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LR16, a compound with potent effects on the oxygen affinity of hemoglobin, on blood cholesterol, and on low density lipoprotein

(oxygen release/allosteric effector/antilipidemic drug)

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ABSTRACT **2-[4-(3,4-Dichlorophenylureido)phenoxy]-2-methylpropionic acid, LR16, combines with two symmetrically related sites in the central cavity of deoxyhemoglobin, 20 Å away from the binding site of 2,3-bisphosphoglycerate, and acts as an allosteric effector synergistic with 2,3-bisphosphoglycerate. LR16 (1 mM) raises P_{50}, the partial pressure of oxygen needed to achieve half-saturation with oxygen of a hemolysate of human hemoglobin, about 50 times more strongly than 1 mM 2,3-bisphosphoglycerate. Oral administration of LR16 (at small doses that produced no ill effects) to rats that were fed a diet rich in cholesterol caused substantial reductions of total serum cholesterol and low density lipoprotein-cholesterol, while high density lipoprotein-cholesterol remained unchanged.**

Perutz and Poyart (1) showed that the antilipidemic drug bezafibrate, a compound related to clofibric acid, lowered the oxygen affinity of human hemoglobin more strongly than the natural effector 2,3-bisphosphoglycerate (DPG) and acted synergistically with it. Bezafibrate passed freely in and out of erythrocytes (1). Their hope that this property of the drug might have clinical application in prolonging the shelf life of stored erythrocytes, in improving delivery of oxygen to ischemic tissues and tumors (2), or in the treatment of shock was not fulfilled because its affinity for hemoglobin proved to be too low. (Its dissociation constant from deoxyhemoglobin is between 0.1 and 1 mM, depending on the chloride concentration.) We became interested in developing other clofibric acid-related compounds in the hope of finding some with higher affinities for hemoglobin and stronger allosteric effects.

We designed and synthesized several additional compounds and investigated their effects on human hemoglobin. Among 19 compounds made in the course of nearly 2 years, two compounds with diphenylurea and *p*-chlorodiphenylurea structures showed activities close to bezafibrate. These findings led to the development of the highly active phenylureido-substituted phenoxyisobutyric acid compound, LR16.

Bezafibrate binds to a site in the central cavity of deoxyhemoglobin, 20 Å away from the DPG binding site, and is in contact with one β and two α subunits. No covalent bond binds the drug to the globin; there are only a multitude of secondary interactions, and many of them are polar (3). We have crystallized LR16 with human deoxyhemoglobin, collected x-ray diffraction data to 2.4-Å resolution, and calculated a difference electron density map. This shows that LR16 binds to the same site as bezafibrate.

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Synthesis

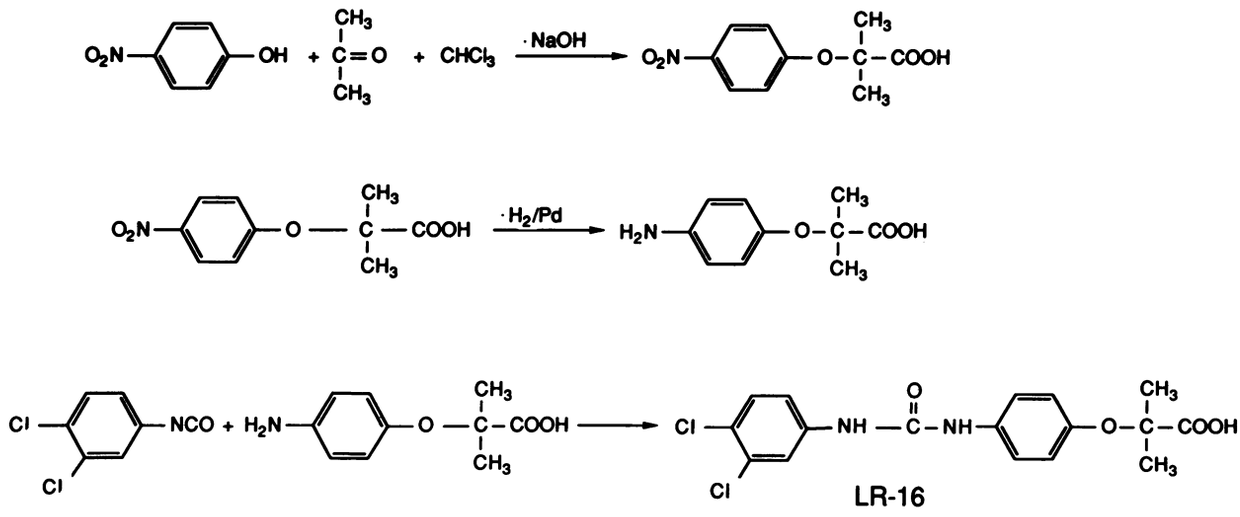
The 2-(4-nitrophenoxy)-2-methylpropionic acid intermediate was prepared by the reaction of chloroform with 4-nitrophenol and sodium hydroxide in dry acetone (4) (Scheme I). The yield was 54% [mp, 121–123°C (cclu)]. 2-(4-Aminophenoxy)-2-methylpropionic acid hydrochloride was prepared by catalytic hydrogenation of the above nitro compound (5). The free amino acid was obtained by sodium hydroxide treatment of the hydrochloride, followed by precipitation with acetic acid as a white crystalline powder (mp, 214–216°C). To prepare 2-[4-(3,4-dichlorophenylureido)phenoxy]-2-methylpropionic acid (LR16), a solution of 0.94 g (5 mM) of 3,4-dichlorophenylisocyanate in 10 ml of tetrahydrofuran was added dropwise to a stirred solution of 2-(4-aminophenoxy)-2-methylpropionic acid [0.975 g (5 mM)] in 5 ml of NaOH cooled to an ice salt bath temperature. Stirring was continued for 2 hr at room temperature. Charcoal in water (20 ml) was added to the reaction product, and it was then filtered. Acidification with hydrochloric acid gave a light-colored crystalline compound. It was recrystallized from aqueous acetone to give large silvery plates [mp, 184–185°C (dec.), yield 82%].

NMR (dimethyl sulfoxide-d_6) δ 1.52 (s, 6H, $2CH_3$), 6.91-7.68 (m, 7H, aromatics), 8.1 (br, s, 1H, NH), 8.8 (br, s, 1H, NH), and 9.15 (s, 1H, COOH). Analysis. Calculated for $C_{17}H_{16}Cl_2N_2O_4$: C, 53.27; H, 4.20; N, 7.31. Found: C, 53.41; H, 4.22; N, 7.46.

Methods

Blood samples were collected in heparin from volunteer laboratory personnel. Erythrocytes were washed three times with saline. Hemolysates were prepared by the addition of 1.5 volumes of water and 0.5 volume of CCl_4 to the packed erythrocytes followed by centrifugation. Hemolysates were stripped of anions by gel filtration on a Sephadex G-25 (fine) column (6). Hemoglobin concentrations were determined spectrophotometrically by using the extinction coefficient of hemoglobin A (7). Bezafibrate (a gift from Boehringer Mannheim) was dissolved in 0.05 M Hepes at pH 7.40. Working solutions of bezafibrate were prepared by diluting the stock solution with appropriate buffers. Oxygen equilibria were determined with an automatic device (Hemox Analyzer, TCS, Southampton, PA) (8). Isotonic buffer at pH 7.40 (0.14 M sodium chloride/0.01 M glucose) was used for erythrocytes at 37°C. Hemoglobin solutions were analyzed at 25°C in 0.05 M [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Bistris) at pH 7.2 (9). Oxygen equilibrium curves were recorded on an XY recorder. Compound LR16 was dissolved by addition of a slight molar excess of $NaHCO_3$ and was kept in

Abbreviation: DPG, 2,3-bisphosphoglycerate.



Scheme I

0.05 M Hepes at pH 7.40 as a 0.1 M stock solution. Erythrocyte suspensions were incubated with different concentrations of the compound for 30 min at 37°C before recording the oxygen dissociation curves. Subsequent to the recording, treated erythrocytes were washed three times with isotonic buffer at pH 7.40 and resuspended in the same buffer used for recording the oxygen dissociation curves.

Human deoxyhemoglobin was crystallized as described by Perutz (10). A 0.2 M solution of LR16 in water was made by addition of a slight molar excess of $NaHCO_3$. LR16 is insoluble in the 2 M ammonium sulfate solutions used to precipitate the deoxyhemoglobin crystals and therefore had to be added to the deoxygenated hemoglobin before addition of the ammonium sulfate. Oxyhemoglobin was deoxygenated

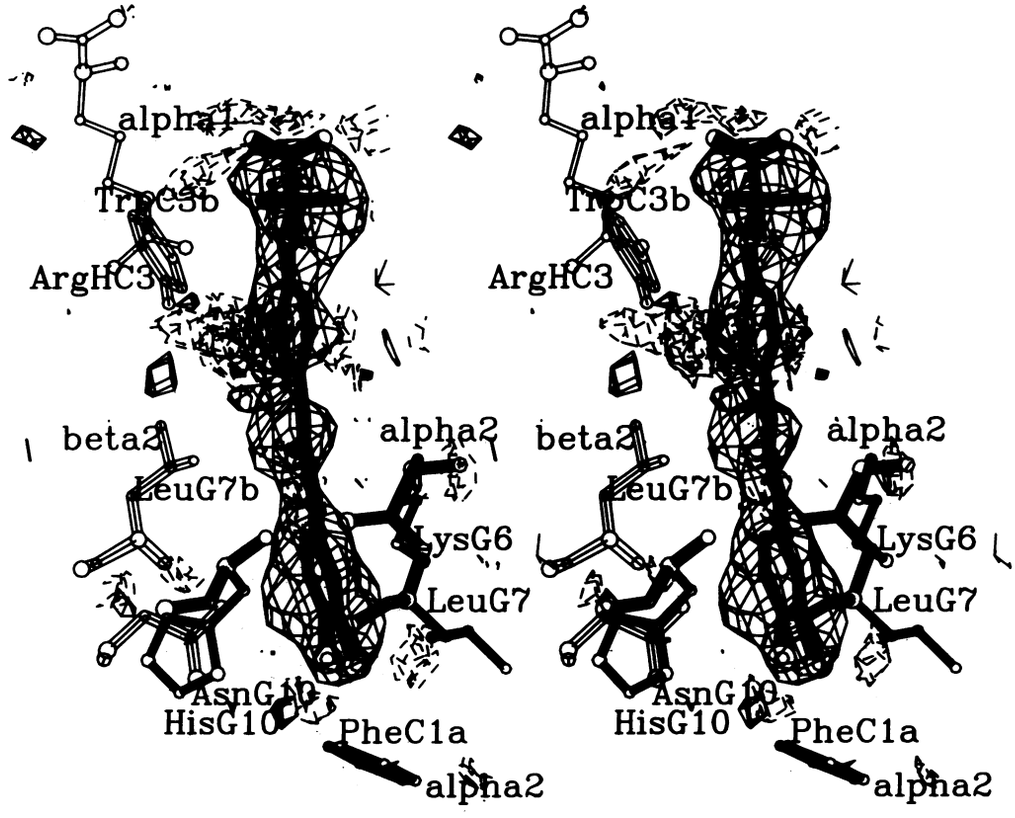


FIG. 1. The difference map, [hemoglobin (Hb) + LR16] − (Hb_{native}), shows one of the two LR16 binding sites. The map is contoured at +0.065 e/Å^3 (solid contours) and −0.065 e/Å^3 (broken contours) or about 3 times the root-mean square density of 0.022 e/Å^3. The atomic model is that of native deoxyhemoglobin (with bonds of increasing heaviness representing the α_1, β_2, and α_2 subunits) and a model of LR16 (heaviest bonds) fitted to the positive peak in the electron density. The difference map showed no significant features away from the immediate vicinity of the binding site. The negative difference peaks surrounding the binding site are probably due to series termination error due to finite resolution. Integration of the positive peaks showed them to contain only about 40–50 electrons, corrected by the Luzzati factor of 2, compared to an expected 182 electrons on heavy atoms in the LR16 structure, so that only about one-fourth or one-fifth of the sites are occupied. This explains why no peaks indicating shifts are seen at Lys G6 α_2, even though the side chain of that residue must move by at least 1 Å to avoid close contact with the lower LR16 benzene ring. The molecules can be fitted to the electron density either as shown or rotated by 180° about its long axis. We cannot distinguish between these orientations or a random mixture of the two.

Table 1. P_{50} values of erythrocyte suspensions

	P_{50}, mm Hg				
		Bezafibrate		LR16	
Exp.	Control	5 mM	0.5 mM	0.5 mM	0.2 mM
1	13	23.5	13.5	33	22.5
2	20	32.5	21	39	35

by alternate evacuation and flushing with nitrogen, and LR16 was then added to aliquots to bring its concentration to 2, 1, 0.5, and 0.25 mM. Each of the solutions was then set up to crystallize with a range of buffered ammonium sulfate solutions (10). After a month, crystals were found at all concentrations of LR16, but those at 2, 1, and 0.5 mM LR16 gave disordered diffraction patterns, whereas at 0.25 mM LR16 good crystals were found. X-ray data from these were collected for *hkl* and $h\bar{k}l$ reflections to 2.4-Å resolution and were corrected by the usual factors. The *R* factor between Friedel pairs was 5.7% on intensity; that between the derivative and native data sets was 6.7% on amplitude. The amplitudes were used to calculate a difference electron density map with ($|F_{\text{native + LR16}}| - |F_{\text{native}}|$) as coefficients.

Results

The difference electron density map shows two peaks in the central cavity related by the molecular two-fold symmetry axis (Fig. 1). The peaks overlap with the two bezafibrate binding sites. Integration over the total difference density shows these binding sites to be occupied by LR16 in only one-fifth to one-fourth of the hemoglobin molecules in the crystal, as compared to about one-half of the sites in the complex with bezafibrate. These occupancies are limited by the drug concentrations that can be added without impairing the quality of the hemoglobin crystals. This low occupancy precludes determination of the displacements of the atoms of the globin by the drug and calculation of interatomic distances, but it is evident from the position of LR16 that its contacts with the protein are similar to those of bezafibrate although they must be different in detail because LR16 is 1.2 Å shorter. There is no indication of additional hydrogen bonds or van der Waals interactions; in particular, the urea moiety forms no hydrogen bonds with the protein, but is probably hydrated in the crystal as it must be in solution.

Table 1 and Fig. 2 show the effect of bezafibrate and LR16 on the oxygen equilibria of erythrocyte suspensions. Table 2 and Fig. 3 show their effect on hemolysates stripped of organic phosphates. The drugs decrease the oxygen affinity of hemoglobin as measured by P_{50}, the partial pressure of oxygen needed to achieve half-saturation with oxygen.

Preliminary experiments done with rats that were fed a cholesterol-rich diet showed that oral administration of LR16 at 30 mg/kg per day for 15 days caused a substantial reduction of total serum low density lipoprotein-cholesterol and cholesterol, while high density lipoprotein-cholesterol remained unchanged. These animals experienced no detectable ill effects. Preliminary toxicity studies showed that mice tolerate the drug well at 100 mg/kg given once intraperitoneally, and rabbits tolerate an intravenous injection of 75 mg/kg.

Dissociation Constants of Drugs from Hemoglobin

The common binding site of LR16 and bezafibrate in deoxyhemoglobin in the T state is in the central cavity. Bezafibrate was found to bind only to the T structures, and the same is likely to be true of LR16, since it binds to the same pair of sites. Hence, their effect on oxygen affinity is probably due to their preferential binding to the T structure, whence the effect on P_{50} of various drug concentrations should depend only on the dissociation constant of the drug from the T structure. Table 2 shows that the P_{50} values calculated from one dissociation constant for each drug are in close agreement with the observed P_{50} values at two different concentrations of each. The dissociation constant for LR16 is about 20 times lower than that of bezafibrate, indicating that the former binds more strongly, with a difference in binding energy of about 1.8 kcal/mol.

The last column of Table 2 shows the combined effect of LR16 and DPG on P_{50}, which can be calculated from the dissociation constant of the two compounds by assuming that they bind independently. Thus LR16 and DPG act synergis-

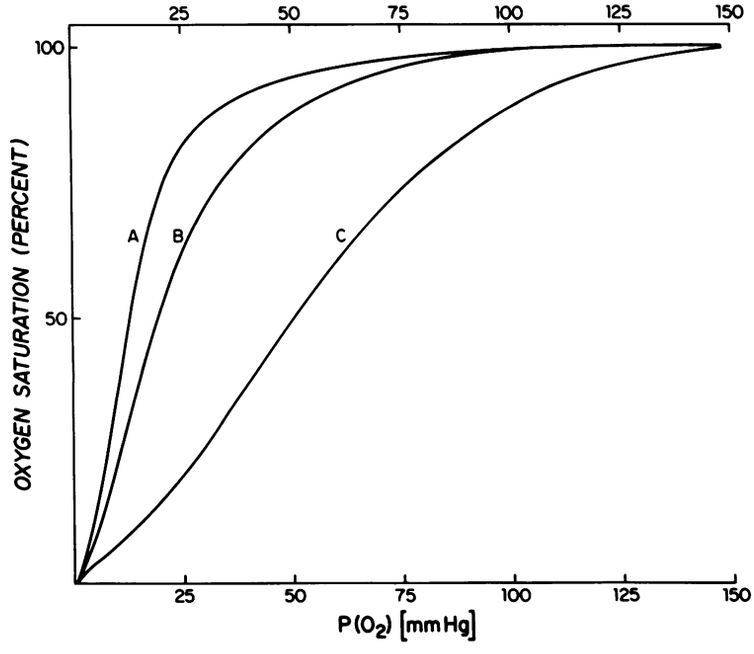


FIG. 2. Oxygen dissociation curves showing the effects of LR16 and bezafibrate on a suspension of fresh erythrocytes. Curves: A, control; B, 5 mM bezafibrate; C, 1 mM LR16.

Table 2. P_{50} values and dissociation constants in hemolysates

	Bezafibrate		LR16		0.2 mM LR16 + 2 mM DPG
	5 mM	0.5 mM	0.5 mM	0.2 mM	
$\Delta\log P_{50}$*					
Observed	0.60	0.12	0.69	0.49	0.85
Calculated†	0.55	0.17	0.68	0.49	0.87‡
Intrinsic dissociation constant§, mM	0.43		0.023		0.060 (DPG)

*Log_{10} of the ratio of the P_{50} value in the presence of the drug to that of the control (hemolysate stripped of organic phosphate); the latter P_{50} was between 2.5 and 3 mm Hg in the various experiments.

†Calculated from the formula $\Delta\log P_{50} = 0.5\log(1 + [X]/D)$, where [X] is the drug concentration and D is the intrinsic dissociation constant. This is derived by assuming that the drug binds only to the T state of hemoglobin, with equal first and second intrinsic dissociation constants, and by applying the relationship $\Delta\log P_{50} = 0.25\,\Delta\log L$ between P_{50} and the allosteric equilibrium constant $L = [T]/[R]$ (11). If $Lx = [T_{total}]/[R]$ is the allosteric constant in the presence of the drug, then $\Delta\log L = \log(Lx/L) = 2\log(1 + [X]/D)$, since $[T_{total}] = [T] + [Tx] + [Tx_2] = [T] + 2[T][x]/D + [T][x]^2/D^2 = [T](1 + [X]/D)^2$. See also footnote §.

‡Calculated from the formula $\Delta\log P_{50} = 0.5\log(1 + [LR16]/D_{LR16}) + 0.25\log(1 + [DPG]/D_{DPG})$. This derived in a manner analogous to that in footnote †, assuming that the binding of DPG to its single site is unaffected by the presence of LR16.

§Calculated to give the best fit to the observed data, except D_{DPG} calculated from the data of Imai (12) for the effect of DPG alone on L. The intrinsic dissociation constant is the dissociation constant adjusted for the entropic factor 2 favoring the singly bound state—i.e., $D = 2[T][x]/[Tx] = 0.5[Tx][x]/[Tx_2]$.

tically on the oxygen affinity of hemoglobin, as shown for bezafibrate and DPG.

Discussion

What causes the high affinity of hemoglobin for LR16 as compared to bezafibrate? The positions of the two molecules in the central cavity superimpose (Fig. 4). *ortho*-Dichlorobenzene has a larger dipole moment (2.25 debye; 1 debye = 3.338×10^{-30} m·C) than chlorobenzene (1.57 debye), which would strengthen the electrostatic interactions between its chlorines and the fractional positive charges on the surrounding residues. These consist of α_1 chain residues Phe-36 (C1), Lys-94 (G8), Leu-100 (G7), and His-103 (G10); unfortunately, the occupancy of LR16 is too low to determine interatomic distances. For example, the lysine side chains can be moved to bring its amino group to within 3.7 Å of the chlorine in position 3, but the electron density map does not show any positive peak there.

At one stage, we wondered if the greater rigidity of the urea moiety bridging the two benzene rings in LR16, as compared to the aliphatic chain in bezafibrate, would reduce the loss of rotational entropy of LR16 on binding to hemoglobin. However, the crystal structures of *N,N'*-diphenylurea and of *N,N'*-bis(3,4-dichlorophenyl)urea show that the phenyl rings are not coplanar with the urea but are inclined to it at angles of up to 43°C. Moreover, the lengths of the bonds from the nitrogen to the phenyl carbon range from 1.40 to 1.42 Å, compared to 1.47 Å for a C–N single bond and 1.34–1.35 Å for the NH–CO bond within the urea moiety, showing that the former have only very weak double bond character.

The greater potency of LR16 compared to that of bezafi-

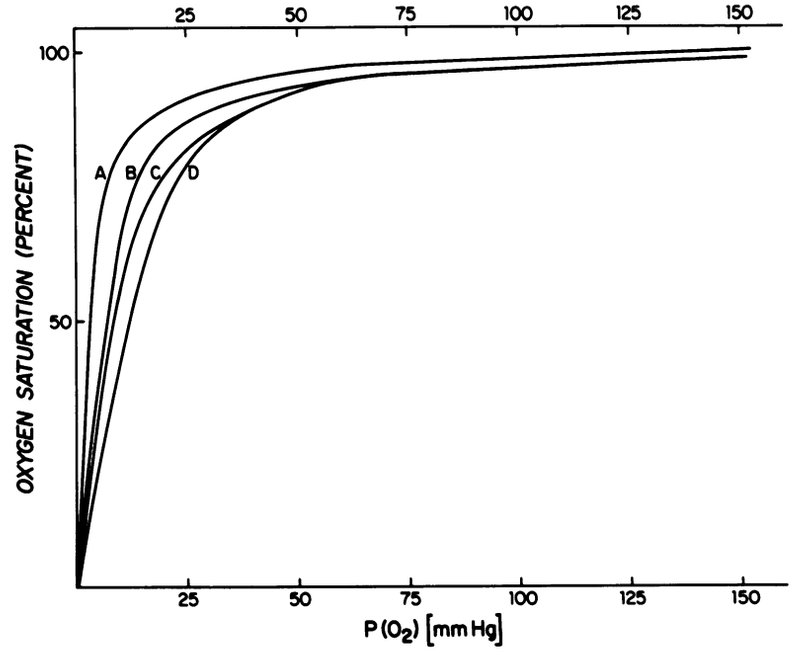


FIG. 3. Oxygen dissociation curves of hemolysates treated with LR16 or bezafibrate. Curves: A, control; B, 0.1 mM LR16; C, 0.2 mM LR16; D, 5 mM bezafibrate.

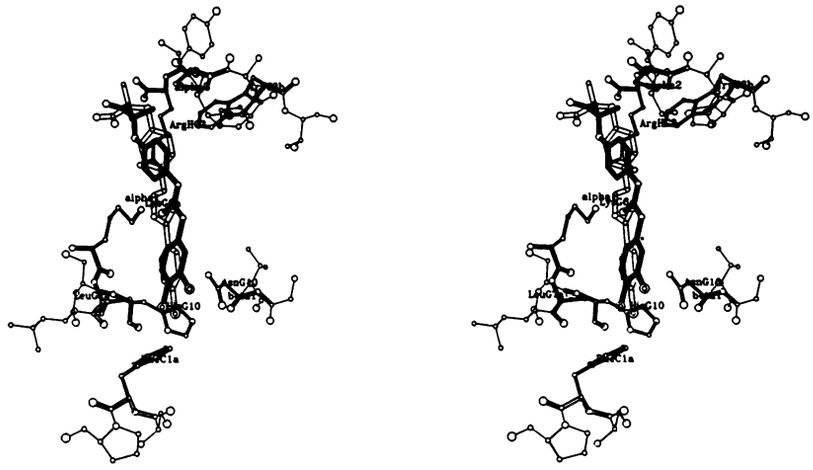


FIG. 4. Superposition of bezafibrate and LR16 in the central cavity of hemoglobin.

brate as an allosteric effector of hemoglobin appears to be paralleled by its greater potency as an antilipidemic agent. This suggests that the binding site or sites of the two compounds on the enzyme whose action they inhibit are also similar to those of hemoglobin. The nature of that enzyme is still unknown.

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