MASTER

WEAK BINDING GASES AS MODULATORS OF HEMOGLOBIN FUNCTION

Benno P. Schoenborn, Anand Saxena and Barry E. North

DISCLAIMER

This book was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

B I O L O G Y D E P A R T M E N T
B R O O K H A V E N N A T I O N A L L A B O R A T O R Y
U P T O N , N E W Y O R K 11973

Research carried out at Brookhaven National Laboratory under the
auspices of the United States Department of Energy.

so
DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

The remarkable phenomenon of anesthesia caused by weakly binding gases has given rise to much interest and speculation because of its physiological significance and because of the challenge of its physical-biochemical interpretation.

Several observations and ideas on possible mechanisms of anesthesia have been presented based on correlations between the anesthetic potency of agents and some of their physical properties such as solubility, polarizability, refractivity, etc.⁽¹⁾ These properties are all intimately interrelated and depend on atomic structure. The so-called inert gaseous agents, such as xenon, cyclopropane, dichloromethane, butene, etc., exert their biological effect without undergoing any change in their chemical structure. It takes little insight to realize that these molecules must interact with some constituents of the body and that the interaction must occur through weak binding forces which leave the agent chemically unaltered.⁽²⁾ Such binding was demonstrated by the x-ray crystallographic analysis of the complexes formed by hemoglobin and myoglobin with xenon, cyclopropane, and dichloromethane.⁽³⁻⁶⁾ These studies showed that the gases bind in interior pockets shielding non-polar areas from polar or even charged groups. In the first study showing the specific binding of an inert gas to proteins ⁽³⁾, the xenon in the xenon-myoglobin complex was found at a location nearly equidistant from the heme linked histidine and from a pyrrole ring of the heme as well as several other groups like phenylalanine, leucine, valine, etc. This xenon site in the interior of the

myoglobin molecule shows the Debye and London types of van der Waals bonds as well as a small contribution from an ion-induced dipole bond with a total binding energy of ~ 10 kcal. The London interactions, which account for $\sim 90\%$ of the total binding energy are particularly strong because the xenon rests in a nearly closed cavity with 32 neighboring atoms less than $5.5 \text{ \AA}$ away. This observation suggested that a number of gases with similar physical properties should bind to enzymes and proteins, since numerous crystallographic investigations have demonstrated that clefts are common features in proteins. Since clefts are often associated with substrate binding sites, it can be predicted that such gas binding should cause changes in many reactions mediated by enzymes, lipoproteins, or lipids. Extensions of this xenon-myoglobin binding study showed that cyclopropane binds at the same site in myoglobin, and that xenon also binds to hemoglobin but at quite a different location. Xenon was found to bind to four sites in met hemoglobin with one site on α chains and one site on β chains.⁽⁵⁾ The α chain site is located in the GH corner close to, but not on, the surface, and the site on the β chain is in the AB corner. Both sites are lined mainly by non-polar groups. Further studies showed that dichloromethane (methylene chloride) does bind to myoglobin and to hemoglobin.^(6,7) Since the xenon and the DCM sites in hemoglobin are close to $\beta 6$, the amino acid responsible for the aggregation of HbS,⁽⁸⁾ it was speculated that structural changes induced in those regions by xenon or DCM might prevent the aggregation of HbS.

Microscopic studies of red cells from homozygous sickle cell patients showed that DCM does prevent sickle cell formation in vitro and does cause reversion of sickled cells to normal after exposure to DCM.⁽⁷⁾ These studies also showed a possible effect of DCM on the red cell membranes which was confirmed by Matthews and James,⁽⁹⁾ who demonstrated that DCM is absorbed by the cell membrane and preferentially lyses sickled cells in addition to inhibiting of hemoglobin S gelation.

The crystallographic study of DCM binding to deoxy Hb showed that DCM is bound to four different sites (Table 1). None of these sites, however, is close to any of the primary contact regions elucidated for the HbS crystal by Wishner et al.⁽¹⁰⁾ Site D1 (Fig 1a) was suggested as the antisickling site since it dislocates Trp 14 α , pushing it further outward and changing the surface configuration in the steplike region bounded by the A, B, and E helices of α chains. That this site is probably responsible for the antisickling effect of DCM is suggested by experiments with amino acid substitutions known to affect sickling.⁽¹¹⁻¹³⁾ Of particular interest are the substitutions of Glu 23 α (B4) and ASP 73 β (E17) by glutamine; both decrease sickling. Benesch et al.⁽¹³⁾ showed that apart from the A, B, and E regions of α chains, the CD corner of α chains is also involved in sickling. This site D1 lies close to the secondary interactive site described by Edelstein in his analysis of multistranded HbS fibers.⁽¹⁴⁾

Another interesting feature of this binding study was suggested by site D3 (Fig 16) near Tyr 145 β in the $\alpha_1 \beta_2$ contact region. Since

this region stabilizes the deoxy structure or, in allosteric terms, the T structure,⁽¹⁵⁾ binding of DCM should affect the oxygen affinity. Subsequent oxygen binding experiments⁽¹⁶⁾ carried out by a tonometric technique (Fig 2) showed that the P_{50} increases from 10 Torr of O_2 to 25 Torr as a function of DCM pressure (Fig 3). Experiments with and without DPG 2,3-diphosphoglycerate showed that the DCM effect is independent of DPG. Although stripped hemoglobin (no DPG) has a higher O_2 affinity than hemoglobin containing DPG, the effect of DCM is similar for both. The maximum effect is recorded at a DCM pressure of 75 Torr, in both cases, and DCM increases the P_{50} by the same fraction, as seen from the plot of P_{50} vs P_{DCM} (Fig 3).

The oxygen equilibrium curves as a function of DCM were further analyzed in terms of the Adair constants, which are the macroscopic association constants for successive steps in the oxygen binding reaction.^(17,18) If the earlier speculation that DCM affects the allosteric equilibrium by favoring the T or deoxy form is correct, then the addition of further bonds between the α and β subunits should leave the Adair constants A_1 and A_4 unchanged while affecting either A_2 or A_3 , or both. According to the theory of ligand binding, the fractional oxygen binding y can be written as a function of the Adair constants:

$$y = \frac{(A_1 p + 2A_1 A_2 p^2 + 3A_1 A_2 A_3 p^3 + 4A_1 A_2 A_3 A_4 p^4)}{4(1 + A_1 p + A_1 A_2 p^2 + A_1 A_2 A_3 p^3 + A_1 A_2 A_3 A_4 p^4)}$$

where A_1 is the equilibrium constant for the first O_2 to be bound, A_2 is that for the second O_2 , etc.

Since heme-heme (allosteric) interactions occur only for the second and third O₂ bound, analysis of these constants will determine the type of effect DCM has on the hemoglobin function. For this case, Adair constants are fitted to the observed O₂ binding curve by a least-squares procedure linked to a gradient search method, a mathematical technique described by Marquardt^(19,20) that was adapted to the analysis of hemoglobin oxygen curves.⁽²¹⁾ The conventional least-squares refinement techniques are unsatisfactory because convergence to the true values occurs only if the initially assumed Adair constants are close to their true values. The gradient search method produces approximate values for the Adair constants which are then further refined by a least-squares technique. Model calculations showed that this technique is useful for calculating Adair constants.

Refinement of the Adair constants with the oxygen equilibrium data for stripped hemoglobin as a function of DCM showed that A₁ and A₄ do not vary significantly but that A₂ and A₃ are strongly influenced by the binding of DCM. It was further noted that A₂ and A₃ can be varied for a given DCM pressure as long as their product is constant, with $A_2 \times A_3 = 0.018$ for DCM = 0 Torr, to 0.0006 for DCM = 75 Torr (Table 2). These observations suggest that the transition from the T to the R state, which most likely occurs when the 2nd and 3rd O₂ are bound, is hindered. The crystallographic binding observations suggest the same since the addition of DCM at site D3 increases the number of bonds between the subunits ($\alpha_1\beta_2$); this,

according to allosteric theory,⁽²²⁾ increases the fraction of molecules in the T state, which would decrease the oxygen affinity.

The presence of DCM therefore has, two opposite effects on gelation: the binding of DCM at site D1 interferes with fiber formation and decreases gelation, whereas binding at site D3 decreases the oxygen affinity, which, under low oxygen tension, increases gelation.

It is possible that gases with similar physical properties might decrease gelation even more without negatively affecting the oxygen affinity. The inhalation anesthetics, aliphatic hydrocarbons are particularly good candidates for possible antisickling agents. A recent study by Poyart⁽²³⁾ has shown that butene behaves very much like DCM and decreases the P_{50} by 45% without altering the cooperativity of ligand binding. It is likely that butene will also decrease HbS gelation and will bind to red cell membranes, since such gases do bind to lipid bilayers⁽²⁴⁾.

1. R. M. Featherstone and C. A. Muehlbacher (1963) Pharmacol. Rev. 15, 97-112.
2. B. P. Schoenborn and R. M. Featherstone (1967) Advan. Pharmacol. 5, 1-18
3. B. P. Schoenborn, M. C. Watson, and T. C. Kendrew (1965) Nature 207, 28-30
4. B. P. Schoenborn (1967) Nature 214, 1120-1122
5. B. P. Schoenborn (1965) Nature 208, 760-762
6. A. C. Nunes and B. P. Schoenborn (1973) Mol. Pharmacol. 9, 835-839
7. B. P. Schoenborn (1976) Proc. Natl. Acad. Sci. USA 73, 4195-4199
8. M. Murayama (1966) J. Cell. Physiol. Suppl. i, 67, 21-31
9. R. M. Matthews and T. L. James (1978) Biochem. Biophys. Res. Comm. 80, 879-885
10. B. C. Wishner, K. B. Ward, E. E. Lathman, and W. E. Love (1975) J. Mol. Biol. 98, 179-194
11. R. Bookchin, R. Nagel, and H. Ranney (1970) Biochim. Biophys. Acta 221, 373-375
12. H. Ranney (1972) Biochimie 54, 633-637
13. R. E. Benesch, S. Young, R. Benesch, and J. Mack (1976) Nature 260, 219-221
14. S. Edelstein, These proceedings
15. M. F. Perutz, Scientific American May 1978, 92-125

16. B.P. Schoenborn and B. E. North (1978) Biochemical and Clinical Aspects of Hemoglobin Abnormalities, Academic Press, New York
17. G. S. Adair (1925) J. Biol. Chem. 63, 529-545.
18. J. M. Baldwin (1975) Prog. Biophys. Mol. Biol. 29, 225-320.
19. P. R. Bevington (1969) Data Reduction and Error Analysis McGraw-Hill, New York pp 215-222
20. D. W. Marquardt (1963) J. Soc. Ind. Appl. Math. 11, 431-441
21. A. Saxena, B. E. North, and B. P. Schoenborn (1979) Eur. J. Biochem., submitted
22. J. Monod, T. Wyman, and T. P. Changeux (1965) J. Mol. Biol. 12, 88-118
23. C. Poyart, E. Bursaux, P. Guesnon and B. Bohn (1979) Eur. J. Biochem. 99, 85-88
24. T. M. Vanderkooi, R. Landesberg, H. Selig and G. G. McDonald (1977) Biochim. Biophys. Acta 464, 1-16
25. G. Fermi (1975) J. Mol. Biol. 97, 237-256

TABLE I. Dichloromethane Binding Sites to Deoxyhemoglobin

Site	Chain	Coordinates			Helix	Amino Acids surrounding the DCM site
		X	Y	Z		
D1	$\alpha 1, \alpha 2$	19 Å	10 Å	- 8 Å	A	Trp 14 α (A12), Gly 15 α (A13), Val 17 α (A15)
					G	Lew 109 α (G16), Thr 108 α (G15), Lew 105 α (G12)
					E	Ala 63 α (E12), Leu 66 α (E15)
					H	Phen 128 α (H11)
D2	$\beta 1, \beta 2$	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	$\alpha 1, \beta 2$ 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* $\beta 2$		10 Å	- 2 Å	20 Å		Heme pyrrole
					E	His 62 β (E6),
					CD	Phe 42 β (CD1)

*This site is not occupied at DCM pressures below 300 mm Hg.

TABLE 2
Product of Adair constants $A(2) * A(3)$
as a Function of DCM Pressure

DCM Pressure	$A(2) * A(3)$
0.0	0.02772
10.0	0.00250
25.0	0.00135
50.0	0.00075
75.0	0.00056

Figure Legends

Fig. 1. Stereoscopic computer drawings of excerpts of the human deoxyhemoglobin structure drawn from atomic coordinates provided by Fermi (25). The amino acid name and sequence number is indicated with numbers; <200 denoting α chain residues, >200 denoting β chain residues eg. PRO 300 represent Proline β 100.

(a) DCM binding site D1

(b) DCM binding site D3

Fig. 2. Effect of DCM on the oxygenation curve of hemoglobin. Solid curves calculated from Adair constants but were derived from the observed data.

Fig. 3. Effect of DCM on the P50 of human hemoglobin. Unstripped hemoglobin $5 \cdot 10^{-5}$ M in 0.1 M phosphate buffer at pH 7.0. Stripped hemoglobin $5 \cdot 10^{-5}$ M in 0.1 M Tris-HCl buffer at pH 7.4.

OXYGENATION DATA - STRIPPED HEMOGLOBIN

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