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Dichloromethane as an antisickling agent in sickle cell hemoglobin

(gas binding to hemoglobin/sickle cell disease)

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ABSTRACT **Microscopic studies of red cells from homozygous sickle cell patients show that dichloromethane does prevent sickle cell formation *in vitro* and does cause reversion of sickled cells to normal after exposure to dichloromethane. X-ray structural analysis of human deoxyhemoglobin crystals exposed to dichloromethane shows four unique binding sites. Arguments are presented to suggest that the binding site close to tryptophan 14α prevents the formation of helical polymers, i.e., prevent sickling.**

It has long been known that sickle cell anemia is caused by distortion of erythrocytes (1). The red cells change from the normal biconcave discs to the stiff elongated crescents giving the disease its name. This abnormality was traced to a gene mutation changing the sixth amino acid of the β chains from glutamic acid to valine (2, 3). This structural change causes aggregations of deoxyhemoglobin into long straight rods resulting in deformation of the red cells (4). In the oxy form sickle cell hemoglobin, hemoglobin S (Hb S), behaves like normal hemoglobin. This behavior can be explained by a lock and key type binding mechanism whereby valine 6β (the altered amino acid) which is located on the surface of the hemoglobin structure, binds to a complementary site on another hemoglobin molecule. The complementary sites are in the correct structural configuration, however, only in the deoxy form. Extensive studies by Perutz on conformational changes of hemoglobin show that oxy and deoxy hemoglobin differ markedly in quaternary structure (5–8).

Any innocuous agent that can be bound to the region involved in sickling should alter the binding site significantly enough to prevent the formation of rods, i.e., prevent sickling. Finch *et al.*, Josephs, and others (9–11) showed that *in vitro* the sickling fibers are rods composed of four, six or eight monofilaments which are helically wound around each other, each monofilament being a string of stacked hemoglobin molecules. This suggests that sickling is not simply caused by a single complementary site, but that other molecular binding sites play a significant role. Interference with any of these contact points might, therefore, prevent sickling.

Certain anesthetics and some other relatively chemically inert gases bind to myoglobin and hemoglobin. In myoglobin crystals, the binding site for xenon, cyclopropane, and dichloromethane is in the interior of the protein and is approximately equidistant from one of the pyrrole rings of the heme group and from the ring of the heme-linked histidine in contact with hydrophobic groups leucine F4 and G5, isoleucine FC4, and phenylalanine H14 (12, 22). In methemoglobin (13), xenon was found to bind quite differently with one site in the GH corner of the α chain and with another site in the AB corner of the β chain. These sites are close to the exterior of the molecule and it was speculated that structural changes in those regions might prevent the aggregation of Hb S. This paper is a report on x-ray crystallographic studies showing the binding site of dichloromethane to deoxyhemoglobin and on observations indicating prevention and reversal of sickle cell formation by dichloromethane.

Abbreviation: DCM, dichloromethane. One millimeter of Hg equals 1.33×10^2 Pa.

Sickle cell prevention studies

For sickle cell prevention studies, fresh heparinized blood from homozygous sickle cell patients was exposed to a gas mixture of N_2, CO_2, and dichloromethane. The samples were flushed 3 times with the gas mixture at 30-min intervals to assure removal of all oxygen. Aliquots were then anaerobically diluted into Dacie's fluid containing 3% trisodium citrate and 1% formalin. At least 300 cells were counted in each aliquot by light microscopy with a standard hemocytometer. The cells were divided into two groups, normal and abnormal, and the latter were subdivided into sickled and other abnormal. Normal cells were those having the regular smooth biconcave shape; sickled cells were those having the characteristic half moon like shape; and other abnormal cells were those having other types of deformation, the most common being "pinched cloverleaf" appearance.

For sickle cell reversal studies, samples were first completely sickled by repeatedly flushing with 90% N_2 and 10% CO_2, and then exposed to gas mixtures with different dichloromethane (DCM) concentrations. Cell counts were performed as described above.

These experiments show that dichloromethane does prevent the formation of sickle cells. Fig. 1 shows that sickle cell prevention depends on the DCM pressure. Data are shown for true sickle cells and for abnormal cells which include true sickle cells. The abnormal cell data are average from two different experiments with blood from two patients. In different samples, at a given DCM gas pressure the number of abnormal cells is quite constant but the true sickle cell count shows considerable variation depending on a number of factors such as total lapsed time and exact composition of the serum. The control samples were treated identically except that the incubation gas was normal air. Sickle cell counts on the controls before during and just after the experiments were identical with 2% sickle cells and 5% abnormal cells.

Data for the reversal experiment (Fig. 2) show that sickled cells revert to normal when treated with DCM. It is noteworthy that at low dichloromethane pressure the number of abnormal cells remains constant but the number of true sickled cells decreases. This indicates the slow transition from true sickle to normal cells. Note that in this experiment the control contained 14% sickle cells and 32% abnormal cells as counted before and after the experiment.

Crystallographic studies

Human deoxyhemoglobin crystals were mounted in thin-walled glass capillaries. Mother liquor was added to the capillaries on one side of the crystals and DCM on the other side. At 20°, DCM has a vapor pressure of 350 mm Hg (4.6×10^4 Pa). Diffraction intensities were collected to a resolution of 4.8 Å on an

Table 1. Dichloromethane binding sites to deoxyhemoglobin

Site	Symmetry related occupancy x,y,2	Symmetry related occupancy −x,y,−2	Chain	Coordinates (Å) X	Y	Z	Helix	Interacting amino acids
D1	0.9	1.0	α_1, α_2	19	10	−8	A	Trp 14α (A12), Gly 15α (A13), Val 17α (A15)
							G	Leu 109α (G16), Thr 108α (G15), Leu 105α (G12)
							E	Ala 63α (E12), Leu 66α (E15)
							H	Phe 128α (H11)
D2	0.5	0.7	β_1, β_2	11	−8	8	G	Arg 104β (G6), Leu 110β (G12), Leu 106β (G8)
							H	Val 134β (H11), Val 137β (H14)
							E	Phe 71β (E15)
D3	0.7	0.5	$\alpha_1\beta_2$ Contact	5	−10	−14	H	Tyr 145β (H22), His 146β (H23)
							F	Cys 93β (F9)
							C	Thr 38α (C3), Lys 40α (C5), Thr 41α (C6)
D4		1.0	β_2	10	−2	20	CD	Heme pyrrole His 62β (E6), Phe 42β (CD1)

automated four circle diffractometer that permitted thermal regulation for the crystal. After the usual corrections, difference Fourier sections were calculated with the use of the phases for the native deoxyhemoglobin structure (8, 14). These maps show seven major binding sites per hemoglobin molecule. Six of these sites occur in pairs related by a noncrystallographic diad axis that relates the two α and the two β chains. The six sites reduce, therefore, to three unique sites, one of which occurs in each of the two α chains, one in the β chains, and one in the interface between the α and β chains. The seventh—now the fourth—unique binding site occurs, however, only in the β_2 chain. This asymmetry is probably caused by the difference in the crystallographic environment of this site that does not allow the molecular rearrangement in β_1 necessary for the binding of DCM. This site, as shown in Fig. 3, lies between a pyrrole ring of heme β_2, histidine $62\beta_2$, and phenylalanine $42\beta_2$. The difference map indicates considerable distortion of the chain near this site with a number of side chain rearrangements, but detailed tabulation of the rearrangements is not possible at this resolution. Characteristics of the binding sites are listed in Table 1.

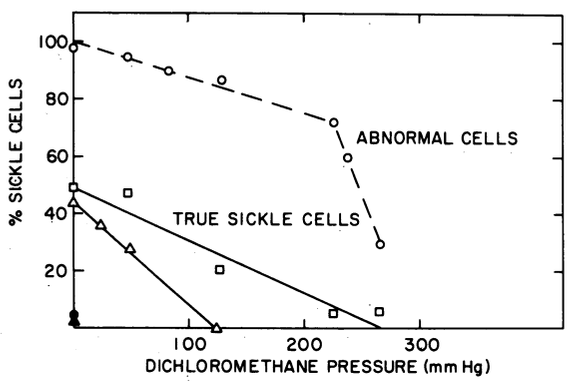

FIG. 1. Prevention of sickling by dichloromethane. The graph shows the percent of observed sickle cells as a function of dichloromethane pressure. Data are presented for "true sickle cells," cells that exhibit the characteristic crescent shape. Considerable sample differences for the "true sickle cell" counts have been observed, and data for the best and worst case are presented. The curve marked abnormal cells includes all cells that are deformed, i.e., all cells that do not exhibit the regular smooth biconcave shape. Abnormal cells do not show the wide variations between samples as observed for "true sickle cells."

Discussion

The observed site D1 dislocates Trp 14α pushing it further outward (Fig. 4a) and changing the surface configuration in the steplike region bounded by the A, B, and E helices of the α chain (see Fig. 5 for hemoglobin notation). That this site is probably responsible for the antisickling effect on DCM is suggested by experiments with amino acid substitutions known to affect sickling (15–17). Of particular interest are the substitutions of Glu 23α (B4) and Asp 73β (E17) by glutamine; both decrease sickling. Benesch *et al.* (17) showed that, apart from the A, B, and E regions of α chains, the CD corner of α chains is also involved in sickling. The DCM binding (site D2) below valine 137β (H15) replaces a water molecule, and is not close to any site known to affect Hb S formation and is not likely to affect molecular function (Fig. 4b). The DCM binding (site D3) near Tyr 145β in the $\alpha_1\beta_2$ contact region (Fig. 4c) does not seem to have any direct effect on the sickle cell phenomenon but may affect the allosteric equilibrium, since the hydrogen bond of the OH of Tyr 145β to the carbonyl of the Val 98β stabilizes the quaternary deoxy structure.

The DCM binding (site D4) between a pyrrole group of heme β_2 and Phe 42β (Fig. 4d) is not likely to be involved in the antisickling effect, although Wishner *et al.* (18) did show that at least in crystals valine (6β) interacts with the β heme group

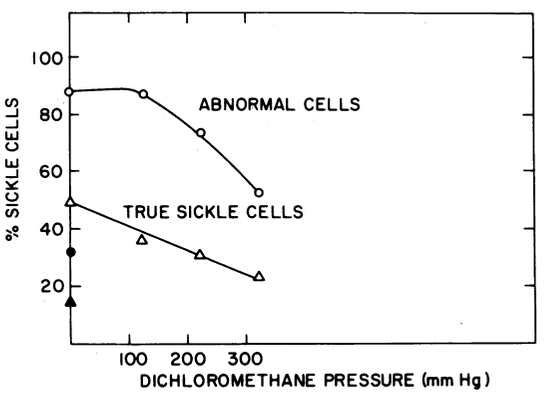

FIG. 2. Reversal of sickled cells to normal cells as a function of dichloromethane pressure. Cell counts for the control measured before and after the experiments were the same (●, ▲); the controls were treated identically except that air replaced the gas mixtures, i.e., the cells were not deoxygenated.

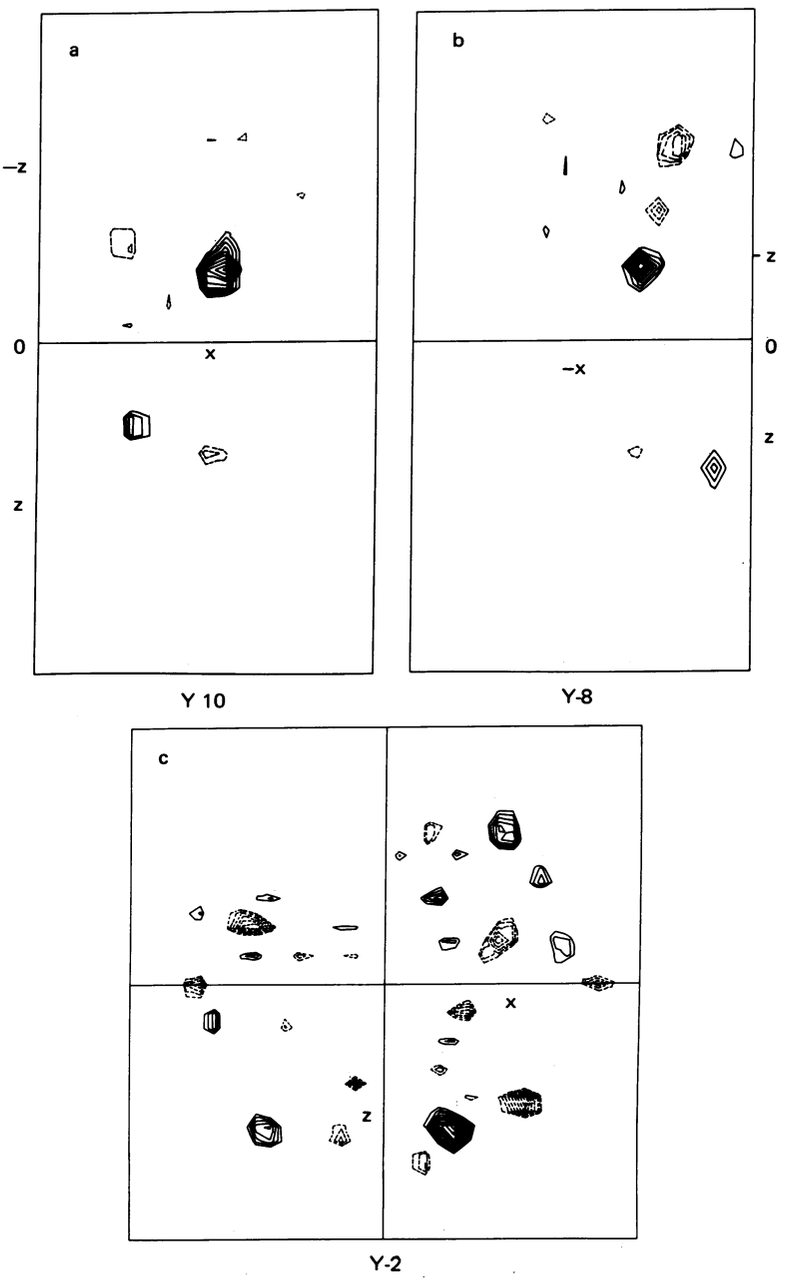

FIG. 3. X-ray difference map between human deoxyhemoglobin and its DCM derivative. Positive densities are solid; negative densities are dashed contours. (a) Depicting the largest observed peak (site D1) lying just below Trp 14α. This map represents the averaged densities for the molecular symmetrical points (noncrystallographic symmetry); the observed difference peak occurs in both symmetry related α chains. (b) Depicting the second DCM site at a water molecule location just below Val 137β. These observed difference peaks are again averaged densities occurring in both β chains. (c) DCM site D4 showing a nonaveraged section depicting the binding of DCM to only one β chain. This map also depicts minor sites and effects of molecular rearrangements.

through contacts with Phe (85β) and Leu (88β). Stereochemical considerations show, however, that site D4 does not directly interfere with the contacts described by Wishner *et al.* (18). From the described experiments it is, however, not possible to assess the small long-range effects caused by DCM binding. Only direct stereochemical interference effects have been considered.

Site D4 could however be responsible for the observed increase in CO affinity of DCM hemoglobin. Note that binding of xenon, cyclopropane, and DCM to myoglobin also involves a pyrrole group and influences the binding of CO. The observed binding of DCM (site D4) only to the β_2 chain is most likely due to the difference in crystallographic environment between the β_1 and β_2 chains because the molecular diad relating the chains is not a crystallographic diad. Settle (19) showed in spectroscopic studies on the effects of DCM on CO hemoglobin in equilibrium, that DCM increases CO binding. Milosz and Settle (20) also showed that addition of DCM to a Hb S solution causes a decrease in absorbance at 504 nm (an isobestic point for hemoglobin) which indicates decreased turbidity, i.e., a decrease in Hb S aggregation.

Prevention and reversal of Hb S aggregation was also observed with propane, ethane, and methane by Murayama (21). These gases have a compact shape and a polarizability similar to that of xenon, cyclopropane, and DCM. Studies of their binding to Hb have not yet been done but it was shown that

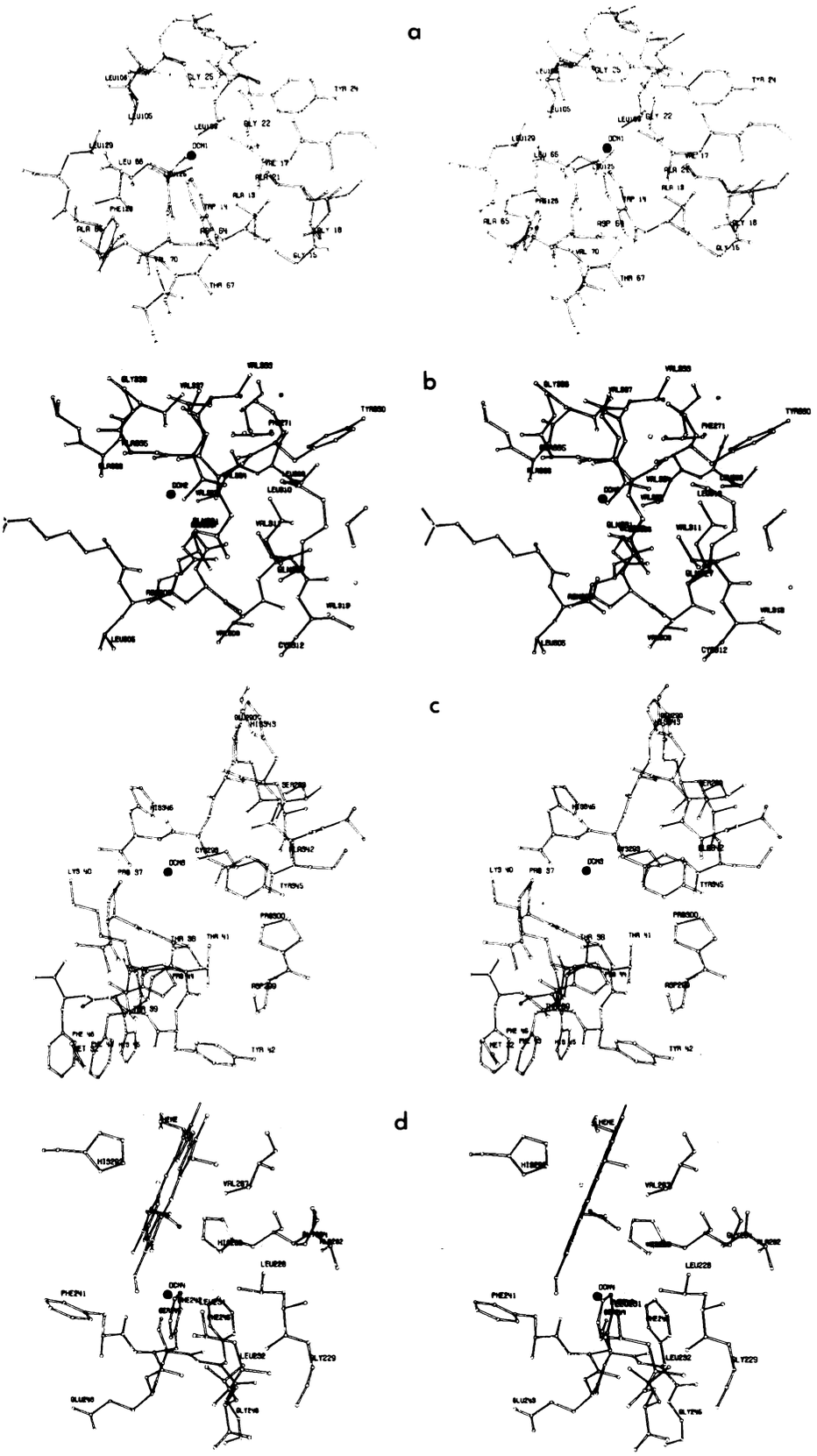

FIG. 4. Stereoscopic drawings of excerpts of the human deoxyhemoglobin structure drawn from atomic coordinates provided by Fermi (14). The amino acid name and sequence number is indicated with numbers <200 denoting α chain residues, and for numbers >200 denoting β chain residues. (a) Dichloromethane binding site D1 indicated as solid sphere. (b) Dichloromethane binding site D2 indicated as solid sphere. (c) Dichloromethane binding site D3 indicated as solid sphere on surface of molecule. (d) Dichloromethane binding site D4 indicated as solid sphere.

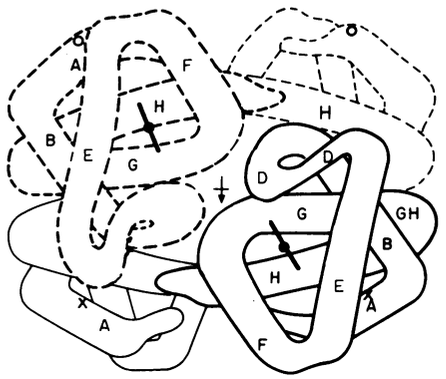

FIG. 5. Schematic drawing of the deoxyhemoglobin structure after Fermi (14). α_1 chain solid heavy outline; α_2 chain solid light outline; β_1 chain dashed light outline; β_2 chain dashed heavy outline. The molecular diad is vertical (arrow). The dichloromethane binding sites D1 (near Trp 14α) are indicated as crosses; the sickle cell mutant sites (6β) as open circles. Helices are indicated by capital letters, and interhelical regions are indicated by double capital letters taken from adjacent helices.

ethane does not bind to crystalline myoglobin at pressures up to three atmospheres (3.03×10^5 Pa).

Finch *et al.* and others (9–11) showed that deoxy Hb S aggregates into long straight rods with each rod made up of long six or eight monofilaments which are wound around the tubular surface with a helical pitch of about 3000 Å. They further showed that each filament is a string of single hemoglobin molecules linked end to end at intervals of about 64 Å. Because such monofilaments are also observed in normal deoxyhemoglobin, Finch *et al.* suggested that the sickling effect is caused by the stabilization of the fibers into rods. This suggestion is supported by a recent crystallographic analysis of Hb S by Wishner *et al.* (18) that shows molecules arranged in pairs of interlocking strands aligned with the *a* axis. The sickle cell causing amino acid substitution Val 6β is involved in these intermolecular contacts. It is, however, not yet possible to consolidate all these findings with a basic molecular interaction mechanism that explains why the amino acid substitution Glu to Val at position 6β causes sickle cell anemia. These findings do however suggest that the prevention and reversal of sickle cell formation by DCM is caused by the binding of DCM close to Trp 14α (A12) (site D1) which prevents the hemoglobin fibers from aggregating into regular helical rods. Studies of the binding of xenon and cyclopropane to methemoglobin and to myoglobin suggest that these gases also prevent sickling but that considerably higher gas pressures are necessary to effect high binding occupancy. While direct clinical application of these results requires further in depth studies, particularly for long-term toxicological effects, some possible uses of dichloromethane and similar gases in the treatment of sickle cell anemia come readily to mind. Because the agents are all anesthetics and exhibit no serious toxic side effect, it is very likely that an effective dose is easily achieved by the use of these gases as inhalents. The effective gas pressure needed to prevent and revert sickle cell formation is well within achievable limits.

While long-term administration of such gases to prevent sickling is unlikely they could be used to treat sickle cell crisis. The effectiveness of such treatment will depend on the exact "gas" composition and duration of gas administration, parameters that only *in vivo* experiments can establish. Apart from the use of dichloromethane and possibly cyclopropane, other gases with similar molecular properties and low toxicity should be considered as potential sickle cell preventives.

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