Hydrophobic Interactions in Proteins. The Alkane Binding Site of β-Lactoglobulins A and B*

Arnold Wishnia and Thomas W. Pinder, Jr.

ABSTRACT: β-Lactoglobulins A and B (β-LG-A and -B), in dilute aqueous solution, bind apolar solutes at a few discrete sites. There are no essential differences in the binding to monomers, to the normal 36,000-mol wt dimers, or to β-LG-A octamers, indicating that: (a) conformation changes, if any, associated with aggregation, are not transmitted to the binding sites; and (b) the structures at the dimer and octamer joints, being inhospitable to butane, pentane, and iodobutane, cannot contain even modest-sized flexible hydrophobic regions. Each β-LG monomer can bind two butanes equally well, two pentanes unequally well, or one iodobutane. In particular, for β-LG-A, pH 2, 25°, the dissociation constants, free energies, enthalpies, and entropies (on a mole fraction basis) are: butane, $K_1 = 0.98 \times 10^{-5}$, $K_2 = 3.76 \times 10^{-5}$, intrinsic $K_0 = 1.92 \times 10^{-5}$, $\Delta F = 6.44$ kcal, $\Delta H = 1.1$ kcal, $\Delta S = -17.8$ cal/deg; pentane, $K_1 = 0.30 \times 10^{-5}$, 7.54 kcal, 2.1 kcal, −18.3 eu, $K_2 = 2.9 \times 10^{-5}$, 6.19 kcal, 0.9 kcal, −17.7 eu; iodobutane, $K_1 = 0.71 \times 10^{-5}$, 7.03 kcal, 4.0 kcal, −10.2 eu, $K_2 = \infty$. All three solutes compete for the same binding site. We conclude that the binding site on each monomer is a single hydrophobic region, near the protein surface, which can accommodate more than 200 but less than 230 ml/mole of additional nonpolar substance.

For some time we have been using hydrocarbons like propane, butane, and pentane as probes to study the nonpolar regions of proteins (Wishnia, 1962). Not only do these hydrocarbons bind quite strongly to some proteins, but the binding characteristics reflect specific aspects of the structure of these proteins also, and may change more or less dramatically when the conformation of the protein is altered (more: the N–F transition in bovine serum albumin, Wishnia and Pinder, 1964a; less: the different states of hemoglobin and myoglobin, Wishnia and Pinder, 1966).

It is clear that in the cases we have studied the alkanes interact directly with hydrophobic regions. However, we are still in the process of defining just what it is we see when we observe a particular sort of binding: to what extent can we infer, from binding, whether there exist large or small, flexible or rigid (in either a thermodynamic or kinetic sense) hydrophobic groupings in a particular protein; to what extent can we use relative and absolute differences in the thermodynamic parameters for binding different hydrocarbons to deduce subtle changes in proteins, and, on the other hand, how far can these parameters be used in computing stable structures of proteins; finally, of course, how can we relate changes in binding to biological function?

We took up the study of β-lactoglobulin because it is well known that the molecule, as it exists above pH 5, is a 36,000-mol wt dimer, which can dissociate cleanly to compact 18,000-mol wt monomers at pH 2 (Green and Aschaffenburg, 1959; Townend *et al.*, 1960; Timasheff and Townend, 1961a). In addition, β-lactoglobulin A, but not B, forms a specific octamer near pH 4.6 at low temperature (Townend and Timasheff, 1960; Timasheff and Townend, 1961b; Witz *et al.*, 1964). Since it had been suggested (Townend *et al.*, 1960) that dimerization involved considerable interpenetration, probably of hydrophobic regions, and since β-LG-A contains two more aspartyl and valyl and two less glycyl and alanyl residues than β-LG-B (Kalan *et al.*, 1962, 1965), we thought that alkane binding studies could illuminate the nature of the several joints between monomer units. When it became apparent that binding occurred at one, or at most two, sites per monomer, we also initiated work with iodobutane, with the notion that this might serve a marker in a future X-ray investigation of the location of this site.

Initial sanguine hopes for differences proved illusory; in what follows we will demonstrate similarities which exclude certain possibilities. We will establish that there is a single site on each β-lactoglobulin monomer capable of binding two molecules of butane (equally well), two molecules of pentane or xenon (unequally well), and one molecule of iodobutane (reasonably well). We suspect that the site is not open to the solvent, but is rather a cleft extending from the surface into the interior of the protein. The interactions leading to dimer and octamer formation do not disturb this site,

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[1] Abbreviations used: β-LG-A and -B, β-lactoglobulins A and B; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; sem, standard error of the mean.

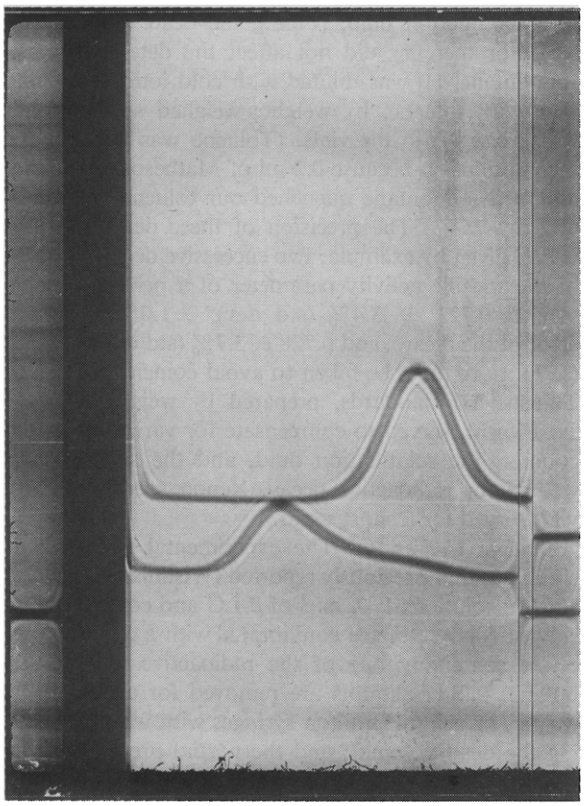

FIGURE 1: Octamer formation by β-LG-A. Upper, β-LG-B, 1.88%, pH 4.70; lower, β-LG-A, 1.42%, pH 4.68; 0.10 μ, acetate; 5.0°; after about 150 min at 50,740 rpm; diaphragm angle 75°.

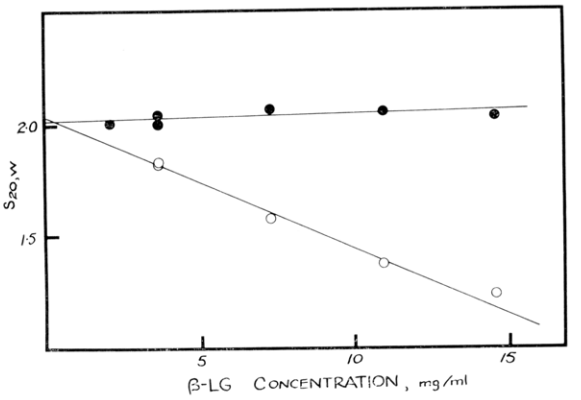

FIGURE 2: Centrifugation of β-LG-A at low pH. ●, pH 2.08, 0.06 μ; ○, solution 1, pH 2.03, diluted as indicated with dialyzate.

and, to that extent, are not far reaching. Our experience with serum albumin prompts us to conclude that an extensive intermeshing of hydrophobic groups between subunits, as proposed for the dimerization, should produce a region favorable to alkane binding; failure to observe changes in the number of sites or their affinities suggests that such intermeshing does not occur.

Experimental Section

Protein Preparations. β-Lactoglobulins A and B were isolated from the milk of homozygous cows according to Aschaffenburg and Drewry (1957) and twice recrystallized. Several preparations were used. Protein concentrations were determined using an absorptivity of 0.96 l. g^{-1} cm^{-1} at 278 mμ (Townend *et al.*, 1960) and a molecular weight of 35,500 for the dimeric, pH 5.3, species (Senti and Warner, 1948). Ultracentrifugation of both A and B (50,740 rpm, 12 mm Kel-F cells, 25°, 1% protein) gave the expected values of $s_{20,w}$: at pH 5.2, 0.10 μ, sodium acetate, 2.9 S; at pH 2.1, 0.1 μ, NaCl, 2.2 S (Timasheff and Townend, 1961a). Electrophoresis at pH 8.6 on sepraphore III and centrifugation at pH 4.65, 0.10 μ, NaCl, and 5° confirmed the identities of β-LG-A (formation of octamer, reaction boundary) and β-LG-B (normal symmetrical dimer peak) (see Figure 1, and Timasheff and Townend, 1961b).

In order to deal with solutions containing primarily monomer, dimer, or octamer, three sets of conditions were chosen: (1) 1.5% protein, pH 2.06 ± 0.03 (*ca.* 0.010 M H^+, 0.027 M Cl^-, in Donnan equilibrium with dialyzate at pH 1.93 ± 0.01, 0.013 M HCl; in passing, this suggests considerable chloride binding); (2) 1.5% protein, pH 5.25 ± 0.05, 0.050 M NaCl; and (3) 3.0% protein, pH 4.65, 0.100 M NaCl. Crystalline β-LG was dissolved at low pH, the pH raised with sodium acetate and ammonia, and the solutions exhaustively dialyzed *vs.* the indicated solutions. The pH was measured at 25°.

Solution 2, by sedimentation velocity and short-column sedimentation equilibrium (Yphantis, 1960), contains pure dimer (a slightly negative B_0 is encountered, *cf.* Townend and Timasheff, 1960). Solution 3 contains, at 0°, about 90% of β-LG-A octamer (see Figure 1, and Timasheff and Townend, 1961b). We must show that solution 1 contains mostly undeformed monomer. A reasonable extrapolation of data in the literature (Townend *et al.*, 1960; Timasheff and Townend, 1961a) suggests that dissociation in solution 1 is favored with respect to pH 2.7, 0.1 μ, by about −3.0 kcal. On this basis the fraction of monomer would be: β-LG-A, 25°, 0.93, 0°, 0.76; β-LG-B, 25°, 0.84, 0°, 0.58). Figure 2 gives the concentration dependence of $s_{20,w}$ in solution 1 and at higher ionic strength. The curve at 0.06 μ corresponds to data at pH 1.6, 0.1 μ, in Figure 2 of Townend *et al.* (1960), which these workers have interpreted as a monomer–dimer equilibrium superimposed on a large concentration dependence of $s_{20,w}$ for both species. The curve derived from solution 1 extrapolates to the same $s^0_{20,w}$ with a somewhat larger concentration dependence of $s_{20,w}$ (doubtless a primary charge effect), indicating, to us, that β-LG-A has dissociated into compact monomers to about the predicted extent. Earlier sedimentation

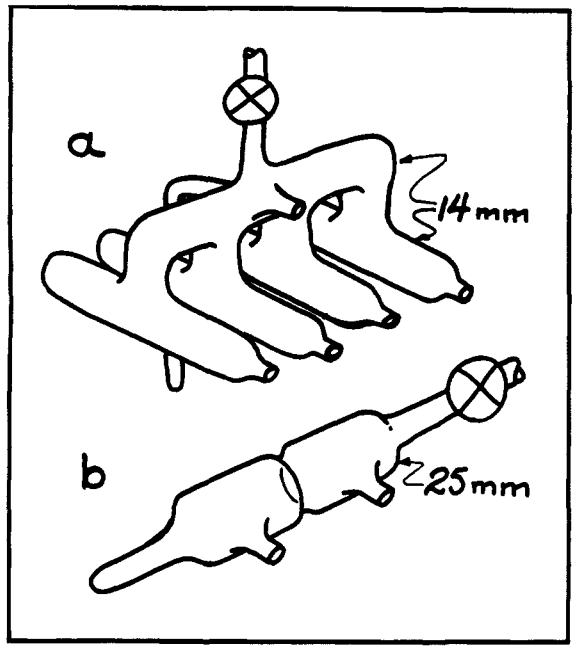

FIGURE 3: Solubility cells. Either precision bore or selected tubing is used for the sampling ports: cell b requires 5.0-mm i.d. tubing and serum stoppers; some versions of cell a used capillaries for 0.067-in. wired Teflon plugs backed by serum stoppers; some used concentric cylinder teflon stopcocks. The gas-inlet stopcocks are Delmar-Urry Teflon needle valves.

equilibrium studies on pooled β-LG showed no great differences between 0 and 25° [somewhat high extrapolated molecular weights (19,000–20,000) and similar, very large B_0]. We have no theoretical estimates of B_0 in these solutions, but if the protein is monomeric at 25° it is also monomeric at 0°.

Radioactive Solutes. Butane-1,2-[^{3}H] and pentane-1,2-[^{3}H], and later butane-1-[^{3}H] and pentane-1-[^{3}H] prepared by decomposition of Grignard reagents (8 mc/g, New England Nuclear), were purified as previously described (Wishnia and Pinder, 1964), except that an intermediate scrubbing with sulfuric acid was introduced. Xenon-133 (Radiochemical Centre) was diluted with xenon-131 (Air Reduction Co., mass spectrographically pure), scrubbed with water, and further diluted. This solute was transferred between reservoirs in Dry Ice–acetone and liquid nitrogen. Iodobutane[^{3}H] (New England Nuclear) was gas chromatographed prior to each run, and the center of the peak was taken.

Counting was done in a Packard Instruments Model 3214 liquid scintillation spectrometer using vials containing 20 ml of scintillation fluid (0.5% PPO, 0.03% POPOP in toluene; Pilot Chemicals) and appropriate amounts of cold protein and/or buffer solution.

Accurate values of specific activity are crucial to these experiments. For the first three substances, aliquots of gas phase of known pressure, temperature, volume, and composition (Dreisbach, 1959; Cook, 1961) in Hamilton gas-tight syringes were extracted into scintillation fluid. (Filling the dead volume with water or mercury did not affect the determinations.) Iodobutane[^{3}H] was diluted with cold iodobutane and then with toluene, by weight; weighed syringe loads were rinsed into the vials. (Toluene was used in the second dilution because 0.2 ml of Matheson, Coleman, and Bell iodobutane quenched our toluene[^{3}H] standards by 75%.) The precision of these determinations is best given by example: two successive determinations of the specific activity parameter of a pentane sample yielded 0.723 ± 3.4% (std dev), ±1.0% (standard error of the mean), and 0.728 ± 3.7% (std dev) ± 1.2% (sem). Care must be taken to avoid coincidence losses. Toluene[^{3}H] standards, prepared by weight for each experiment, served to compensate for variations in the counter, the scintillation fluid, and the solutions, as well as for radioactive decay. Xenon specific activity vials served for a short series.

Binding Curves. (a) The experimental technique is similar to that previously reported (Wishnia and Pinder, 1964a): solutions 1, 2, or 3 of β-LG and corresponding solvent (dialyzate) are equilibrated with a common gas phase containing one of the radioactive solutes, say butane; 0.2-ml aliquots are removed for counting (by weight, or using Hamilton syringes with water occupying the dead volume); and the partial pressure of the butane is changed for the next point. The counting rate in the solvent gives the free concentration of the butane, c_{BUT}, the excess in the protein solution gives the number of moles bound per mole of protein, r_{BUT}.

The cells were redesigned to minimize the mass of rubber in contact with alkane (some versions of cell 3a used wired Teflon plugs) and to promote mixing of gas between legs, which seemed to us to be the limiting factor in reaching equilibrium. Even so, while a solution in cell 3b (Figure 3) reached equilibrium with the gas in its leg in <2 min, mixing between legs, especially at low partial pressures of alkane, was slower. We now pump the gas phase back and forth with syringes midway between samplings, as well as when the concentration is changed. In this way, 20 min of rocking or 15 min of rolling is more than adequate.

Butane, xenon, and sometimes pentane were condensed into evacuated cells. Iodobutane was introduced as a liquid. Concentrations were reduced by stepwise replacement of gas phase with air. We found it desirable, when running pentane at 0°, to *add* alkane vapor by syringe.

(b) Analyzing the data according to the model of independent equivalent sites [$K = (N - r)c/r$] has the advantage that N and K are not too sensitive to systematic errors; the real protein, however, may not have independent equivalent sites; in particular, weaker sites may be overlooked. We chose, instead, to fit our data to the more general form

$$r = \frac{\sum_{n=1}^{N} \left(nc^n \Big/ \prod_{m=1}^{n} K_m \right)}{1 + \sum_{n=1}^{N} \left(c^n \Big/ \prod_{m=1}^{n} K_m \right)}$$

TABLE I: Dissociation Constants.[a]

	Iodobutane			Pentane		Butane		
	Temp, °C	K_1	K_2	K_1	K_2	K_0[b]	K_1	K_2
Dimer (35,500 mol wt)	25	0.354	∞	0.141	1.61	1.03	0.59	1.72
				0.153	1.70	0.87[c]	0.45[c]	1.69[c]
Monomer	25	0.417	19.4	0.166	1.71	1.06	0.54	2.08
		(0.371)		0.166	1.51	0.90[c]	0.45[c]	1.83[c]
				0.118[c]	1.25[c]			
Dimer	0	0.222	67.7	0.077	2.02	0.81	0.30	2.1
				0.126	4.4			
Monomer	0	0.235	∞	0.130	1.17	0.91	0.48	1.71
		0.189	8.3	0.13	1.6	0.87	0.48	1.60
Octamer	0			0.105	1.50			

[a] Calculated values refer to independent monomers (see text). Units of K are millimoles per liter. [b] Calculated for two independent equivalent sites per monomer. The results of duplicate experiments are listed separately. [c] Denotes β-LG-B.

An iterative least-squares algorithm (Margenau and Murphy, 1956), suitable for any number of dissociation constants, was programmed for a GE-235 computer. We could then test many models: independent subunits, interacting subunits, any number of binding sites. The program underweights the lower values of r, since the relative precision is really the same at all values; in practice, this was not serious. However, the program does throw the weight of systematic errors onto the weaker constants. In particular, if the maximum solute concentration falls far short of saturating all the sites, and if the amount of binding is systematically overestimated, the early flattening of a hyperbola may force the introduction of a nonexistent weaker site. We had to satisfy ourselves that the errors in specific activities and in protein concentrations did not produce such an effect.

(c) Inhibiton. We wanted to measure the effect of high concentrations of one solute on the binding of another, to see whether the sites interacted, or coincided. Using, for example, pentane[^{3}H], we could easily measure c_P and r_P in the presence or absence of nonradioactive iodobutane, but we were limited, perforce, to the iodobutane concentrations attained in the presence of liquid organic phase. (This concentration is less than the solubility of iodobutane at the temperature in question by the factor 1 − (c_P/pentane solubility.)

The inhibition expected for different models can be calculated, using the known dissociation constants and the experimental solute concentrations, and compared with the experimental values.[2]

Results and Discussion

Examples of the binding curves are displayed in Figures 4–7. The salient feature is that they are curves: one immediately concludes that with β-LG, unlike with bovine serum albumin (Wishnia and Pinder, 1964b), binding of butane, pentane, iodobutane, and xenon occurs at a small number of discrete sites.

A second conclusion is also inescapable: as far as binding is concerned, the monomers must be treated as independent, even when they are associated into dimers or octamers. Dissociation constants computed on this basis are listed in Table I. The values for monomer, dimer, and octamer are close to identical. [If one requires a fit for an even number of sites per dimer (two for iodobutane, four for butane or pentane) the result is merely to split the monomer constants according to the statistical factors. Since the limited solubility of pentane prevents binding in excess of 2.5 moles/36,000 g, a forced fit to three constants per dimer

[2] We know the solubilities in water, c_i^0 (*i.e.*, the concentration in water in equilibrium with the pure solute at its saturated vapor pressure p_i^0), for butane, pentane, and iodobutane. If it is assumed that the three solutes obey Henry's law in water ($c_i = p_i c_i^0/p_i^0$) and Raoult's law in solutions of each other in the side arm ($p_i = x_i p_i^0$), then it is easily shown that the water concentration of the nonradioactive solute, say iodobutane (c_I), at the experimentally determined concentration of radioactive solute, say pentane (c_P), is given by $c_I = (1 - c_P/c_P^0)c_I^0$.

The experimental value of r_P (inhibited) is determined, as usual, from the counting rates. For the case of two distinct sites, only one of which binds iodobutane (case 1, below)

$$r_P \text{ (inhibited)} = \frac{\left(\frac{c_P}{K_{P_1}} + \frac{2c_P^2}{K_{P_1}K_{P_2}} + \frac{c_I c_P^*}{K_I K_P'}\right)}{\left(1 + \frac{c_P}{K_{P_1}} + \frac{c_P^2}{K_{P_1}K_{P_2}} + \frac{c_I}{K_I} + \frac{c_I c_P^*}{K_I K_P'}\right)}$$

For butane we took $K_P' = K_0$, the intrinsic constant, since the statistical factor no longer applies when one (distinguishable) site is occupied by iodobutane; for pentane, $K_P' \cong K_{P_2}$. For case 2, the starred terms in the numerator and denominator do not occur.

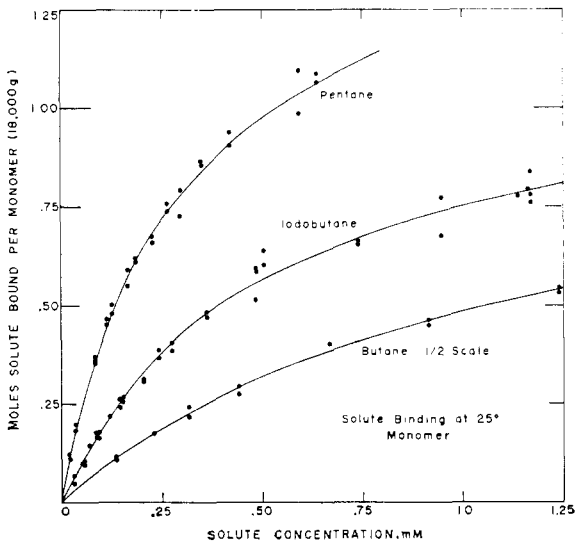

FIGURE 4: Binding of pentane, iodobutane, and butane to β-LG-A monomer at 25°. The curves are calculated from the constants in Table I.

can be achieved; however, not only is there no improvement in fit (root mean square error), but, as is already obvious from Figure 5 and Table I, exactly the same fit can be forced for fictitious dimers in solution 1.] There are two corollaries: if configuration changes occur during association they are not transmitted to the binding sites, nor does binding of these compounds change the state of aggregation of the protein; more significantly, the structures of the dimer and octamer joints are inhospitable to butane, pentane, and iodobutane. We shall return to this point later.

We would like now to discuss the nature of the binding sites. We propose to conclude that all the solutes are bound at a common site on each β-LG monomer, and to estimate its size; further, to show that this site is interior and hydrophobic, in the sense that the solute is removed from contact with water and placed in contact with an apolar region of the protein. The first proposition is based primarily on the inhibition data and the ratios between the first and second dissociation constants for the different solutes. The second depends on the strength of the binding, and the relative affinities

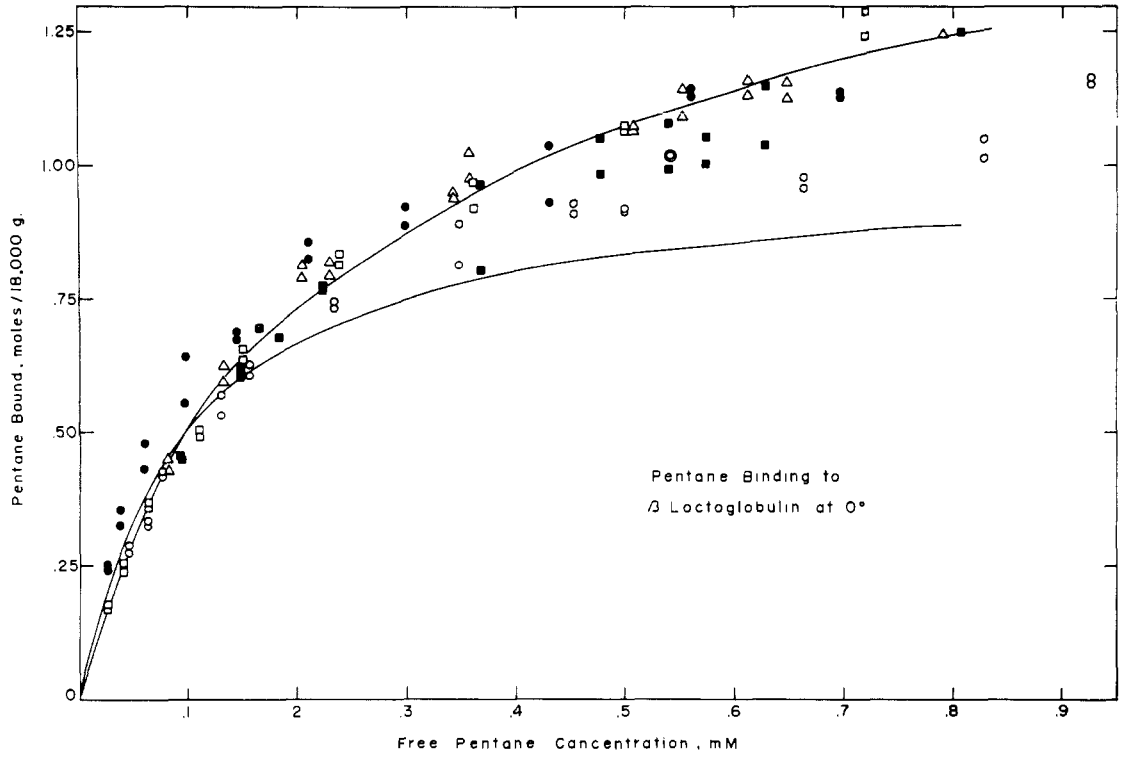

FIGURE 5: Pentane binding and the independences of subunits, 0°. β-LG-A dimer ●, ○ (separate experiments on solution 2); monomer, ■, □ (separate experiments on solution 1); octamer, △ (solution 3). Experimental values of the dissociation constants for the five runs are listed in this order in Table I. The upper curve, calculated for $K_1 = 0.12 \times 10^{-3}$ M, $K_2 = 1.4 \times 10^{-3}$ M; and the lower curve, for $K_1 = 0.10 \times 10^{-3}$, $K_2 = \infty$, are shown for comparison.

and thermodynamic parameters for the different solutes, as compared to binding to dodecyl sulfate micelles. The arguments, however, cannot be separated quite so neatly.

Given the limitations of the inhibition experiments, we made calculations for only two models: (1) a common strong site for all solutes and a second independent (and for pentane, weaker) site; (2) a single large site, in which binding of one kind of solute completely prevents binding of another kind. Models in which all sites are different, but interacting, seem, to us, inherently implausible. A variant, 3, permitting inhibitory interaction between sites, would span the range between 1 and 2.

Strong inhibition is indeed observed (Table II). The mutual inhibition of iodobutane and pentane binding is consistent with 2 or 3. The decrease in butane binding in the presence of iodobutane is even greater than what is expected for 2 (perhaps exposing the limitations of the technique), but is obviously closer to 2 than anything else: the binding of one molecule of iodobutane is competitive with the binding of two molecules of butane.

TABLE II: Competitive Inhibition of Binding.[a]

Solute; Concn (mM) (Inhibitor)	Temp, °C	% Inhibtn[b]	Amount Bound Calcd/Exptl Model 2	Model 1
Iodobutane; 0.021 (pentane)	25	81 ± 1	0.99	0.99
Pentane; 0.039 (iodobutane)	25	70 ± 1	0.99	1.26
	0	76 ± 1	0.92	1.24
Butane; 0.099 (iodobutane)	0	84 ± 2	1.11	3.69
Butane; 1.36 (iodobutane)	0	50 ± 6	1.22	1.41

[a] pH 2. [b] Experimental, ± std dev, four determinations each.

Now, the curve in Figure 6 is a beautiful hyperbola: for all the butane data the ratio between K_2 and K_1 is close to 4, the statistical factor. For pentane, K_2/K_1 is between 10 and 15; for iodobutane the ratio is immeasurably large. It cannot be a question of two independent sites, which is incompatible with the inhibition data; moreover, it is difficult to picture two sites indistinguishable to butane which would behave so differently with pentane and iodobutane. The question is, rather, what sort of interaction is involved.

A great deal can be explained in steric terms. We suspect, although we do not yet know, that we have been studying the site, discovered long ago (McMeekin *et al.*, 1949), which binds a molecule of dodecyl sulfate very strongly. The molar volumes of CH_2 and CH_3 in liquid alkanes, at 25°, are 16 and 34 ml, respectively (see Dreisbach, 1959); the volumes of butane, pentane, and dodecyl are 100, 116, and 210 ml, respectively. Presumably there is a region in β-LG which can readily expand to include one dodecyl radical (there is certainly no preexisting hole), leaving the negative sulfate and exposed to solvent. Two molecules of butane can fit into such a region without strain, but two molecules of pentane are too much. The large second dissociation constant then reflects either a stress on the site or, more simply, that the equivalent of one methyl or two methylene groups of the pentane remains, or of the protein becomes, exposed to water. (The equilibrium constant, or partition ratio, for the transfer of alkanes from water

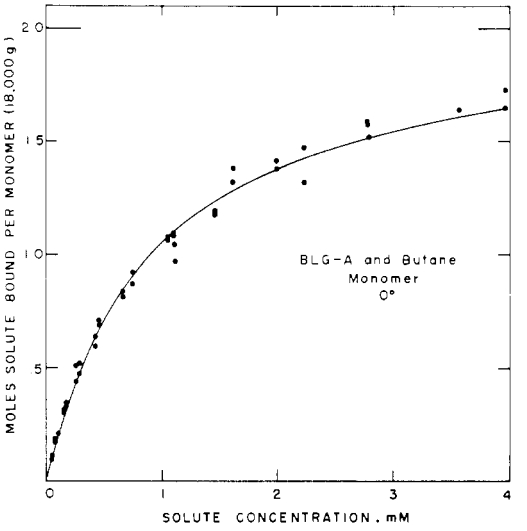

FIGURE 6: Butane binding to β-LG-A monomer, solution 1, 0°.

to a variety of hydrophobic environments increases by three- to fourfold/CH_2 group, as is well known: *e.g.*, Wishnia, 1963.) An iodide residue is larger than a methyl group, so that the effect on a second iodobutane molecule would be greater still. (This is not so straightforward. The actual molar volume of iodobutane is only 115 ml, which may or may not reflect interactions in the pure liquid which are not relevant for binding. We had originally thought that the large dipole moment of iodobutane would effectively prevent the head from leaving the solvent, but the micelle data seem to rule this out: the transfer of iodobutane from water to dodecyl sulfate micelles is quantitatively predicted from the pentane data and the water solubilities of the two compounds, and the dissociation constants are in the same ratio; pentane and iodobutane appear to be good analogs. However, it is still possible that the constraints of the site impose unfavorable dipole–dipole configurations on two bound iodobutane molecules

TABLE III: The Transfer of Hydrophobic Solutes from Water to β-LG-A and SDS Micelles at 25°.

	Relative Affinity	ΔF^a (kcal)	ΔH (kcal)	ΔS^a (cal/deg)
β-Lactoglobulin				
Pentane, K_1	1	−7.54	−2.1	18.3
K_2	0.10	−6.19	−0.9	17.7
Iodobutane	0.43	−7.03	−4.0	10.2
Butane	0.16	−6.44	−1.1	17.8
Xenon	(0.011–0.031)[b]	−(4.8–5.5)	...	...
Dodecyl Sulfate				
Pentane	1	−5.82	−1.1	15.8
Iodobutane	0.46	−5.36	...	...
Butane	0.31	−5.13	0.0	17.2
Xenon	0.0081	−2.96	...	...

[a] Calculated on a mole fraction basis, for transfer from water to protein or micelle. [b] Different conditions; see text.

which weaken the second binding beyond the steric effect.)[3]

We have been proceeding as if it were clear that the alkanes do not bind at some special trough right at the surface of the lactoglobulin molecule. This requires justification. We have discussed at some length (Wishnia and Pinder, 1964a) the values of ΔH and ΔS expected if either the making or breaking of gas hydrate-like cages were involved in binding; these are not encountered in this work. Instead, the thermodynamic parameters have the values one expects for the transfer of an apolar solute to any hydrophobic milieu; the order of binding of pentane, iodobutane, butane, and xenon to β-LG and to dodecyl sulfate micelles is the same, and the magnitudes are, within reason, quite similar (see Table III). (The standard states are not strictly comparable, so that both ΔF and ΔS contain arbitrary terms. Still, if one treats the interaction between the bound solute and both apolar environments in terms of a cell model, the same van der Waals interactions, and hence the same potentials, and thus the same factors in the partition coefficients, appear (Hill, 1956). One might expect the entropy of an alkane and its β-LG binding site to be close to the entropy of an alkane in a micelle. As is well known, the major contribution to the entropy change comes from the water–alkane interaction, which is, of course, the same in both cases.)

It is still possible that the site consists of exposed apolar residues which are not surrounded by specific icebergs, but which only shift the distribution of bond types in neighboring water molecules the way free alkanes do. It is not enough to say that exposure of such residues on the scale required is improbable on thermodynamic grounds, since the existence of specific binding sites is also improbable. The fact that the intrinsic dissociation constant for butane is larger than it ought to be (six, rather than three or four, times the first pentane constant) may reflect a cooperative folding of the site, most favorable at the level of one pentane or iodobutane. However, perhaps out of prejudice, we feel that binding at a site of this kind would not remove enough hydrophobes from contact with water to produce the observed strong affinities. [The dissociation constants for lactoglobulin are the smallest we have found in *ca.* 12 proteins. The constants for hemoglobin and myoglobin, involving interior, hydrophobic (if somewhat special) sites near the hemes, are three to six times larger (Wishnia and Pinder, 1966).] Our view of the site as a hydrophobic region, close to the surface, but not exposed to solvent (*e.g.*, a normally closed cleft), is certainly consistent with the data.

Dimer Joint. We cannot describe what the dimer joint is, but only what it is not. It is probably not a large structure of any sort, and it is certainly not a large hydrophobic structure. If the hydrophobic interfaces between subunits of serum albumin, which are also disrupted at low pH, bind six molecules of pentane at the vapor pressure, similar structures in a lactoglobulin dimer ought to bind three. There is no evidence that binding to the dimer exceeds the monomer

[3] Before we leave this subject we must discuss a xenon-binding result which, if literally applicable, knocks the preceding argument into a cocked hat. As an afterthought to another project (Wishnia and Pinder, 1966) we did one smooth run on β-LG-A at 0°, pH 7, in 2 M ammonium sulfate (partly with an eye to crystallographic possibilities [Schoenborn *et al.*, 1965] and partly because the salting out of xenon gives more acceptable protein–solvent counting ratios). The fit for two disparate constants ($K_1 = 5.3 \times 10^{-3}$ M, $K_2 = 47 \times 10^{-3}$ M) is measurably better than the fit for two noninteracting sites with a single intrinsic constant ($K_0 = 14.4 \times 10^{-3}$ M) (corrected for xenon activity coefficient), whereas xenon should occupy less than half the volume of butane. Two not entirely compatible lines suggest a way out: (1) Ammonium sulfate salts out hydrocarbons more effectively than it does xenon; only the more compact configurations of the site may now have favorable energies, and its capacity to expand may be more limited. (2) In hemoglobin and myoglobin, where an interior site is involved (Schoenborn *et al.*, 1965; Wishnia and Pinder, 1966), xenon and pentane behave as if nearly equivalent. However, the magnitude of the dissociation constant is in line with the other values for β-LG and dodecyl sulfate, making it unlikely that the xenon–protein interaction is peculiar.

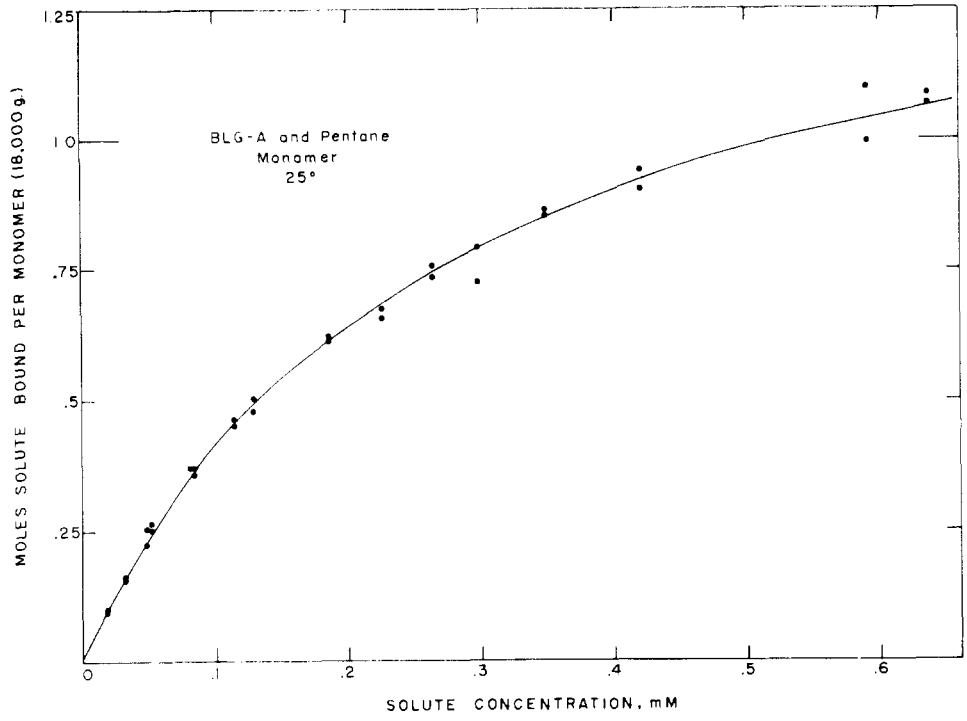

FIGURE 7: Pentane binding to β-LG-A monomer at 25°.

values at all, certainly not by as much as 0.1 mole/18,000 g.

We do not think it is necessary to account for the discrepancy between the observed entropy of dissociation, 24 eu, and the value of 95 eu one can estimate for the gain in rotational and translational entropy, on the basis of the immobilization of water by the newly exposed monomer surface (Timasheff and Townend, 1961a). [It may be fatuous to apply a theory, appropriate to diatomic molecules in a gas, to the calculation of either the rotational or the vibrational entropy of protein dimers in solution, but it is certainly wrong to think that the excess vibrational entropy is negligible. One can evaluate the parameters for a Lennard-Jones potential between two molecules (*e.g.*, any of the C_4–C_5 alkanes) from gas critical data (Hill, 1956), and compute the force constants and frequencies for small-amplitude vibrations of two alkanes in contact under van der Waals forces. A naive extension to β-LG, allowing from 1 to 10 pairwise interactions, gives 13–11 eu for one of the six additional vibrational modes the dimer must have. Vibrational entropy may recoup much or all of the translational and rotational entropy loss on dimerization.][4] Moreover, such a mechanism leaves the observed ΔH, +12 kcal, unexplained. Entropy decreases of the proposed magnitude associated with the nucleation of real ice cages around the exposed groups would be accompanied by large negative enthalpies (−10 to −20 kcal). A less specific exposure akin to dissolving alkanes in water would give small enthalpy changes. If one wants to think in these terms, and if the other effects actually canceled, a ΔH of 12 kcal and a ΔS of 24 eu could be obtained if association resulted in the formation of a single gas hydrate-like cage on the surface of the dimer.

Similar arguments can be applied to octamer formation, although Townend and Timasheff (1960) are almost certainly correct in suggesting that the additional aspartyl residue of β-LG-A plays a significant role. It is a curious fact, for which we offer no explanation, that β-LG-B, which does not form an octamer, and which has a more stable dimer, also shows consistently higher affinities for butane and pentane than β-LG-A. It gives food for thought to recall that the unique residues of β-LG-B, glycine, and alanine, permit the greatest range of peptide configurations, whereas the constraints imposed by aspartate and valine, the unique residues of β-LG-A, are, for different reasons, among the most stringent.

[4] It is well to realize that vibration and translation are not necessarily distinct concepts, when one is dealing with noncovalent interactions in condensed phases. Most theories of the liquid state are really applicable to crystals; if the van der Waals forces are the same, then solid and liquid differ by only the small communal entropy term, at most 2 cal/deg; if the number, type, and distance of near neighbors change, other terms change also.

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