Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 2209–2211, March 1991
Medical Sciences

X-ray diffraction study of the binding of the antisickling agent 12C79 to human hemoglobin

(drug design/Schiff base adduct/aldehyde/molecular symmetry and binding)

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Communicated by M. F. Perutz, December 20, 1990

ABSTRACT **The hemoglobin binding site of the antisickling agent 12C79 has been determined by x-ray crystallography. 12C79 is recognized as one of the first molecules to reach clinical trials that was designed, *de novo*, from x-ray-determined atomic coordinates of a protein. Several previous attempts to verify the proposed Hb binding sites via crystallographic studies have failed. Using revised experimental procedures, we obtained 12C79-deoxyhemoglobin crystals grown after reaction with oxyhemoglobin and cyanoborohydride reduction to stabilize the Schiff base linkage. The difference electron-density Fourier maps show that two 12C79 molecules bind covalently to both symmetry-related N-terminal amino groups of the hemoglobin α chains. This is in contrast to the original design that proposed the binding of one drug molecule that spans the molecular dyad to interact with both N-terminal α-amino groups.**

12C79 [5-(2-formyl-3-hydroxyphenoxy)pentanoic acid] (formerly known as BW12C79) (1) is being investigated as a candidate for treatment of sickle cell anemia (2). To date, no drugs have been approved for therapy that inhibit the basic cause of the disease, the polymerization of deoxygenated sickle hemoglobin (HbS). 12C79 is generally regarded as the first description of a drug designed, *de novo*, from receptor-based molecular modeling that has reached clinical trials (3). A decade of research has attempted to verify whether the molecule binds as originally proposed (3–5).* This manuscript presents x-ray crystallographic evidence that demonstrates that the molecule does not bind as proposed. However, the initial concepts that targeted the N-terminal amino group of the α chains as the prime locale for reaction, with subsequent shift of the allosteric equilibrium toward the relaxed state (R state), were correct.

The basic concept in the modeling of 12C79 was to stabilize the soluble oxygenated R-state conformation of HbS, thereby reducing the concentration of deoxygenated tense-state (T-state) HbS available for polymerization (6). The crystal structures available at the time for horse deoxyhemoglobin, (7) and horse methemoglobin (oxy-like) (8) revealed that the α-subunit terminal amino groups are separated by ≈20.7 Å in the deoxy form but by only 12.4 Å in the methemoglobin (oxy) form. It was postulated (6) that these observed differences in distance were sufficient for 12C79 to preferentially bind to the oxy form, where a single 12C79 molecule would span the water cavity of oxyhemoglobin and be tied down at both ends by the symmetry-related α-chain terminal amino groups. It was envisioned that a Schiff base reaction of the 12C79 aldehyde would occur with one of the HbS α-subunit N-terminal amino groups, while the 12C79 carboxylate would form a salt bridge with the other α-subunit N-terminal nitrogen (Fig. 1). Additional stabilization of the complex was also proposed via hydrophobic interactions between the 12C79 side-chain methylene groups with the two 77α prolines and hydrogen bonding of the phenolic hydroxyl of 12C79 with the α-amino terminus. Previous attempts to crystallize the drug–hemoglobin complex and determine the binding site crystallographically have failed (3–5).*

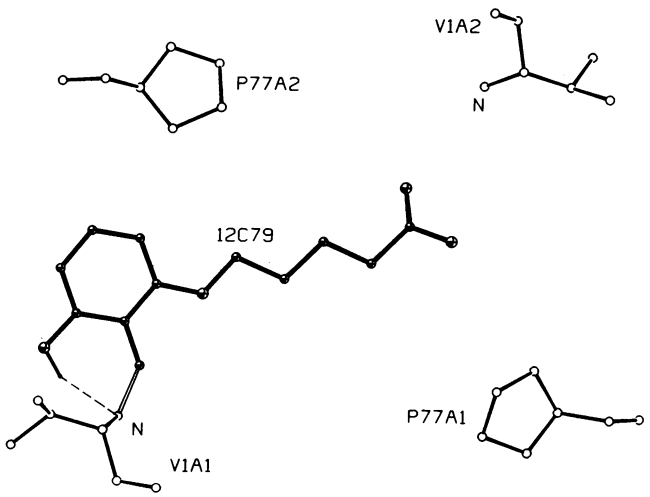

FIG. 1. 12C79 has been placed, using native human oxyhemoglobin A coordinates (9), as close as possible to that depicted in the original design (6) showing the phenyl ring of 12C79 planar perpendicular to the twofold axis. V1A1, Val-1α1; V1A2, Val-1α2; P77A1, Pro-77α1; P77A2, Pro-77α2. The molecule is not long enough to span the dyad in human hemoglobin and the prolines are not in contact with the 12C79 methylene side chain. The amino nitrogens are separated by 14.3 Å and the closest Pro-77α1–Pro-77α2 contact is 11.7 Å. Movement of the protein side chains by computer graphics and modern programs to permit the optimization of the 12C79 fit has produced a better orientation (Figure 8 in ref. 3).

METHODS

Hemoglobin was prepared from human blood as described by Perutz (10). The reaction and reduction to stabilize the Schiff base adduct were carried out under aerobic (R state) conditions and the x-ray diffraction study was carried out with T-state crystals to avoid technical problems observed with the R-state crystals (4).* Concentrated hemoglobin in 0.01 M ammonium phosphate buffer (pH 7.0) was diluted to 6 gm% (60 g/liter) with 50 mM potassium phosphate buffer (pH 7.4). A freshly prepared solution of 12C79 in potassium phosphate

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Abbreviations: HbS, sickle cell hemoglobin; T state, tense state; R state, relaxed state.

*See also unpublished results on reduced 12C79, M. F. Perutz, personal communication to D.J.A., March 23, 1990; D.J.A. and M. F. Perutz, unpublished results with unreduced 12C79, July 21, 1981; D.J.A., unpublished results indicating poor data collection by diffractometer due to the long 195 Å axis for R-state crystals.

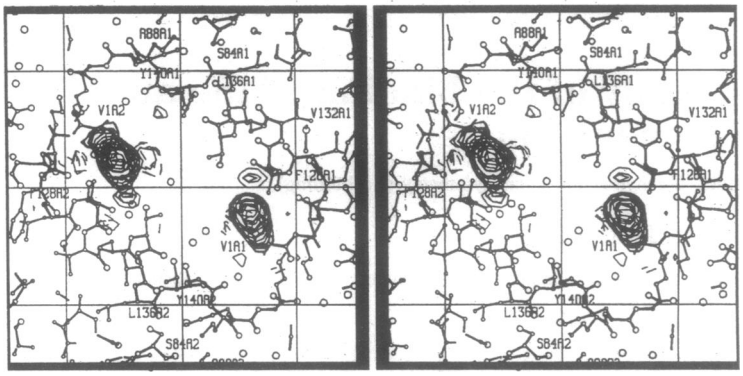

FIG. 2. A stereoview of the 4.0-Å electron-difference density Fourier map looking along the twofold symmetry axis of adult human hemoglobin/12C79 from inside the cavity toward the opening between the α chains. Two symmetry-related binding sites are observed. The map is contoured starting at +0.04 electron (e)/Å^3 (solid contours) and −0.04 e/Å^3 (broken contours), over 3 times the root-mean-square density (0.012 e/Å^3). No other high occupancy binding sites are apparent at this drug/hemoglobin ratio. Note the position of the terminal Val-1α amino group that extends into the density. The binding sites are separated at the closest point by ≈7Å. The farthest point of separation is ≈11 Å. The N-terminal nitrogens are separated by 16.2 Å.

buffer was incubated with the hemoglobin (60 g/liter) at a 2:1 (12C79/oxyhemoglobin tetramer) molar ratio for 15–20 min. Reaction of 12C79 with hemoglobin was monitored by using cation-exchange HPLC. Reacted and unreacted hemoglobin elute at different retention times and the concentration of each species is proportional to the area under the peak. Sodium cyanoborohydride, as a freshly prepared solution in potassium phosphate buffer, was added in 100-fold molar excess of hemoglobin tetramer to reduce the Schiff base adduct. Reduction was allowed to proceed for ≈2 hr at room temperature. The reduced 12C79–oxyhemoglobin complex was then deoxygenated by alternate flushing with nitrogen and evacuation without first desalting on a G-25 Sephadex column. This appears to be important as crystals and electron-density maps obtained from desalted samples either did not contain or had lower concentrations or occupancies of 12C79. The solution was then placed in tubes for crystallization following the procedures described by Perutz (10). Suitable crystals were obtained after 2–3 weeks. X-ray data were collected to 2.8 Å resolution for hk ± 1 reflections and their Friedel pairs using ω-scan on a Rigaku (Molecular Structure, The Woodlands, TX) AFC5R rotating anode diffractometer operating at 9 kW power and equipped with a 60-cm-long evacuated beam tunnel. A total of three crystals were used for the data collection. Data collection was controlled by TEXRAY software from Molecular Structure Corporation (11). Corrections for radiation damage, absorption, and Lorentz and polarization factors were applied to the data and the intensities from symmetry-related reflections merged to generate a unique set. Difference electron-density maps were calculated by using the known phases of deoxyhemoglobin (12) and the structure amplitudes ($|F_{\text{native+compound}}| - |F_{\text{native}}|$) as coefficients. Computations after initial data processing were done with CCP4 programs (13). A model of 12C79, initially created with MACROMODEL (14) and minimized with an MM2 force field, was fitted to the electron-density by using FRODO (15) on an Evans and Sutherland (Salt Lake City) PS390 graphics station.

RESULTS

Difference Fourier maps revealed two symmetry-related electron-density regions encompassing the Val-1α ammonium ions (Fig. 2). The fit of 12C79 to the 2.8-Å electron-density map demonstrated covalent binding to both symmetry-related α-valine terminal amino groups (Fig. 3). The Val-1α N-terminal amino atoms were found extending into the density of the drug almost overlapping the aldehyde

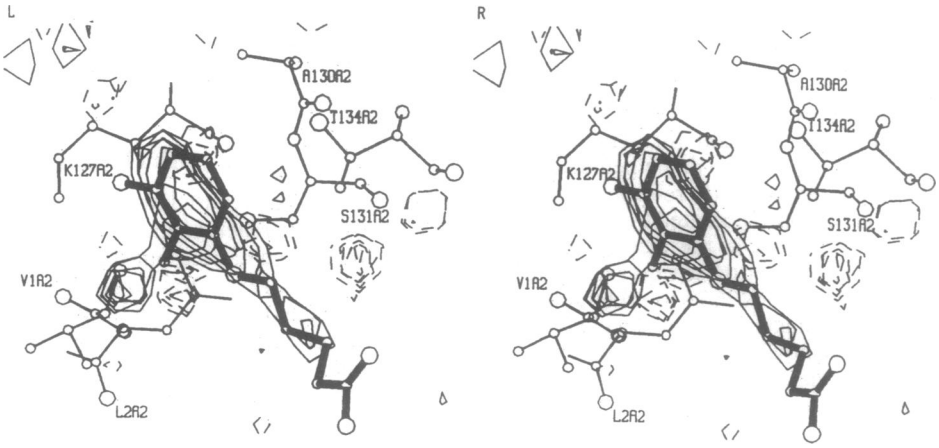

FIG. 3. The α2 12C79 site fit to the 2.8-Å electron-density map. The map is contoured starting at +0.08 electron (e)/Å^3 (solid contours) and −0.08 e/Å^3 (broken contours), at 3 times the root-mean-square density (0.026 e/Å^3). The drug orientation indicates a covalent bond has been formed with the N-terminal α-amino group. The distance and angles around the reduced Schiff base linkage are standard. Only one torsion angle adjustment (22°) to the N-terminal peptide chain brings the protein into an ideal tetrahedral position for bonding. Adjustments to four torsion angles (≤20°) in the N-terminal peptide chain permit a Schiff base trigonal geometry that would be pertinent to the chemistry *in vivo*. The α1 site density has the acid moiety shifted more toward the lining of the cavity. The map was not symmetry averaged so some shape differences in electron densities are observed between the α1 and α2 binding sites. The closest contact between the two molecules is ≈7 Å.

oxygen of 12C79. This confirms covalent binding of the aldehyde groups where the N-terminal amino atoms of the protein would replace the oxygens of the drug aldehyde to form a covalent Schiff base double bond. The fit of 12C79 to the 2.8-Å electron-density map shows hydrogen bonds between the N-terminal amino Val-1α amino group and the phenolic hydroxyl of 12C79 and between the Ser-131α hydroxyl and the ether oxygen of the drug. Thr-134α, Leu-2α, and Lys-127α make close contacts with the 12C79 phenyl ring, aldehyde carbon, and phenolic hydroxyl, respectively. The two 12C79 carboxylic acid moieties and methylene side chains point toward the top of the central water cavity and do not appear to be engaged in any obvious polar interaction with charged protein residues. A comparison of the α1 and α2 binding-site electron densities indicates a slightly different orientation for the 12C79 acid and methylene side chain. The electron density was not symmetry averaged. The remainder of the difference electron-density map is generally featureless and additional binding sites are not apparent at the drug/hemoglobin ratio used for this study.

DISCUSSION AND CONCLUSIONS

Merrett *et al.* (4) reacted hemoglobin under oxygenated conditions with the same molar ratio of 12C79/hemoglobin (2:1) used in our study and identified two strong binding sites from their Scatchard plot. However, they concluded that the original design hypothesis was correct; therefore, they inferred that the two binding sites cannot be related by the molecular dyad of hemoglobin. The electron-density maps clearly reveal two symmetry-related sites separated by ≈7 Å at the closest point (Fig. 2). The crystallographic determination of the binding sites for 10 different organic acid molecules bound to hemoglobin (even at low ratios of compound to hemoglobin) have always exhibited symmetry-related binding in this region of the α-subunit central water cavity (5, 16–18). Solution binding studies conducted with a number of these agents are consistent with the x-ray observations and show the same even number of drug binding sites per tetramer as observed in the electron-density maps (ref. 19; G. Joshi and D.J.A., unpublished results).

The α-chain N-terminal amino groups in horse hemoglobin are separated by 20.7 Å in the T state and 12.4 Å in the R state. In adult human hemoglobin, they are separated by 16.2 Å in the T state and 14.3 Å in the R state. In retrospect, it is logical to expect, since room is available for binding two 12C79 molecules in human R-state hemoglobin, that two covalent bonds with the two terminal α-amino nitrogens (≈40 kcal per bond or 80 kcal; 1 cal = 4.184 J) would be highly preferred over a single molecule spanning the cleft with one covalent linkage (≈40 kcal) and a noncovalent salt interaction (5–10 kcal).

In conclusion, we have shown that two symmetry-related 12C79 molecules bind to the N-terminal α-amino groups, in contrast to the original proposed mode of binding. However, the molecule did bind selectively, as originally suggested, to the oxyhemoglobin Val-1 α-amino groups and has functioned as envisioned, shifting the allosteric equilibrium toward the R state. Review of the literature (20–24) suggests that functional groups of molecules that bind across the hemoglobin dyad possess internal symmetry or pseudosymmetry that corresponds to the symmetry of the interacting functional groups of the protein. Redesign of 12C79 with this in mind and utilizing human hemoglobin coordinates with modern molecular graphics systems should produce a molecule that binds as originally proposed.

12C79 powder was supplied by Burroughs Wellcome. We thank Drs. M. F. Perutz and G. Fermi (Medical Research Council Laboratory of Molecular Biology, Cambridge, U.K.) for coordinates and helpful discussions. The financial support of this work was provided by National Institutes of Health Grant HLBI-RO1-32793 and the School of Pharmacy and the Medical College of Virginia, Virginia Commonwealth University.

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