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Substrate Specificity at the Alkane Binding Sites of Hemoglobin and Myoglobin*

Arnold Wishnia

ABSTRACT: Binding of the ligands (L) iodobutane, pentane, neopentane, butane, and xenon, to ferrihemoglobin and ferrimyoglobin, and of pentane to oxy- and deoxyhemo- and myoglobin (H is one subunit), at pH 7, 0°, is accurately described by two equilibria, $HL = H + L$, $HL_2 = HL + L$, with dissociation constants K_1 and K_2, as found earlier for β-lactoglobulin. Apomyoglobin requires three. The constants vary (*e.g.*, for pentane and β-lactoglobulin, apomyoglobin, oxyhemoglobin, oxymyoglobin, K_1 = 0.12, 0.28, 0.34, 0.8, all $\times 10^{-3}$ M). K_2 may approach $4K_1$ (*e.g.*, xenon and butane) or may be very large (*e.g.*, ferrihemoglobin pentane, and iodobutane; ferrimyoglobin and iodobutane). The ratios of K_1 for different ligands and a given protein roughly approximate the ratios calculated from partition between water and dodecyl sulfate micelles (pentane:neopentane:iodobutane:butane:xenon = 1:1.7:2:3.8:120). An exception is neopentane, which binds moderately well to ferrihemoglobin, but is almost excluded from β-lactoglobulin and ferrimyoglobin. The xenon constants fall in the expected range. In both ferrihemoglobin and ferrimyoglobin, pentane and xenon compete for the same two interacting sites. It is concluded that each protein has a single, localized, but not strictly discrete, hydrophobic binding region (*e.g.*, a circle has localized but continuous binding sites for two semicircles), which probably correspond to the xenon loci of ferrihemoglobin and ferrimyoglobin described by Schoenborn.

In early studies of alkane binding to hemoglobin (Wishnia, 1962) there appeared to be differences in the affinity of sodium dodecyl sulfate, bovine serum albumin, and Hb[1] for butane and propane suggestive of a size limitation, if not discrete binding, in Hb. In subseqeunt work the binding region of bovine serum albumin was shown to be large (Wishnia and Pinder, 1964), while βLG proved to have a small discrete site (Wishnia, 1964; Wishnia and Pinder, 1966a). When improvements in the technique promised both feasibility and precision, the study of the functional derivatives of hemoglobin (Hb, HbO_2, and Hb^+) was taken up again, in the hope that one of two possibilities would occur: (1) that binding would be to the interfaces between subunits as in bovine serum albumin, and would therefore be very sensitive to the state of the heme (Perutz *et al.*, 1968), even if a bit nonspecific with respect to alkane; the Mb derivatives would then serve as null binding controls. (2) Binding to Hb and Mb would occur at the same site in the structurally similar molecules—the Mb derivatives would be controls for the existence or nonexistence of interaction between subunits somewhat as βLGA monomer was a standard for βLGA dimer and octamer, and estimates of the strength and range of the internal stresses that accompany oxygenation could be made.

When binding proved to be discrete, a series of ligands,

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[1] Abbreviations used are: βLG, β-lactoglobulin; Hb, deoxyhemoglobin; Hb^+, ferrihemoglobin; Hb^+CN^-, ferrihemoglobin cyanide; HbO_2, oxyhemoglobin; Mb, deoxymyoglobin; Mb^+, ferrimyoglobin; Mb^+CN^-, ferrimyoglobin cyanide; MbO_2, oxymyoglobin; ApoMb, apomyoglobin.

iodobutane, pentane, neopentane, and butane, were studied, to describe the capacity and adaptability of the site or sites, as had been done with βLG. When Mb^+ and Hb^+ were shown to have fixed, but different, binding sites for xenon (respectively, hydrophobic pockets on the distal side of the heme and between the AB and GH corners of each subunit (Schoenborn *et al.*, 1965; Schoenborn, 1965)), the interaction of xenon and pentane binding was investigated, since I was convinced that xenon was a typical hydrophobe, and that the complex, unique aspects of the solution behavior of these systems might thereby be related to known structures.

Experimental Section

The provenance, purification, and characterization of the [^{3}H]alkanes and ^{133}Xe-containing xenon, and the techniques of measuring binding in protein solutions, have been described (Wishnia and Pinder, 1966a).

Protein starting materials were human oxyhemoglobin, isolated and crystallized according to Drabkin (1950) and commercial (Seravac) sperm whale ferrimyoglobin, dialyzed against water at concentrations around 15%, and centrifuged. In subsequent manipulations the protein concentrations were brought to 3–10% in 0.09 M phosphate buffer (pH 7.3). Deoxyhemoglobin (Hb) was prepared from HbO_2, as needed, by deoxygenation with wet nitrogen followed, for insurance throughout a run, by addition of equivalent amounts of $Na_2S_2O_4$ dissolved in deaerated buffer. Ferrihemoglobin (Hb^+) was prepared from HbO_2 by oxidation with a slight excess of potassium ferricyanide. Deoxymyoglobin (Mb) was prepared from Mb^+ by reduction with a twofold excess of $Na_2S_2O_4$ in deaerated buffer; oxymyoglobin (MbO_2) was prepared by exposing the Mb solution to air. By using high protein concentrations, and deaerated, well-buffered solutions, the worst difficulties attending use of dithionite were avoided. Apomyoglobin (ApoMb) was prepared from Mb^+ by extraction with 2-butanone according to Breslow (1964).

Apomyoglobin concentrations were determined by dry weight, referred to a molecular weight of 17,200. Myoglobin was determined either as Mb^+, in pH 6 phosphate–EDTA, using $\epsilon_{280} = 3.13\ cm^{-1}\ mM^{-1}$ (Breslow, 1964), or as Mb^+CN^-, in pH 7 ferricyanide–cyanide–phosphate, using the secondary value, $\epsilon_{540} = 10.2\ cm^{-1}\ mM^{-1}$. Hemoglobin was determined as Hb^+CN^-, using the value $\epsilon_{540} = 11.1\ cm^{-1}\ mequiv^{-1}$, referred to pyridine hemochromogen content (Keilin and Hartree, 1951). (The value of 11.5, customarily referred to Drabkin and Austin (1935–1936), and used by many workers (*e.g.*, Dalziel and O'Brien (1954), Benesch and Benesch (1962), and Guidotti (1967)), was actually determined in 1932, using a subjective spectrophotometer, and whole blood, and referred to oxygen binding capacity.) On this basis, the millimolar absorptivities of HbO_2, Hb, and Hb^+, to ±1%, are: HbO_2, 14.80, 8.37, and 14.00 at 576, 560, and 541 nm; Hb, 13.10 at 555 nm; and Hb^+, 8.98 and 3.77 at 500 and 630 nm, in good agreement with Drabkin (1950), and Dalziel and O'Brien (1954). (Benesch *et al.* (1965) report similar relative values for the three compounds, but much higher absolute values; these authors do not, however, give the analytical basis for their absolute scale.)

As a routine check, undiluted samples taken from the solubility cell at the beginning and end of each run were characterized by their visible spectra in short-path-length cells. For example, HbO_2 samples showed no significant conversion to Hb^+. Hb, protected by dithionite, showed no conversion to HbO_2; moreover, subsequent aeration produced the expected HbO_2 spectrum. All spectra were obtained with a Cary 14 spectrophotometer.

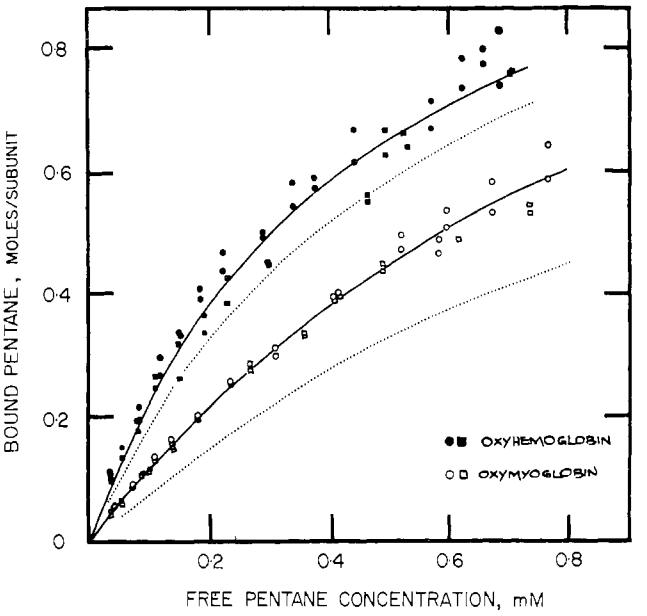

FIGURE 1: Pentane binding to hemoprotein derivatives. Complete data and calculated curves are given for HbO_2 (upper) and MbO_2. The dashed lines are calculated curves for Hb^+ and Mb^+. The deoxy data fall between, lying closer to the ferri curves at low pentane and to the oxy curves at high pentane concentrations, as is clear from the calculated constants. The number of sites does not change.

Results

The result of any given binding run is a set of pairs: R_L, moles of ligand bound per mole of protein subunits, at C_L, the free ligand concentration. Figures 1–3 show such binding isotherms, at 0°, for, respectively: Pentane and the hemoprotein derivatives; pentane and ApoMb, butane, and Hb^+ or Mb^+; and xenon and Mb^+ or Hb^+. The binding curves for iodobutane, neopentane, and Mb^+ or Hb^+, which are straightforward, are not illustrated.

Except for the butane–Mb^+ isotherm, there is an obvious curvature in each plot, which, in the usual way, must be attributed to the progressive saturation of a small number of binding sites. Dissociation constants, K_m, for the reaction $HL_m = HL_{m-1} + L$, were calculated by fitting the binding data to the functions, R_L, using a standard nonlinear least-

$$R_L = \sum_{n=1}^{N}(nC_L{}^n/\prod_{m=1}^{n}K_m)/\{1 + \sum_{n=1}^{N}(C_L{}^n/\prod_{m=1}^{n}K_m)\} \qquad (1)$$

squares algorithm (Margenau and Murphy, 1956). N was usually required to take on values of 1, 2, or 3, with no constraints on the relative values of the K_m, although other schemes were sometimes tested. The fit for $N = 2$ was always adequate, and, in fact, usually the best (see below). The computed dissociation constants are given in Tables I and II.

In the tracer method, the relative errors in R_L, rather than

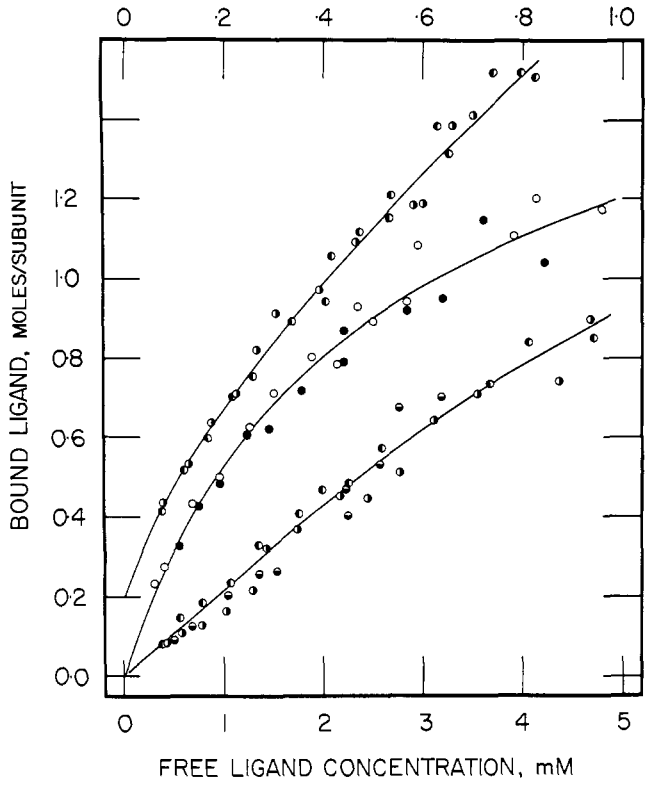

FIGURE 2: Butane and pentane binding. Mb^+ and butane, lower half-filled circles. Hb^+ and butane, filled and open circles. Free butane concentration, lower abscissa. ApoMb and pentane, upper half-filled circles (displaced upward by 0.2 mole/mole). Free pentane concentration, upper abscissa. Since the relative error is normal, the absolute errors decrease toward (0, 0), through which, as the best point, all curves must go; the observed curvature is therefore real.

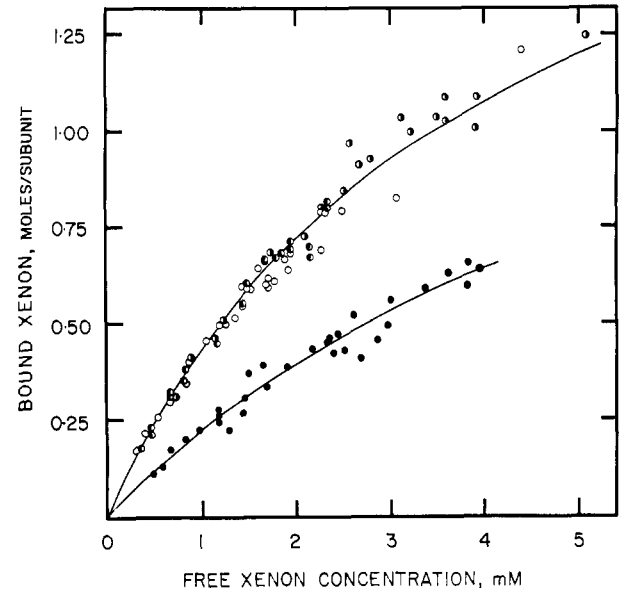

FIGURE 3: Xenon binding. Mb^+, 2 M $(NH_4)_2SO_4$, upper curve, several runs. Hb^+, 1.8 M $(NH_4)_2SO_4$, lower curve, two runs. The abscissa is xenon concentration, uncorrected for activity coefficients. The curves shown are calculated for two interacting (or nonequivalent) sites. The fits for one site are bad in the expected systematic way. As might be expected for the ratios K_2/K_1, the fits for two independent equivalent sites are rather good: *e.g.*, for Mb^+, the curve for $K_0 = 20$ mM is 4% above the curve for $K_1 = 10.6$, $K_2 = 35$ mM at the lowest binding, 2% below at the highest, and closer between.

the absolute errors, are normally distributed: that is, a 5% error in R_L is equally likely for $R_L = 0.1$ or 1.0. However, the apparent absolute errors, $R_L^{obsd} - R_L^{calcd}$, are more sensitive to systematic errors (*e.g.*, in protein concentration, specific activity of the ligand), not the least of which is an incorrect value of N. Thus, although the results reported are based on fits minimizing $\Sigma(1 - R_L^{calcd}/R_L^{obsd})^2$, the fits minimizing $\Sigma(R_L^{obsd} - R_L^{calcd})^2$ were also routinely computed. The values of K_m for those species HL_m which contribute significantly (say 0.2 mole/mole) to the total binding in at least some portion of the binding curve are known to the precision which the subsequent analysis will require.

Pentane binding to the myoglobins is uncomplicated. The curvature of the isotherms requires two dissociation constants (Table I). The hyperbola for $N = 1$ shows characteristic deviations with all three derivatives: the lower experimental values of R_L lie systematically below, and the higher values above, the calculated curves. The changes

TABLE I: Pentane Binding to Hemoprotein Derivatives.[a]

Hemoglobin	K_1	K_2	K_1[b]
Oxy-	0.34	7.4	0.30
Deoxy-	0.42	4.0	0.34
Ferri-	0.40	10	0.36
Myoglobin			K_3
Oxy-	0.77	6.2	
Deoxy-	1.14	3.1	
Ferri-	1.20	6.0	
Apo-	0.28	3.2	0.6

[a] Millimolar dissociation constants, 0°, 0.09 M phosphate, pH 7.3. [b] Calculated for one site.

TABLE II: Dissociation Constants of Ligand–Protein Complexes.[a]

	Hb^+		Mb^+		βLG[b]	
	K_1	K_2	K_1	K_2	K_1	K_2
Pentane	0.40	10	1.20	6.0	0.13	1.4
c	0.29	11	1.27			
Butane	1.24	7.9	4.8	6.8	0.48	1.65
Iodobutane	0.61		1.75		0.21	
Neopentane	1.6	7	16		6	
Xenon	19	89	10	55		
c	18	97	10.7	35	5.3	47

[a] Millimolar dissociation constants, at 0°. [b] Wishnia and Pinder (1966a). βGLA monomer. [c] $(NH_4)_2SO_4$ solutions, 1.8 M for Hb^+, 2.0 M for Mb^+, βLG.

following chemical modification at the heme are clear and reproducible, if not dramatic. Interaction between the first and second ligands to MbO_2 is mildly antagonistic, to Mb^+ less so, and to Mb mildly cooperative; since, for independent sites, $K_2/K_1 \geq 4$, the last result, taken literally, makes a model of two noninteracting sites impossible, so at least unlikely.

The hemoglobin data were analyzed on the basis of independent, equivalent, subunits, despite the obvious anomaly, because greater complexity was not justified by the data. For example, pentane binding to any of the derivatives can be fitted reasonably well using either one site per subunit or a strong and a weak site per subunit (the best fits are obtained with a model of one strong and a large number of very weak sites whose contribution to R_P can be approximated by a single term proportional to C_P providing perhaps 5% of the binding at the upper end of the curves). Nonequivalent or interacting subunit schemes are not satisfactory: the fit to two sites per dimer is systematically poor, and the fit to three or four sites per dimer is odd, primarily because the species H_2P_3 and H_2P_4 do not contribute appreciably to R_P at any point.

Differences in pentane binding to HbO_2, Hb, and Hb^+, at 0°, are smaller than to the Mb compounds. The clearest distinction among the hemoglobin derivatives is in the temperature dependence of K_1 (Wishnia, 1969a).

Hb^+ behaves like βLG with respect to butane binding (Wishnia and Pinder, 1966a): two molecules are bound per subunit, and the fit for $K_2/K_1 = 4$, the statistical factor for independent, equivalent sites, is almost as good as for $K_1 = 1.24$ and $K_2 = 7.9$. On the other hand, the butane binding "curve" for Mb^+, which barely deviates from a straight line even at 0.8 mole/mole, would normally be interpreted in terms of a large weak site or a large collection of weak sites; if we want, for consistency, to limit binding to two or three molecules, cooperative ligand interaction is necessary. It can be argued that the required sigmoidal curve is observed.

A major difference between Hb^+, and Mb^+ and βLG, is in the binding of neopentane. The ideal ratio of dissociation constants for neopentane and pentane, predicted from binding to dodecyl sulfate micelles, is 1.7. In Hb^+, neopentane binding is slightly weaker than this, but on the other hand two molecules can be accommodated almost equally. Mb^+ and βLG discriminate strongly against neopentane by factors of 8 and 30 above the ideal ratio.

Iodobutane binding is straightforward. Hb^+, Mb^+, and βLG behave alike, albeit with different constants: a simple hyperbola with $N = 1$ gives very good fits. If N is set to 2, then for some experiments $K_2 \sim 20K_1$, and for others K_2 diverges.

Further analysis of the hydrophobic binding regions hinges on the detailed interpretation of the xenon binding and xenon–pentane interaction data. Xenon binding to Mb^+ and Hb^+ was studied in dilute phosphate buffer and in 2.0 and 1.8 M $(NH_4)_2SO_4$. The latter solutions were studied, first, to approximate the crystallographic environments, and second, because the salting-out effect of ammonium sulfate yielded much more favorable ratios of bound to free xenon at moderate protein concentrations. Ligand activity coefficients (for xenon, 4.7 and 5.6; for pentane, 12.2 and 16.3, in 1.8 and 2.0 M solutions) were calculated from the ligand solubilities in these solutions relative to water. The dissociation constants, suitably corrected for activity coefficients, are not materially different, in low and high salt (Table II); to this extent, at least, the proteins preserve their integrity on the way to crystallization.

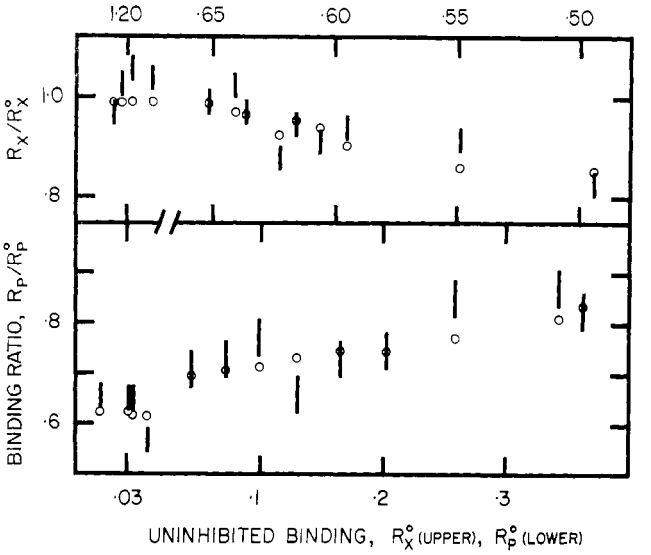

FIGURE 4: Interaction of xenon and pentane binding in Mb^+. The abscissae are R_L^0, calculated ligand binding appropriate to the observed C_L in the absence of the other ligand (R_X^0, upper; R_P^0, lower), arranged so that simultaneous xenon–pentane points are in the same sequence (there is no other horizontal relation between points). The circles are ratios R_L/R_L^0, calculated for a least-squares fit allowing K_X^* to vary with K_{1P}, K_{1X}, and K_{2X} fixed at 1.27, 10.6, and 35 mM (two runs are computed together; separately they yielded $K_X^*/K_{1X} = 1.38$ and 1.80). The bars are ratios of observed to uninhibited binding (R_L^{obsd}/R_L^0), with the average error for all points distributed about the means of independent duplicates. If all constants are allowed to vary the new values of K_{1P}, K_{1X}, K_{2X}, and K_X^*/K_{1X} become 1.28, 9.2, 37 mM, and 1.90.

The Xe–Hb^+ curve gives a substantially better fit with two constants than with one. Since xenon binding to Mb^+ exceeds 1 mole/mole, it is not surprising that the Xe–Mb^+ curve absolutely requires two constants. For both proteins $K_2/K_1 = 4 \pm 1$, and, in fact, the hyperbolas for $N = 2$ and K_2 constrained to be $4K_1$ fit the data very well. We are thus not dealing with major and minor sites, but with two substantially equivalent, and probably interacting, sites.

Pentane binding to Hb^+ in 1.8 M $(NH_4)_2SO_4$ is exactly like binding in dilute salt: one strong site and something weaker. Binding to Mb^+ in 2.0 M $(NH_4)_2SO_4$ is somewhat different: K_2 is increased, but, because the species HP_2 now contributes so little to the total pentane binding, the value of K_2 is not firmly established.

Finally, given the properties of ^{133}Xe and ^3H, it was possible to determine xenon and pentane binding, R_X and R_P, simultaneously, in all the accessible portions of the C_X–C_P plane. (Because all four values are experimentally determined, it is theoretically unnecessary, as well as practically difficult, to keep one ligand concentration constant while the other is varied over the requisite range, but it is awkward to present these data graphically.)

If, using the results in Table II, one calculates the binding of xenon, R_X^0, or of pentane, R_P^0, at the concentrations C_X or C_P, to be expected if the other ligand were absent, it is immediately obvious that the coupling of xenon and pentane binding in both Mb^+ and Hb^+ is very clear and very strong:

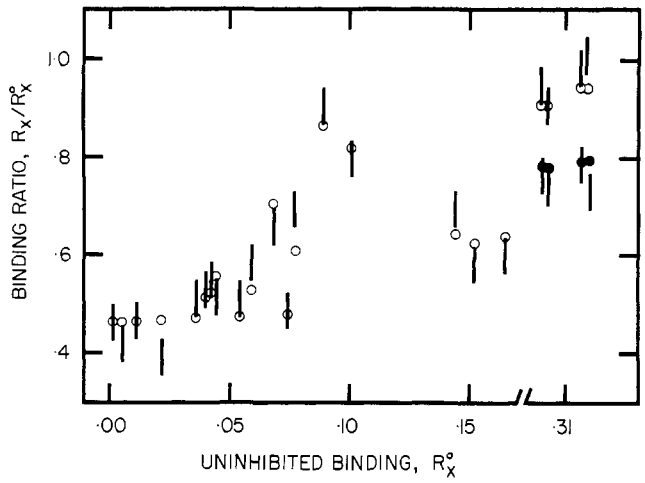

FIGURE 5: Interaction of xenon and pentane binding in Hb^+. Open circles indicate xenon data; the few filled circles are pentane data at low R_P arbitrarily placed under the corresponding xenon points (the rest of the pentane data, with R_P between 0.5 and 0.7 mole per subunit, clusters near the binding ratio of 1.0, and is omitted). Otherwise the significance is as in Figure 4. Two runs gave K_X^*/K_{1X} = 2.6 and 3.5, with K_{1P}, K_{2P}, K_{1X}, and K_{2X} fixed at 0.29, 11, 18, and 97 mM. When the parameters were allowed to vary, K_{1P}, K_{1X}, K_{2X}, and K_X^*/K_{1X} became 0.31, 20, 72 mM, and 5.0.

e.g., in Figure 4, R_P/R_P^0 for Mb^+ is reduced to 0.6 when R_X is near 1 mole/mole; in Figure 5, R_X/R_X^0 for Hb^+ is reduced to 0.45 when R_P is near 0.55 mole/subunit. To go any further in the analysis it is necessary to examine specific models of ligand and site interaction. The least-squares program was suitably modified, and fits minimizing $\Sigma[(1 - R_X^{calcd}/R_X^{obsd})^2 + (1 - R_P^{calcd}/R_P^{obsd})^2]$ were obtained. First, K_{1X}, K_{2X}, K_{1P}, and K_{2P} were fixed at their Table II values, allowing only the new parameters to vary, and the usual criteria of goodness of fit were applied. Now, models of pure competition between two ligands for the same site with no interaction between sites have some leeway in the assignment of intrinsic constants at each site, but are otherwise strictly determined, whereas models permitting interaction between sites provided one or two variable parameters to be determined by the program, and so have better fits. As another test, the previously fixed constants were treated as parameters, to see how well their values were preserved.

It was found that satisfactory fits could be achieved with one new phenomenological dissociation constant, K_X^*, describing the reaction(s) HXP = HP + X (HXP = HX + P is important, but not independent). In particular, the touchstone species, HX_2P, required by models in which the xenon and pentane sites do not occupy the same space, but only interact, does not occur (HXP_2 and HX_2P_2 would not be significant species in any case). In the only noninteracting site model which approaches a fair fit the observed deviation of the K_{2X}/K_{1X} ratios from 4 is disregarded, and the xenon sites are treated as both independent and equivalent, requiring $K_X^* = 2K_{1X}$. The observation is that, for Mb^+, $K_X^* = (1.60 \pm 0.3)K_{1X}$, and for Hb^+, $K_X^* = (3.0 \pm 0.5)K_{1X}$, with the other constants fixed. K_{1X}, K_{2X}, and K_{1P} do not drift significantly when the constraints on them are removed (see Figures 4 and 5). The conclusion is that xenon and pentane compete for the same region(s), but that in Hb^+ the first ligand bound interferes with the binding of the second, while in Mb^+ binding of the second ligand is somewhat facilitated.

Discussion

Our analysis must be directed at two kinds of problems. The first, while important, is narrowly technical: are binding sites, on different derivatives, or for different ligands, or for successive molecules of the same ligand, independent and spatially distinct, or do they interact, overlap, or coincide. The notion of a discrete site should not be pushed too far: these clearly localized, limited binding sites are not likely to be discrete in the strictest sense of having delimitable regions of which one can assert that they always contain either exactly zero or exactly one molecule of any ligand. When these questions are settled we can begin on the fundamental problem, understanding the structural basis of the detailed behavior of the complexes.

It does not take Occam's Razor ("It is vain to do with more what can be done with fewer" (Bernal, 1965)) to require a unitary hypothesis for the binding of butane, pentane, and iodobutane to Mb^+ or to Hb^+. For β-lactoglobulin, we know, from the way iodobutane, pentane, and butane binding interact (Wishnia and Pinder, 1966a), and from the way 1 mole of dodecyl sulfate prevents binding of butane, pentane, and neopentane (Wishnia, 1969b), and other considerations, that there is a single hydrophobic binding region in each subunit of this protein with a capacity of no more than 230 ml of properly shaped nonpolar ligands. A major feature of the behavior of this structure, the marked dependence of the ratio K_2/K_1 upon ligand volume (97, 118, and 129 ml for butane, pentane, and iodobutane, at their boiling points), also occurs for Hb^+ and Mb^+ (Table II). Such behavior must be justified *ad hoc* for two independent sites, but follows naturally for one continuous binding region. Binding occurs the way a child's knobby balloon inflates, in spurts. Under some constraints a particular segment may always inflate first, under others the first spurt may start anywhere; the final shape and volume are fairly well determined. Each spurt will reorder the side chains in a characteristic way, since distinct holes do not occur, and may or may not change the relative "free volume" (that is, the average interaction energy (Wishnia, 1969a)).

Occam's Razor is needed to argue, from the fact that xenon and pentane binding are coupled, that these binding regions correspond to the xenon loci described by Schoenborn. It is in order, therefore, to ask whether xenon is a typical hydrophobe, so that, other things being equal, we can expect xenon and pentane to gravitate naturally to the same environment, or whether xenon binding is a separate phenomenon. The notion (Schoenborn *et al.*, 1965) that xenon binding is stabilized by strong London forces arising from the high polarizability of xenon, *and is therefore* not a hydrophobic interaction, misses the point. The molar polarizability of xenon is not exceptional when compared, say, with ethane: both substances have similar boiling points and similar energies of vaporization per unit volume (Hildebrand and Scott, 1964); both are most soluble in alkanes (it is noteworthy, however, that the solubility of xenon in alkanes is almost double the ideal solubility), and become less soluble as solvent polarity increases (Saylor and Battino, 1958; Thomsen and Gjaldbaek, 1963); it is the transfer *to water* which is unusual, and which provides the quintes-

sential feature of a "hydrophobic interaction," with its characteristic ΔH, ΔS, and ΔC_p. The hydrophobe seeks any port in a storm, to get out of the wet.

The xenon sites are properly hydrophobic. The site in Mb^+ to which xenon (Schoenborn *et al.*, 1965), cyclopropane (Schoenborn, 1967), and the much larger anion, HgI_3^- (Kretsinger *et al.*, 1968), bind is a pocket on the distal side of the heme, with contacts to a pyrrole ring and the side chains of leucine, isoleucine, phenylalanine, and histidine. The xenon bound to Hb^+ (Schoenborn, 1965) is found 6 Å in from the surface of each subunit, between the AB and GH corners, with probable contacts to phenylalanine, valine, and leucine. (This site is far from the sliding contacts of the $\alpha_1\beta_2$ interface, and on the side of the G and H rods facing away from the stable $\alpha_1\beta_1$ interface formed by closely intermeshed side chains of the G, H, and B rods (Perutz *et al.*, 1968)). It is too early to say whether these sites would necessarily exhibit the observed or postulated steric and thermodynamic properties in detail.

There remains the question whether, in Mb^+, we have apparent noninterference between two xenon atoms in the same region, a compensation phenomenon seen also for butane and βLG, or whether there are, in fact, two spatially distinct noninteracting equivalent sites. (The latter is certainly not the case for Hb^+.) The results (strong interactions between butanes, the other ligand relations, suggestions of xenon–xenon and xenon–pentane interaction) collectively favor the former, but no single result is decisive. Schoenborn's observations present no problem: xenon binding at 1.5 atm and 0° could easily be double the binding at 2.5 atm and room temperature, nor would two-dimensional difference Fourier maps at 2.8-Å resolution readily distinguish between 0.75 xenon atom at singly occupied sites and 0.47 so distributed plus 0.28 randomly oriented as double occupants of the site.

The behavior of apomyoglobin is instructive. Removal of the heme produces real but minor changes in secondary structure (Breslow *et al.*, 1965) and allows binding of one moderately sized hydrophobic probe, 1-anilino-8-naphthalenesulfonate (Stryer, 1965), at the same site. The Mb^+ dual pentane site does not survive unmodified (if we substract binding to unperturbed sites the residuum cannot be accounted for by any model), nor is a large, dedifferentiated, region produced or added; the observed curve can only result from strong binding of one molecule of pentane plus somewhat weaker, complex binding of two or more others (if the three pentane model, which is adequate, is correct, the second and third add almost simultaneously). The new sites are certainly in the heme region; it is very likely that the old sites were, also.

An effect of oxygenation on pentane binding to Mb is therefore not unexpected. The modest, relatively subtle changes in the hemoglobins are probably part of the internal mechanism of "heme–heme interaction" that ultimately produces the large-scale displacement at the $\alpha_1\beta_2$ interfaces of Hb upon oxygenation.

The basis of the strength of binding and of selectivity of these hydrophobic sites (comparable with the active sites of enzymes in these respects) is discussed in detail elsewhere (Wishnia, 1969a). We have a model of ideal hydrophobic behavior, dodecyl sulfate micelles, which provides normative values of the magnitude and temperature dependence as well as of the ratio of dissociation constants (1:1.7:2:3.8:120 for pentane, neopentane, iodobutane, butane, and xenon) for the reaction ligand (aqueous) + unstrained hydrophobic region → (water) + unstrained hydrophobic complex. The key to understanding the specific behavior of these proteins and ligands lies in this: all of the binding observed is *stronger* than ideal, by an excess heat of dissociation. The source of anomalies is a strained, nonideal unoccupied binding site, and the odd interactions arise from the degree to which successive ligands afford relief, in a nonlinear fashion, of fractions of this strain, by reordering and optimizing side chain–side chain and side chain–ligand interactions. This is why pentane is bound so strongly, why neopentane binds moderately well to Hb^+ but relatively poorly to βLG and Mb^+, why xenon, with a molar volume of 43 ml, behaves like the functional equal, not of methane, but of pentane (and probably why the xenon constants fall in the expected range, but the order $Hb^+ > Mb^+ > \beta$LG, differs from the order for *n*-alkanes, $Mb^+ > HB^+ > \beta$LG). The behavior of apomyoglobin toward pentane suggests that binding of heme is strengthened by a similar strain mechanism.

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On the Thermodynamic Basis of Induced Fit. Specific Alkane Binding to Proteins*

Arnold Wishnia

ABSTRACT: Small alkanes bind to β-lactoglobulin, ferrihemoglobin, and ferrimyoglobin, in each case, to a localized, interior, hydrophobic site with high affinity and nontrivial stereospecificity. In order to understand this behavior, the thermodynamic parameters for the transfer of pentane, neopentane, and butane to water, from ideal solutions, dodecyl sulfate micelles, ferrimyoglobin, ferrihemoglobin, and β-lactoglobulin, between 0 and 40° or 50°, were determined. ΔC_p for the proteins, and for all solutes, is comparable with the dodecyl sulfate values; this variation of ΔH with temperature derives from the unusual behavior of water, and is typical of "hydrophobic" interactions. The heat of dissociation of butane and pentane from β-lactoglobulin and ferrihemoglobin is 3–4 kcal higher than from dodecyl sulfate micelles over the whole temperature range, and is the source of the high alkane affinity of these proteins. For neopentane, ΔH for β-lactoglobulin is more negative, and for ferrihemoglobin more positive, by 1–2 kcal, than the dodecyl sulfate values: β-lactoglobulin makes a clear distinction between pentane and neopentane, while ferrihemoglobin does not. The excess ΔH cannot arise from the solute–water interaction, from a nonoccurring displacement of water, or from exceptionally strong solute–protein interactions, but must come from suboptimal interactions in the unoccupied site (plausibly, abnormally large molar and "free" volumes), which are normalized by strictly local rearrangements in the butane and pentane, but not (for β-lactoglobulin) the neopentane, complexes.

The origin of the high affinity and dramatic stereospecificity of enzymes for their substrates is a major problem of biochemistry. Whatever general model one assumes, any particular active site must, of course, still be constructed to interact with the substrate in modes dictated by the structure of the substrate: monopole or dipole attractions, London interactions, hydrogen bonding, covalent bonding, or whatever. In the classical "lock and key," or template, model, appropriately complementary groups are rigidly positioned for maximal interaction with the substrate, and no other, molecule; it is usually implicit that the shape and nature of the cavity will provide good, close, van der Waals contacts. The favorable local free energies sum to the overall free energy of binding. Koshland (see Koshland and Neet, 1968) has long proposed that the complete active site may arise only by an "induced fit:" in the act of binding, the enzyme structure is modified to achieve the interactions required by the specific substrate. It is not required, but it is often implicit, that the changes be large: for example, the known shift of tyrosine-248 when glycyltyrosine is bound to carboxypeptidase A (Lipscomb *et al.*, 1968) and the speculative postulated rearrangement of β-amylase (Koshland *et al.*, 1962). It also seems to be assumed that, since the new configuration is not observed in the absence of substrate, the contribution of the rearrangement itself to the overall free energy of binding must be positive (*i.e.*, unfavorable). In both models the native enzyme tends to be thought of as a well-behaved, stable structure, not only generally, but locally. I would like to add a third model, for which experimental examples are given below, in which the active site is so constructed that the free energy of binding of the right substrate is more negative than one thought one had the right to expect.

Alkanes bind to β-lactoglobulin (Wishnia and Pinder, 1966), ferrimyoglobin, and ferrihemoglobin (Wishnia, 1969), in each case, to a localized, interior, strictly hydrophobic site, with high affinity and nontrivial stereospecificity. For this type of interaction where exist explicit normative models for maximal, ideal, interaction: transfer of alkanes from aqueous solution to liquid alkanes (see, *e.g.*, Kauzmann, 1959) or, for convenience, to the interior of detergent micelles (Wishnia, 1963). The thermodynamic parameters to be expected for the progressively weaker interactions of alkanes with solvents of increasing polarity (*e.g.*, hexane, benzene, and dioxane, Thomsen and Gjaldbaek, 1963; methanol, ethanol, and 2-propanol, Kretschmer and Wiebe, 1952; among others) are also known. Since the binding of some alkanes to βLG,[1] Hb^+, and Mb^+ is, in fact, *stronger* than to quasi-ideal systems like dodecyl sul-

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[1] Abbreviations used are: βLGA, β-lactoglobulin A; βLGA-1, βLGA monomer at pH 2.0; βLGA-2, βLGA dimer at pH 5.3; Hb, deoxyhemoglobin; Hb^+, ferrihemoglobin; Hb^+CN^-, ferrihemoglobin cyanide; HbO_2, oxyhemoglobin; Mb^+, ferrimyoglobin; and ApoMb, apomyoglobin.