DICHLOROMETHANE AS AN ANTISICKLING AGENT IN SICKLE CELL HEMOGLOBIN

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Introduction

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 (1,2). In methemoglobin (3), 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.

In previous crystallographic experiments (4), the DCM binding to deoxyhemoglobin was established at a DCM^{*} pressure of 350 mm Hg. In that analysis four different DCM sites were observed (Table 1) and arguments were presented that site D1 which dislocated Trp 14 α is responsible for the observed antisickling effects (4) (Fig. 1 and 2). DCM at site D2 replaces a water molecule and is not thought to have any effect on the sickling effect or on hemoglobin function. Binding site D3 in the $\alpha_1\beta_2$ contact region does not seem to have any direct effect on sickling phenomena but may affect the allosteric equilibrium since this region stabilizes the quaternary deoxy structure. Site D4 located between a heme pyrrole group (β_2) and phenylalanine (42 β_2) is asymmetric, it occurs only in the β_2 chain and causes considerable

^{*} dichloromethane = DCM

molecular reorganization or crystal disorder. In order to evaluate that finding, the x-ray diffraction study was repeated at half the previous DCM pressure. As a first step in the investigation of DCM effects on the functional properties of hemoglobin by site D3, oxygenation studies in the presence of varying partial pressures of DCM were undertaken.

Materials and Methods

Crystallographic Binding Studies: Deoxyhemoglobin crystals grown from ammonium sulfate 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. Diffraction intensities were collected to a resolution 4.8 Å on an automated four-circle diffractometer that permitted thermal regulation of the crystal at 4°C with a resultant DCM pressure of 172 mm Hg. After the usual corrections, difference Fourier sections were calculated with the use of the phases for the native deoxyhemoglobin structure (5,6).

Hemoglobin Oxygenation Curves: Fresh heparinized blood was obtained from normal donors. After washing the blood four times with 0.9% sodium chloride at 4°C, the red blood cells were hemolyzed by shaking with a volume of water and 0.4 volume of toluene for 3 min. A clear hemoglobin solution was obtained by centrifuging at 117,000 x g for 30 min.

The oxygen binding experiments were carried out in tonometers similar to the design used by Benesch et al. (7) which permitted

spectrophotometric examination of the hemoglobin solution. Hemoglobin was deoxygenated by passing a stream of 90% N_2 and 10% CO_2 over the solution at $0^\circ C$ with gentle stirring. The deoxygenated hemoglobin was anaerobically transferred to the tonometers which contained degassed buffer and either nitrogen or nitrogen and dichloromethane vapor. The spectrum was recorded to assure complete deoxygenation and absence of methemoglobin.

The absorbance was measured at 541, 560 and 576 nm using a GCA-McPherson spectrophotometer. The temperature of the solution was maintained at $20^\circ C$. After each addition of a measured quantity of air to the tonometer and thorough equilibration, the absorbance was again recorded. The percent oxygenation and the oxygen pressure in the tonometer were calculated by the method of Benesch et al. (7).

Results

The difference Fourier maps based on data with DCM at 172 mm Hg show little change at sites D1 and D2 but show a decrease of the DCM occupancy at site D3 and an absence of DCM at site D4 (Fig. 3 to 5). The region around site D4 is now quite clear and shows no evidence of any crystal disorder or molecular rearrangements as was observed in the case with DCM at twice this pressure.

The effect of dichloromethane on the oxygen affinity of hemoglobin can be seen in Fig. 6, which shows the binding curves for untreated hemoglobin as well as for hemoglobin in the presence of dichloromethane, at several partial pressures. With increasing partial pressure of DCM the binding curve shifts to the right, indicating a decrease in oxygen

affinity of the hemoglobin. Under the conditions of these experiments, no further shift is observed when the dichloromethane pressure is increased from 50 to 100 mm Hg, indicating that saturation of the hemoglobin at the responsible dichloromethane sites has occurred at the lower pressure. Up to this pressure, the lowering of the oxygen affinity as indicated by the increase in the P_{50} appears to be proportional to the partial pressure of the dichloromethane (Fig. 7).

Discussion

These observations show that DCM does have a significant effect on the oxygen binding properties of hemoglobin. At DCM pressures high enough to prevent or reverse sickling (4), DCM would lower the oxygen affinity of hemoglobin, therefore reducing oxygen transport at low oxygen pressure. This decrease in oxygen affinity might, however, increase the oxygen availability to tissue as long as a sufficiently large lung P_{O_2} is maintained.

The crystallographic studies show that site D4 has a much lower affinity for DCM than site D3 while sites D1 and D2 show a higher affinity.

Site D1 results in an altered configuration of the protein surface in the step-like region bounded by the A, B and E helices of the α chains (see Fig. 8 for hemoglobin notation) and as previously described (4) is probably responsible for the antisickling effect of DCM as suggested by experiments with amino acid substitutions known to affect sickling. Of particular interest are the substitutions of Glu 23 α (B4) and

Asp 73 β (E17) by glutamine; both decrease sickling. Benesch et al. (10) showed that, apart from the A, B and E regions of α chains, the CD corner of the α chain is also involved in sickling. The DCM binding site D2 below valine 137 β (H15) replaces a water molecule, and is not close to any site known to affect Hb S formation. The observation that the oxyhemoglobin P_{50} does not change further after a DCM pressure of 50 mm Hg suggests at first instance that site D3 might not be involved in the oxygen binding mechanisms, since at DCM pressures below 50 mm Hg, no binding of DCM at that site is expected, judging from the crystals data. From the extensive studies of Perutz (11), the above results seem surprising, since site D3 in the $\alpha_1\beta_2$ contact region would be expected to have an effect on the oxygen affinity. Low oxygen affinity has been observed for Hb Kansas (12,13) in which the replacement of Asn 102 β (G4) by threonine prevents the formation of a hydrogen bond across the $\alpha_1\beta_2$ interface in oxy Hb. The binding properties of DCM to hemoglobin might be different in solution and in crystalline form so that in solution, binding of DCM to site D3 may occur at ^{lower} partial pressures. In order to clarify these observations, the number of DCM sites involved in the oxygen effect will be established from more accurate hemoglobin oxygenation studies as well as binding studies of DCM to oxyhemoglobin in solution. To elucidate the involvement of site D1 in the sickle cell phenomena neutron scattering studies (14) on oriented sickle cell fibers (15) exposed to deuterated DCM will attempt to establish the intermolecular interaction of such helical fibers (16,17,18) in relation to that site. It is also planned to study the effect of DCM on the function of the red cell membrane, since in prolonged exposures of whole blood to DCM, cells were observed to lyse.

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Table 1. Dichloromethane binding sites to deoxyhemoglobin

Site	Symmetry related occupancy for $\rho_{\text{DCM}} = 350 \text{ mm Hg}$		Chain	Coordinates			Helix	Interacting Amino Acids
	x, y, z	$-x, y, -z$		X	Y	Z		
D1	0.9	1.0	α_1, α_2	19 Å	10 Å	- 8 Å	A	Trp 14 α (A12), Gly 15 α (A13), Val 17 α (A15)
							G	Leu 109 α (G16), Thr 108 α (G15), Leu 105 α (G12)
							E	Ala 63 α (E12), Leu 66 α (E15)
							H	Phen 128 α (H11)
D2	0.5	0.7	β_1, β_2	11 Å	- 8 Å	8 Å	G	Arg 104 β (G6), Leu 110 β (G12), Leu 106 β (G8)
							H	Val 134 β (H11), Val 137 β (H14)
							E	Phen 71 β (E15)
D3	0.7	0.5	α_1, β_2 Contact	5 Å	-10 Å	-14 Å	H	Tyr 145 β (H22), His 146 β (H23)
							F	Cyh 93 β (F9)
							C	Thr 38 α (C3), Lys 40 α (C5), Thr 41 α (C6)
D4	1.0	1.0	β_2	10 Å	- 2 Å	20 Å	E + Op	Heme pyrrole His 62 β (E6), Phe 42 β (CD1)

Figure Legends

Fig. 1. Prevention of sickling by dichloromethane (4). The graph shows the percent of observed sickle cells as a function of dichloromethane pressure. Data is presented for "true sickle cells", cells that exhibit the characteristic crescent shape. The curve marked abnormal cells includes all cells that are deformed, i.e. all cells that do not exhibit the regular smooth biconcave shape.

Fig. 2. Reversal of sickled cells to normal cells as a function of dichloromethane pressure (4).

Fig. 3. X ray, not averaged difference maps between human deoxy Hb and its DCM derivative at a partial pressure of 132 mm Hg. Positive densities are solid, negative densities are dashed contours. This map section at $y = 10 \text{ \AA}$ depicts the largest observed peak with site D1 lying just below Trp 14 α .

Fig. 4. X ray, not averaged difference maps between human deoxy Hb and its DCM derivative at a partial pressure of 132 mm Hg. Positive densities are solid, negative densities are dashed contours. This map section at $y = 9 \text{ \AA}$ shows sites D2 and D3.

Fig. 5. X ray, not averaged difference maps between human deoxy Hb and its DCM derivative at a partial pressure of 132 mm Hg. Positive densities are solid, negative densities are dashed contours. This map section at $y = -2 \text{ \AA}$ shows the region of binding site D4 which is unoccupied at this DCM pressure of 132 mm Hg.

Fig. 6. Effect of dichloromethane on the oxygenation curve of human hemoglobin. Hemoglobin concentration, $2 \text{ to } 3 \times 10^{-4} \text{ M}$ as Fe equivalents in 0.01 M NaCl and 20°C . O, untreated hemoglobin; +, dichloromethane pressure 10 mm Hg ; $\square$, dichloromethane pressure 23 mm Hg ; Δ , dichloromethane pressure 50 mm Hg ; X, dichloromethane pressure 100 mm Hg .

Fig. 7. Effect of dichloromethane on the P_{50} of human oxyhemoglobin. Conditions same as in Fig. 6.

Fig. 8. Schematic drawing of the deoxyhemoglobin structure after Fermi (6). 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 14 α) are indicated as crosses; the sickle cell mutant sites (6 β) as open circles. Helices are indicated by capital letters, interhelical regions are indicated by double capital letters taken from adjacent helices.

Fig. 1
8-216-76

Fig. 2
8-217-76

HE BECKY DUNSD 101 100 100 100 NOT AVERAGED 4.34
HEBECKY DUNSD 100 100 100 100 NOT AVERAGED 4.34

Fig. 3

Fig 4

PL. DENSITY DENSITY OUT 183 IN 50 NOT AVERAGED 4.99

W. NUMBER 50 NOT OVER 1.00

EFFECT OF DICHLOROMETHANE ON THE OXYGENATION CURVE OF HEMOGLOBIN

Fig. 6
9-1279-77

EFFECT OF DICHLOROMETHANE ON THE P_{50} OF
HUMAN HEMOGLOBIN

Fig. 7
9-1282-77