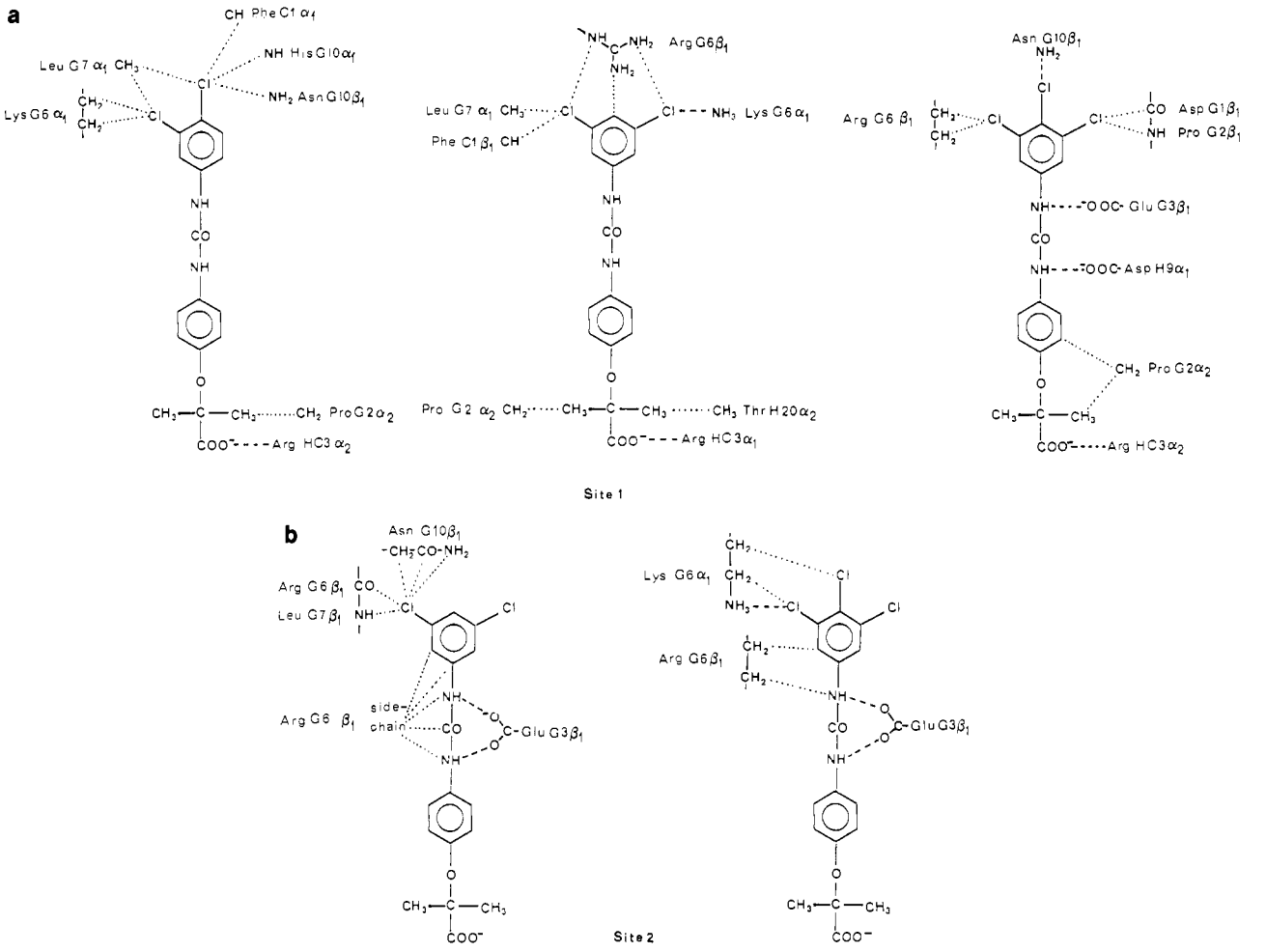

FIGURE 11: (a) Contacts between, from left to right, LR16, L35, L345, and the protein in site 1. (b) Contacts between L35 and L345 and the protein in site 2.

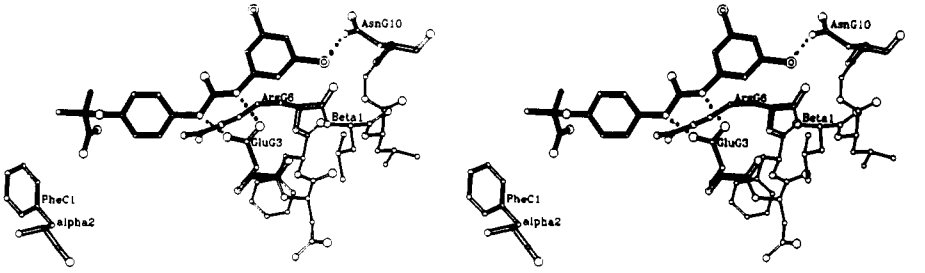

FIGURE 12: View of L35 at site 2 viewed approximately parallel to the molecular symmetry axis. Note the hydrogen bonds between Glu G3β and the urea moiety of L35.

that small chemical modifications of a drug may produce large changes in its affinity for different binding sites. They may also produce large changes in its physiological effect, even when the drugs bind to the same or similar sites.

For example, LR16 lowers the oxygen affinity 2.4 times more strongly than bezafibrate, and L345 lowers it 2.3 times more strongly than L35 (Table I). In each case the increased potency is associated with the substitution of a chlorine for a hydrogen. Hansch and Leo (1979) have shown that the partition coefficients of organic compounds between *n*-octanol and water increase with every chlorine added to these compounds, because chlorines make them more hydrophobic. It seems that hemoglobin and probably many other drug-binding proteins behave like drops of organic solvents that scavenge hydrophobic molecules from aqueous solutions.

At one stage we wondered whether rotation of the phenyls about the urea bridge is hindered; such hindrance would reduce the loss of rotational entropy on binding to hemoglobin and therefore account for the higher affinity of LR16 compared with bezafibrate (Lalezari et al., 1988). We abandoned that idea when we found that in the crystal structures of *N*,*N*′-diphenylurea and *N*,*N*′-bis(3,4-dichlorophenyl)urea the two phenyl rings are inclined to the urea plane by angles of up to 43°. Contrary to these structures, we now find that the chlorophenyl groups of bezafibrate, LR16, L35, and L345, in both sites 1 and 2, are always coplanar with the amide group, while the opposite phenyl groups are not coplanar with it. This suggests that rotation about the chlorophenyl–N bond in these four compounds is indeed hindered. The two phenyl rings in LR16, L35, and L345 can therefore rotate relative to each other only about the other phenyl–N bond. On the other hand, the phenyl rings in bezafibrate can rotate relative to each other about the three bonds (NH–CH_2–CH_2–Phe). This difference would reduce the loss of rotational entropy on binding to

hemoglobin of the urea-bridged compounds as compared to bezafibrate and account in part for their higher affinity for hemoglobin.

The nearest distance between any of the drugs and organophosphates bound to deoxyhemoglobin is about 20 Å. In the absence of any overlap the drugs and organophosphates stabilize the T structure synergistically. L35 and IHP combined lower P_{50} from 1.9 mmHg in stripped hemoglobin (pH 7.2) to 244 mmHg, a more than 100-fold reduction (Table III). It corresponds to a reduction of K_T from 10 to >250 $mmHg^{-1}$. Yet the structure of deoxyhemoglobin with the effectors is the same as without, except for small shifts of the atoms surrounding the effectors. This may seem surprising in view of the more than 50-fold rise in P_{50} produced by L345 alone, but Kitagawa's resonance Raman measurements prove that the heme iron is barely perturbed by L345 in the unliganded T state: there is evidence of only slight stretching of the Fe–N_ϵ bond. Many other experiments have shown that the oxygen affinity of the T structure can vary over a wide range, depending on the constraints placed on it by allosteric effectors, without causing more than minute changes in the T structure itself. On the other hand, the presence of effectors can manifest itself in the liganded T state by magnetic and other changes (Perutz et al., 1987). The strain caused by the effectors is probably localized at the heme iron only in liganded high-spin or mixed-spin derivatives where the Fe–N bonds are weak; in low-spin derivatives where the Fe–N bonds are strong, the strain may be taken up by the globin.

We had hoped that the new allosteric effectors might find clinical use to promote oxygen delivery to infarcted tissue or to tumors before irradiation, but these hopes were dashed by our finding that the effectors lose their activity in the presence of physiological concentrations of serum albumin, even when palmitin or tripalmitinglyceride was added as decoy. These lipids either bind to different sites or bind more weakly than our synthetic compounds. There may be ways of overcoming this problem. Meanwhile, we hope that our new allosteric effectors may be useful as research tools.

ACKNOWLEDGMENTS

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Registry No. L35, 121809-80-1; L345, 121809-82-3; LR16, 117011-50-4; HbA, 9034-51-9; IHP, 83-86-3; DPG, 138-81-8; azidomet HbA, 9072-23-5; Cl^-, 16887-00-6; CO, 630-08-0; oxygen, 7782-44-7; bezafibrate, 41859-67-0.

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