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Aromatic Hydrophobes and β-Lactoglobulin A. Thermodynamics of Binding†

Kenneth A. Robillard, Jr.,* and Arnold Wishnia‡

ABSTRACT: The protein β-lactoglobulin A forms complexes with toluene, α,α,α-trifluorotoluene, and hexafluorobenzene. By equilibrium solubility studies it is shown that there are two distinct forms of binding: a strong association which occurs at a single, localized hydrophobic region within the protein monomer; and, a weaker, multiple, association which occurs at one or several other sites on the protein. The strong binding site has an accessible volume of 200–230 ml/mole, which can accommodate two molecules of toluene or α,α,α-trifluorotoluene, or one molecule of hexafluorobenzene. The dissociation constants (millimolar units) free energies (kilocalories), enthalpies (kilocalories), and entropies (entropy units), at 25°, for strong binding are: $K_1 = 2.20$, $K_2 = 17$, $\Delta G_1^\circ = 6.0$, $\Delta H_1^\circ = 2.8$, $\Delta S_1^\circ = -11$ for toluene; $K_1 = 2.39$, $K_2 = 32$, $\Delta G_1^\circ = 6.0$, $\Delta H_1^\circ = 2.8$, $\Delta S_1^\circ = -11$ for trifluorotoluene; and, $K = 0.64$, $\Delta G^\circ = 6.78$, $\Delta H^\circ = 1.6$, and $\Delta S^\circ = -17$ for hexafluorobenzene. The weaker dissociation can be described by the equation $r = (1/k) \cdot C$, where r is (moles of ligand bound per mole of protein monomer) and C is the free ligand concentration. For toluene at 25° $k = 7.7$ mM, for α,α,α-trifluorotoluene $k = 8.0$, and for hexafluorobenzene $k = 17$. Binding studies on the separated monomer subunits indicate that dimerization does not affect either the strong or the weak associations.

β-Lactoglobulin A (BLG-A)[1] has been shown to have a distinct hydrophobic region of limited capacity (200–230 ml of ligand/mole of protein monomer) and unusually high and stereoselective affinity for small alkanes (Wishnia and Pinder, 1966). ΔG° for strong ligand binding is comparable to the "ideal" ΔG° for transfer of the ligand from water to the pure liquid, and more negative than the ΔG° for transfer from water to the interior of dodecyl sulfate micelles. The ΔC_p° for the three processes is similar, which is a diagnostic for so-called hydrophobic interactions; but ΔH° for ligand binding is more negative by 3–4 kcal over the entire range from 0 to 50°. This last was attributed to a normalization, when appropriate ligands are bound, of some suboptimal interaction or "strain" in the *native* molecule; one plausible source of strain is that the packing of the side chains in the hydrophobic site, determined by the surrounding framework, is less dense than normal by 50–80 ml/mole, precluding optimal van der Waals contacts, whereas the ligand–site complex has a normal density and good contacts (Wishnia, 1969a).

Since thermodynamic studies provide no direct information regarding the identity or flexibility of the amino acid moieties that constitute the site, or the motional constraints upon the bound ligand molecule, and no data at all on the kinetics of binding, we thought it useful to reinvestigate ligand–protein associations by two complementary techniques, equilibrium binding studies and nuclear magnetic resonance spectroscopy (see Robillard and Wishnia, 1972). Therefore, we studied the interaction between βLG-A and the aromatic compounds toluene, α,α,α-trifluorotoluene, and hexafluorobenzene. (The latter two, with several equivalent fluorine nuclei, both have a single line ^{19}F nuclear magnetic resonance (nmr) spectrum, an important advantage at low concentrations (2–3 mM), where sensitivity is critical.) In the binding studies, we were primarily looking for three properties of these ligands. (1) Do they bind to the same site as the alkanes (does the binding of one mole of dodecyl sulfate per mole of protein prevent aromatic binding as it does alkane binding)? (2) Are the thermodynamic parameters similar? (3) Does the substitution of fluorine atoms for the three methyl hydrogens of toluene change the ligand's interaction with the protein?

† From the Department of Chemistry, State University of New York at Stony Brook, Stony Brook, New York 11790. *Received September 16, 1971.* Supported by funds from National Science Foundation Grant GB-16060 and taken from a dissertation submitted by K. R. in partial fulfillment of requirements for the Ph.D. degree, State University of New York at Stony Brook, 1971. Presented in part at the 62nd Annual Meeting of the American Society of Biological Chemists, San Francisco, Calif., June 1971.

‡ National Defense and Education Act Predoctoral Fellow, 1966–1969. Present address: Research Laboratories, Eastman Kodak Co., Rochester, N. Y.

[1] Abbreviations used are: βLG-A, β-lactoglobulin A; $PhCF_3$, α,α,α-trifluorotoluene; PhF_6, hexafluorobenzene; in subscripts, DS, DDS, and TDS are decyl, dodecyl, and tetradecyl sulfate.

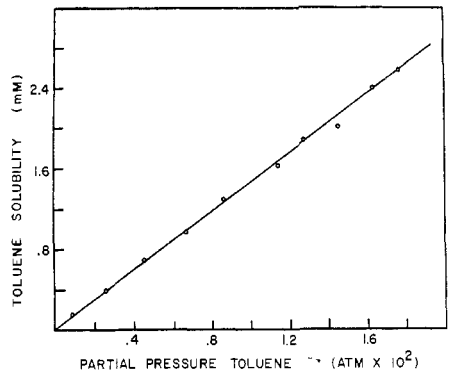

FIGURE 1: Henry's law behavior of toluene. The solubility of toluene in 0.1 M sodium acetate solution at 25° as a function of toluene partial pressure.

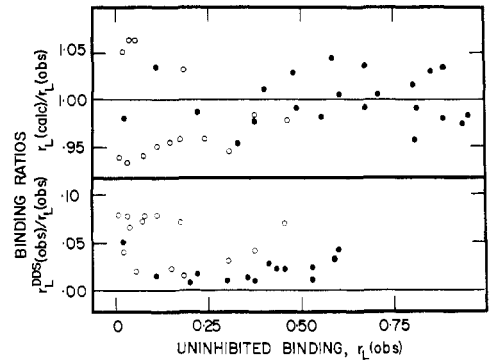

FIGURE 2: Deviation and inhibition at 0°. Upper chart shows the deviation of calculated from observed binding. (●) Pentane; K_1 = 0.124 mM, K_2 = 5.06 mM. (○) Cyclohexane; K_1 = 1.92 mM, K_2 = 0.82 mM. (25° values are 1.93 mM, 0.89 mM.) Lower chart, the remaining binding with 1 mole of dodecyl sulfate added per mole of βLG-A monomer.

Experimental Section

Materials

β-Lactoglobulin A was isolated from the milk[2] of homozygous cows according to the procedure of Aschaffenburg and Drewry (1957). The protein was twice recrystallized, mixed with toluene, and stored at 4° until use. Aqueous solutions were prepared by dissolving the protein–toluene paste in the appropriate buffer and exhaustively dialyzing to remove the toluene. 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 species (Senti and Warner, 1948). Equilibrium solubility studies of the dimer were conducted at pH 5.8; and on the monomer at pH 2.0 and low salt (Townend *et al.*, 1960; Timasheff and Townend, 1961).

Sodium dodecyl sulfate (gift, Alcolac Chemical Corp.), sodium decyl sulfate, tetradecyl sulfate (gifts, Lever Brothers Laboratory), hexafluorobenzene (>99.5% pure by chromatography) (Pierce Chemical Co.), and *n*-heptane (Allied Chemicals) were used without further purification. Octane (Eastman Organic Chemicals) was washed three times with sulfuric acid and with water, dried over $MgSO_4$, and fractionally distilled.

[α-^{3}H]Toluene (New England Nuclear) was purified by washing with sulfuric acid, potassium permanganate, and water (Wishnia, 1963). The specific activity of the toluene was determined by gravimetric dilution of the tritiated toluene into cold toluene. The reproducibility of this method was better than 0.5%.

α,α,α-[3-^{3}H]Trifluorotoluene was synthesized from 0.18 mole of 3-bromo-α,α,α-trifluorotoluene (Aldrich Chemical) *via* decomposition of the Grignard reagent in ^3H_2O, using standard methods (Cason and Rapoport, 1962). The main fraction (2.2 g, bp 103°) was shown to be pure by gas–liquid chromatography and infrared spectroscopy. The specific activity was determined as for toluene.

Methods

Solubility Studies. Experimental techniques and data reduction methods for obtaining ligand-binding isotherms have been described (Wishnia and Pinder, 1966). The solubility cell was modified to promote interchamber gas exchange. Aliquots of equilibrated buffer and protein solutions were withdrawn using weight-calibrated Hamilton syringes with precisely defined intake strokes. Concentrations of [^{3}H]ligands were determined by scintillation counting, as before; the overall sampling and counting error was 2%. Hexafluorobenzene was determined by analytical vapor phase chromatography (*cf.* Mohammadzadeh-K. *et al.*, 1967). Aliquots were delivered into vials charged with 1 ml of water and 1.00 ml of stock octane containing 0.1 mM heptane as an internal standard (previously calibrated with known dilutions of PhF_6). When extraction was complete (98 ± 2%), 1-μl samples of the octane solution were analyzed (5 ft of 3% SE-30 on 100–120 Varaport 30, Varian Model 1200 chromatograph, flame detector). Relative peak heights (PhF_6–heptane) replicated to 3%.

Activity Coefficients. It is assumed in the subsequent analysis that the activity coefficient of the hydrophobic ligand in a given aqueous solvent is constant throughout its solubility range. A study of the solubility of toluene in water as a function of its partial pressure (calculated from counting rates of samples of the gas phase) showed that Henry's law is indeed obeyed (Figure 1). The activity coefficient of toluene depends on the ionic strength of the solution: between 0.020 and 0.10 M ionic strength it increases by 10%.

Results

Typical binding curves for alkanes, toluene, trifluorotoluene, and hexafluorobenzene are shown in Figures 2–6. The isotherms for toluene and $PhCF_3$, unlike those for PhF_6, or for alkanes (Wishnia and Pinder, 1966; Wishnia, 1969a), show relatively low curvature. (In our technique *relative* errors are normally distributed, with sampling replication errors under ±2% and general standard deviations from correct curves under ±5%; this curvature, although small, is quite real and must not be underestimated.) Calculated curves require that more than two equilibria be involved (forced one- and two-site fits show characteristic systematic deviations), but fits with three or more successive equilibria produce unreasonable ratios of successive constants. Benzene binding to βLG (Mohammadzadeh-K. *et al.*, 1969) exhibits this problem in a more extreme form.

Interpretation of the isotherms is achieved if one separates the binding sites into two classes. *It is the central assumption of this study*, which we will justify below, that the binding of 1 mole of dodecyl sulfate/mole of βLG-A monomer effectively

[2] Generously provided by Dr. Charles Kiddy, Dairy Cattle Research Branch, U. S. D. A., Beltsville, Md.

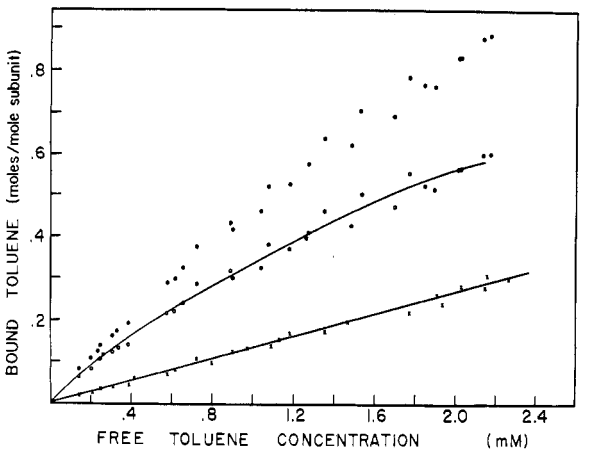

FIGURE 3: Toluene binding to βLG-A dimer at 25°, pH 5.8, and μ = 0.1. (●) Binding to the native protein; (×) binding to the βLG-A–sodium dodecyl sulfate complex; (○) binding to the strong binding site. The solid line is the calculated binding curve for a model of two successive dissociation constants with K_1 = 2.20 mM and K_2 = 17.0 mM.

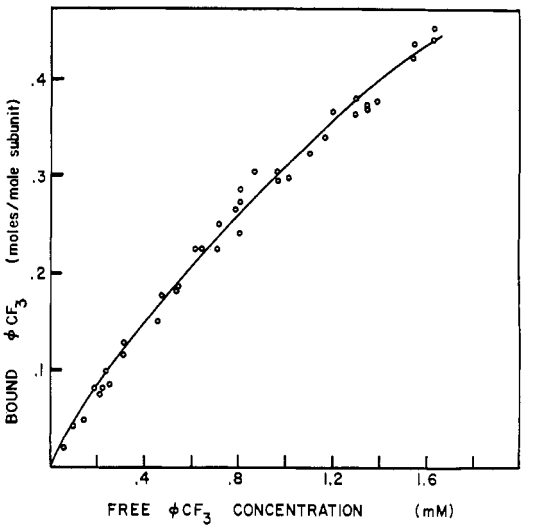

FIGURE 5: $PhCF_3$ binding to the strong binding site of βLG-A, at 25°, pH 5.8, and μ = 0.1. The solid line is the calculated binding curve for a model of two successive dissociations with K_1 = 2.39 mM and K_2 = 32.3 mM.

precludes binding of the other ligands to one class, the "strong" site(s), while leaving the second, "residual," sites essentially unperturbed. The operational application of this assumption is that binding to the residual sites, denoted by r_L(res), molecules of ligand L per subunit, at free ligand concentration c_L, can be obtained independently: namely, r_L(res) = r_L^{DDS}(obsd); then r_L(strong) = r_L(obsd) − r_L^{DDS}(obsd).

The binding of toluene to the decyl sulfate, dodecyl sulfate, and tetradecyl sulfate complexes of βLG-A, at 25°, is shown in Figure 4. The isotherms for dodecyl sulfate–βLG-A and tetradecyl sulfate–βLG-A are virtually identical and, like the isotherms for dodecyl sulfate–βLG-A for all ligands and temperatures, do not depart sensibly from the straight lines $r_L = c_L/k_L$ over the observable range of c_L. The values of k_L are given in Table I.

The derived isotherms for strong-site binding have unambiguous curvatures and yield readily to familiar, straightforward analysis. At most two successive equilibria are required: PL = P + L, K_1 = (P)(L)/(PL); PL_2 = PL + L (K_2); P denotes the 18,000 molecular weight βLG-A subunit. We obtain the best values of K_1 and K_2 from nonlinear least-squares fits of the equation $r_L(\text{strong}) = (c_L/K_1 + 2c_L^2/K_1K_2)/(1 + c_L/K_1 + c_L^2/K_1K_2)$. For PhF_6, only one equilibrium is needed: in forced fits to two, K_2 often diverges and is always negligibly large. For toluene, the observable range of binding, $0 \leq r_L(\text{strong}) \leq 0.6$, is quite adequate to establish that a second, weaker, association is occurring (*cf.* Wishnia and Pinder, 1966). The computed values of K_1 are precise to 3% and, moreover, insensitive to the number of additional equilibria one chooses to invoke. The uncertainty in K_2 is perhaps 20–30%. K_3, if invoked, typically diverges (Figure 3). The $PhCF_3$ data behave similarly. It should be noted that for the conclusions we wish to draw we only require that K_2 be substantially larger than $4K_1$, which is the case. The values of the dissociation constants at 0 and 25° are listed in Table I.

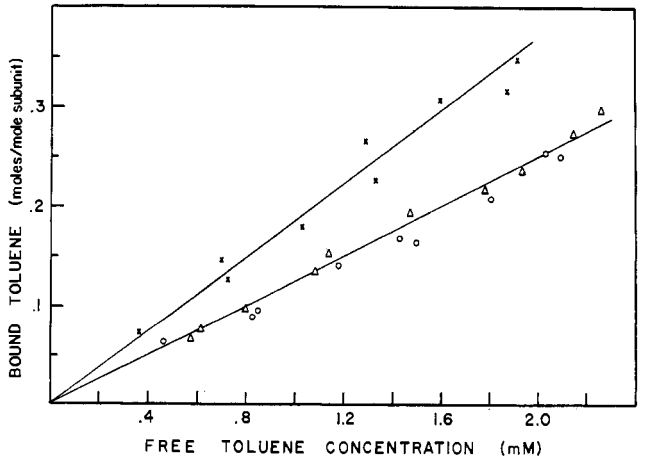

FIGURE 4: Toluene binding to βLG-A–detergent complexes at 25°, pH 5.8, and μ = 0.1. (×) βLG-A–decyl sulfate; (△) βLG-A–dodecyl sulfate; and (○) βLG-A–tetradeycl sulfate.

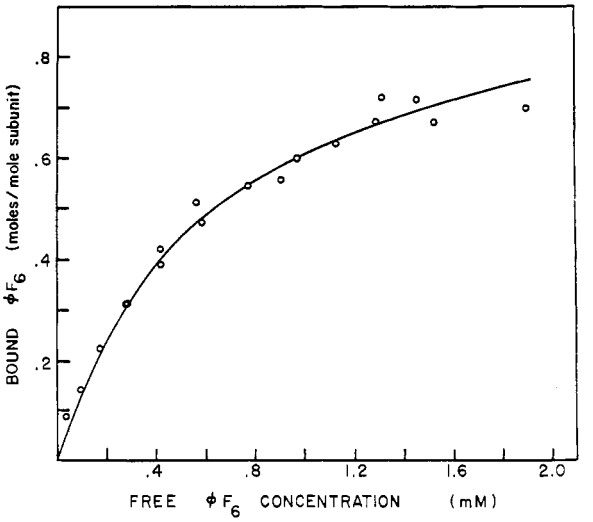

FIGURE 6: PhF_6 binding to βLG-A at 25°, pH 5.8, and μ = 0.1. The solid line is the calculated binding curve for a model of one dissociation with K = 0.64 mM.

TABLE I: Binding of Aromatic Ligands to βLG-A Dimer.

Ligand	Temp (°C)	K_1 (mM)[a]	K_2 (mM)[a]	k (mM)[a]
Toluene	25	2.20	17.0	7.7
	0	1.76	12.4	9.5
$PhCF_3$	25	2.39	32.3	8.0
	0	1.79	14.5	10.8
PhF_6	25	0.64	$\gg K_1$	17.0
Toluene binding to βLG-A monomer				
	25	2.43[b]	15.9[b]	7.0[b]

[a] Dissociation constants at pH 5.8, ionic strength 0.10. [b] Dissociation constants at pH 2, ionic strength 0.02.

TABLE II: Thermodynamic Quantities for Transfer of Ligands from Hydrophobic Environment to Water.[a]

Ligand–Hydrophobic Environment	$\Delta G°$ (kcal/mole)	$\Delta H°$ (kcal/mole)	$\Delta S°$ (eu)	$\Delta C_p°$ (cal/(mole deg))
Toluene–βLG-A[b]	6.00	2.8	−11	82
Toluene–sodium dodecyl sulfate micelle	4.84	0.3	−15	53
Toluene–liquid toluene	5.47	−0.1	−19	87
$PhCF_3$–βLG-A[b]	5.95	2.8	−11	83
$PhCF_3$–sodium dodecyl sulfate micelle	5.29	0.4	−17	82
PhF_6–βLG-A[b]	6.78	1.6	−17	55
PhF_6–sodium dodecyl sulfate micelle	4.76	−1.5	−21	116
Pentane–βLG-A[c]	7.60	3.3	−15	140
Pentane–sodium dodecyl sulfate micelle[c]	5.82	−0.4	−21	103
Butane–βLG-A[c]	6.54	2.9	−12	71
Butane–sodium dodecyl sulfate micelle[c]	5.01	−0.7	−19	67

[a] All values are at 25°. [b] Binding to the dimer; only binding to the strong site is considered. [c] Values are taken from the work of Wishnia (1969a).

The temperature dependence of K_1 was examined more closely (5° intervals between 0 and 40° for toluene (Figure 7) and $PhCF_3$, 10–40° for PhF_6). In addition, the partition of the ligands between water, dodecyl sulfate micelles, and vapor phase, in the range 10–50°, was studied. Using the same mole-fraction-oriented standard states as previously (Wishnia, 1969a), the standard free energies for the transfer of ligand from the hydrophobic environment (strong site, micelle interior, neat liquid) to water were computed. From the fitting of the cubic equation $\Delta G° = A + BT + CT^2 + DT^3$, one derives $\Delta S°$, $\Delta H°$, and $\Delta C_p°$ as usual. The values of these quantities at 25° are given in Table II.

In all respects the behavior of toluene is almost indistinguishable from that of $PhCF_3$. Both exhibit substantial binding to dodecyl sulfate–βLG-A (r_L(res)/r_L(strong) reaches 0.5), in contrast to other ligands (for PhF_6 the ratio is 0.05 at low c_L, and reaches 0.2 only when $c_L \gg K_1$; for pentane, butane, and cyclohexane the ratio ranges from 0.00 to 0.05 at low c_L (Figure 2)). The curvature of the r_L(strong) component is sufficiently large to establish that $4K_1 < K_2 \ll K_3$. The behavior of βLG-A and dodecyl sulfate–βLG-A at pH 5.8 (dimer) is not significantly different from their respective behavior at pH 2.0 (essentially monomer). (Incidentally, this vitiates any model in which the complexities of the toluene–$PhCF_3$ results are attributed to subunit interactions.) For all three ligands the behavior of K_1 produces the large $\Delta C_p°$, with increases in $T\Delta S°$ compensating increases in

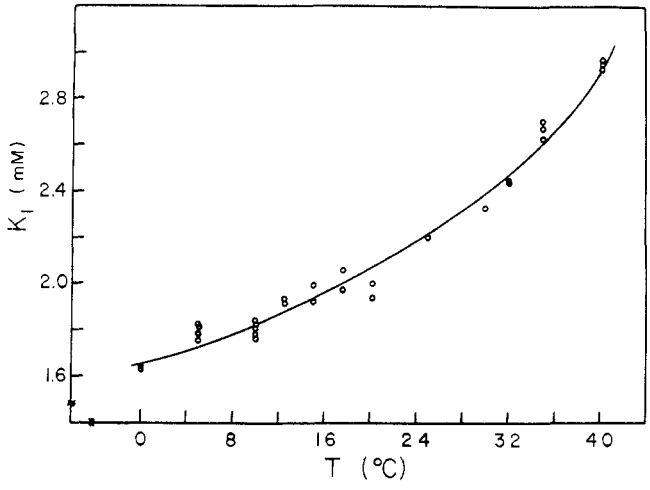

FIGURE 7: The temperature dependence of K_1 for toluene binding to βLG-A, pH 5.8 and $\mu = 0.1$.

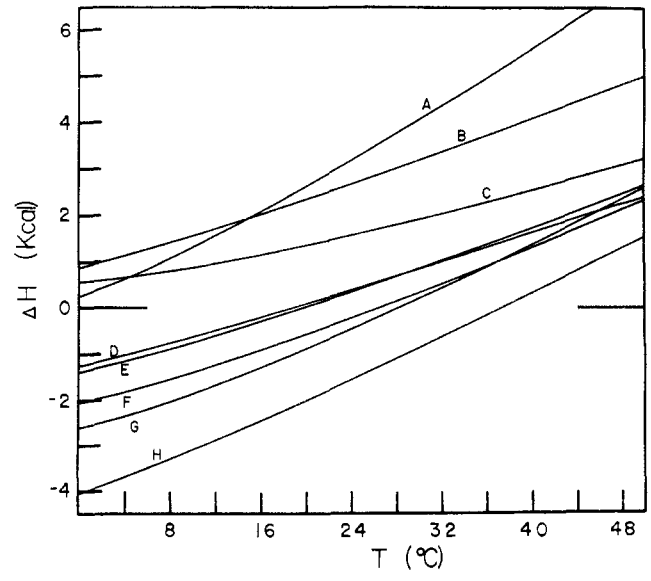

FIGURE 8: Enthalpies for the transfer of ligand molecules from a hydrophobic environment to water. Ligand–hydrophobic environment: (A) pentane–βLG-A (from Wishnia (1969a)); (B) $PhCF_3$–βLG-A and toluene–βLG-A; (C) PhF_6–βLG-A; (D) $PhCF_3$–sodium dodecyl sulfate micelles; (E) toluene–sodium dodecyl sulfate micelles; (F) toluene–liquid toluene; (G) pentane–sodium dodecyl sulfate micelles (from Wishnia (1969a)); and (H) PhF_6–sodium dodecyl sulfate micelles.

$\Delta H°$, characteristic of transfers of hydrophobes from hydrophobic environments to water. The *excess* enthalpy of transfer of ligand, $\Delta H°$ (strong-site → water) − $\Delta H°$ (micelle → water), is several kilocalories, and nearly temperature independent, as previously observed for butane and pentane (Wishnia, 1969a), although the difference is 3.1 kcal for PhF_6, and 2.5 kcal for toluene and $PhCF_3$, at 25°, rather than 3.6 kcal (Table II, Figure 8).

Discussion

The behavior of PhF_6 and, if one grants our fundamental assumption, toluene and $PhCF_3$, clearly parallels that of iodobutane or pentane, both with respect to dodecyl sulfate inhibition and in thermodynamic parameters; in particular, the excess enthalpies of dissociation are comparable. We want to conclude, first, that dodecyl sulfate, alkanes, and the aromatics bind to the same restricted site, and second, that the aromatics must share the same mechanism (*viz.*, the reordering and optimizing of side-chain–side-chain and side-chain–ligand contacts) which produces the unexpectedly strong binding of some favored alkanes to this region (Wishnia, 1969a).

It seems necessary to review some of the properties of the βLG-A–dodecyl sulfate complex. The stable complex contains one molecule of detergent per subunit (McMeekin *et al.*, 1949; Lovrien and Anderson, 1969; Seibles, 1969). Circular dichroic spectra and chemical reactivity differ slightly or negligibly from βLG-A itself (Seibles, 1969). Ultracentrifuge studies show the monomer, the dimer, and the monomer–dimer equilibrium to be largely unaffected, although the dimer–octamer association is virtually wiped out (Wishnia, 1969b); similar conclusions may, with hindsight, be drawn from electrophoretic studies (*vide* McMeekin *et al.*, 1949). The dodecyl sulfate association constant, at 25°, pH 5.4, is reportedly 1.2×10^5 M^{-1} (Ray and Chatterjee, 1967), but calculated from spotty data and for a model of three equivalent sites per dimer. Minimum values compatible with the effects of dodecyl sulfate on pentane and PhF_6 binding are at least an order of magnitude larger, perhaps lying between 10^6 and 10^7 M^{-1} at 25°.

The effect of dodecyl sulfate on ligand binding is best understood as strict competitive inhibition. Earlier mutual inhibition studies (Wishnia and Pinder, 1966) had led to the conclusion that the alkane-binding "sites" formed a continuous region, so that the ratios of successive dissociation constants for a given ligand depended mostly on ligand volume: for hexane (W. Stone and A. Wishnia, unpublished observations), iodobutane, and PhF_6, $K_2 \gg 4K_1$; for pentane (and, in our view, for the strong binding of toluene and $PhCF_3$), $K_2 > 4K_1$; for butane, $K_2 = 4K_1$; for cyclohexane, the binding is cooperative, with $K_2 < K_1$. For orientation, the molar volumes of the neat liquids at 25° (which can only approximately reflect specific interactions at the hydrophobic site) are: butane, pentane, hexane, cyclohexane, benzene, toluene, $PhCF_3$, and PhF_6, 100, 116, 132, 107, 89, 107, 125 (Dreisbach, 1959), and 117 ml (Counsell *et al.*, 1965), respectively. The inclusion of 210 ml/mole of dodecyl would exhaust the expansive capacity of the specific site, preventing further binding to the βLG-A–dodecyl sulfate complex. Dodecyl sulfate and βLG-A are present in stoichiometric amounts, at 1 mM (N.B. adding 5 and 10% excess dodecyl sulfate produces no significant change); any displacement of dodecyl sulfate by other ligands would produce free concentrations of dodecyl sulfate several to manyfold larger than its dissociation constant ($K_{DDS} \leq 10^{-6}$ M), a far larger ratio than is available to the other ligands. There seem to be only local perturbations of the protein structure; certainly no new general hydrophobic sites appear.

The free-energy increment per methylene group for transferring *n*-alkanes from unstressed hydrophobic regions to water is 0.8 ± 0.1 kcal/mole (*vide*, *e.g.*, Wishnia, 1963). The specific hydrophobic region of βLG-A is not unstressed. However, the equivalence of the first and second butane "sites" requires that the relief-of-strain contribution also be smoothly incremental, as is also true in the butane–pentane–hexane series. The average increment per CH_2 is 1.07 kcal/mole. (One CH_3 is the equivalent of two CH_2.) The enthalpy of dissociation of βLG-A–dodecyl sulfate, +8.8 kcal/mole at 25° (Lovrien and Anderson, 1969), somewhat more than double the pentane value, suggests that dodecyl sulfate, and so necessarily decyl sulfate, also share the same relief-of-strain mechanism. In that case, K_{DS} should be 30–50 times larger than K_{DDS}. The increment for the additional CH_2CH_2 of tetradecyl sulfate, following the pentane data, would be less, perhaps 0.5 kcal/mole of CH_2.[3]

To repeat, there are *two* odd aspects to the interaction of βLG-A with toluene and $PhCF_3$: the modest curvature of the binding isotherms (which imposes three-parameter fits, whereas the sharply curved isotherms for PhF_6 and *n*-alkanes require at most two), and the substantial binding of toluene and $PhCF_3$ to the βLG-A–dodecyl sulfate complex. Our hypothesis is that the residual sites preexist, are distinct from, and do not interact with the specific site. This simplest model has the nontrivial virtue that the second abnormality normalizes the first. The three conceivable models for extensive residual binding which do not share this virtue are: strict competition (no mixed βLG-A–dodecyl sulfate–ligand complexes); special creation of new, distant, sites for aromatic molecules only; partial competition: the toluene–$PhCF_3$ sites interact with, or overlap, but do not coincide with, the PhF_6–alkane–dodecyl sulfate site. Strict competition fails categorically on two grounds: the markedly different ^{19}F nmr chemical shifts for ligands in residual as compared to strong sites *require* mixed βLG-A–dodecyl sulfate–ligand complexes (Robillard and Wishnia, 1972); and, if a gamut of compounds, whose single or multiple affinities for βLG-A are all greater than those of toluene or $PhCF_3$, cannot displace dodecyl sulfate from its site, then neither, *a fortiori*, can the latter. Special creation does violence to Occam's criterion: where previously one hypothesis rendered all the data self-consistent, we now need three separate, *ad hoc*, and not particularly straightforward, explanations for toluene binding to the βLG-A–dodecyl sulfate complex, for the peculiarities of toluene binding to βLG-A, and for the much greater binding

[3] The role of the head group is problematic. The dodecyl radical is larger than pentane by six equivalent methylene groups, contributing factors of 2×10^3 (unstressed), or 5×10^4 (stressed) in binding constant; the actual ratio is 10^2–10^3. Thus the sulfate does not help, and may hinder, binding of dodecyl sulfate. The issue is complicated, however: inhibition studies have been carried out for a series of alkyltrimethylammonium bromides, RMe_3NBr (K. A. Robillard and A. Wishnia, unpublished observations). The toluene-binding isotherm, in the presence of one equivalent of dodecyltrimethylammonium bromide, is indistinguishable from the curve for pure βLG-A. Tetradecyl-Me_3NBr produces about 40% inhibition (less than decyl sulfate), while hexadecyl-Me_3NBr produces about 70% (slightly less than dodecyl sulfate). Parallel effects on the dimer–octamer equilibrium are observed. The large cationic head group apparently fouls something strongly enough to keep the nearest five or six CH_2 groups out of the site.

of benzene.[4] Moreover, dodecyl sulfate binding would have to produce several new toluene sites without changing most of the observable properties of βLG-A.

The model of partial competition between interacting sites seems more plausible. First, since new equilibria are postulated (PDL = PD + L, K_1'; PDL_2 = PDL + L, K_2'; *etc.*), providing two or three additional parameters, fits of the lines in Figure 4 are successful. Second, the mixed complexes required by the nmr results are permitted. The primary objection is still the benzene binding.[4] In addition, for toluene, the quantitative features of the model are implausible. Having fitted for K_1', K_2', (K_3'), one may calculate the related *detergent* dissociation constants (PDL = PL + D, $K_D^{(1)}$; PDL_2 = PL_2 + D, $K_D^{(2)}$). It turns out that the binding of 1 or 2 moles of toluene decreases the free energy of dissociation of dodecyl sulfate by only 0.9 and 1.5 kcal per mole. The net interaction with tetradecyl sulfate must be identical; that with decyl sulfate (DS) must be somewhat weaker (perhaps 0.7 and 1.1 kcal per mole). Further, the temperature dependence of K_1K_2 for this model is more or less the same as that of K_1 for the strong site in the residual-site model, yielding similar enthalpies and entropies. To accept the partial competition model one has to accept the following coincidences. There exists in βLG-A a hydrophobic region whose binding capacity is exhausted somewhere between dodecyl sulfate and tetradecyl sulfate (TDS), which, within that capacity, binds *n*-alkanes and the hexagonal molecules PhF_6 and cyclohexane uniquely well, but which is incapable of binding toluene, $PhCF_3$, or, probably, benzene. There exists another, parallel, hydrophobic region, of virtually the same capacity, which binds toluene, $PhCF_3$, and, presumably, benzene, which shows very similar relief-of-strain thermodynamic behavior between 0 and 40°, but which is incapable of binding PhF_6 or aliphatic compounds. The two regions interact, but so marginally that the equivalent overlap is smaller than one CH_2 per ligand.

[4] Old observations of ours (A. Wishnia, unpublished), at very low r_L and c_L, show an initial slope of 1/(2.00 mM) for the 25° isotherm. From the data of Mohammadzadeh-K. *et al.* (1969), we calculate that at 20°, in the presence of liquid benzene, c_L = 14 mM, r_L(obsd) = 5.0 molecules/subunit. Their isotherm is curved, but has not become flat. Five molecules of benzene cannot conceivably be accommodated at the main hydrophobic site; still less could the necessarily larger number of equilibria be accepted. Both sets of data are quantitatively accounted for if K_1 = 4, K_2 = 16, and k_L = 4 mM, values entirely compatible with the results for toluene, 2.20, 17, and 7.7 mM. In any case the number, *N*, of residual sites for benzene binding must exceed r_L(res,obsd) > 3. For toluene, cooperative binding with N = 2,3 could produce approximately linear isotherms in the range $0 \leq r_L(\text{res}) \leq 0.3$. Otherwise, the minimum value of *N* is necessarily greater than 3.

It seems to us that the residual-site model, in which all the ligands studied are bound in about the same way at the strong site, and in which there are a number of weak sites for aromatic molecules (affinities, benzene > toluene > trifluorotoluene > hexafluorobenzene), is overwhelmingly preferable. (Quantitative requirements are $K_{DDS} \leq 1 \times 10^{-6}$ M, $K_{TDS} \leq K_{DDS}$, $K_{DS} = 2.3 \times 10^{-5}$ M. There is then no effect of excess dodecyl sulfate, and slight dissociation of βLG-A–decyl sulfate accurately produces the additional toluene binding.) The consequences of choosing this model were discussed above.

References

Aschaffenburg, R., and Drewry, J. (1957), *Biochem. J. 65*, 273.

Cason, J., and Rapoport, H. (1962), Laboratory Text in Organic Chemistry, Englewood Cliffs, N. J., Prentice-Hall, Inc., p 449.

Counsell, J. F., Green, J. H. S., Hales, J. L., and Martin, J. F. (1965), *Trans. Faraday Soc. 61*, 212.

Dreisbach, R. R. (1959), Physical Properties of Chemical Compounds, Vols. I and II, Washington, D. C., American Chemical Society Publications.

Lovrien, R., and Anderson, W. (1969), *Arch. Biochem. Biophys. 131*, 139.

McMeekin, T. L., Polis, B. D., Della Monica, E. S., and Custer, J. H. (1949), *J. Amer. Chem. Soc. 71*, 3606.

Mohammadzadeh-K., A., Feeney, R. E., Samuels, R. B., and Smith, L. M. (1967), *Biochim. Biophys. Acta 147*, 583.

Mohammadzadeh-K., A., Feeney, R. E., and Smith, L. M. (1969), *Biochim. Biophys. Acta 194*, 246.

Ray, A., and Chatterjee, R. (1967), *in* Conformation of Biopolymers, Vol. I, Ramachandran, G. N., Ed., New York, N. Y., Academic Press, p 235.

Robillard, K. A., Jr., and Wishnia, A. (1972), *Biochemistry 11*, 3841.

Seibles, T. S. (1969), *Biochemistry 8*, 2949.

Senti, F. R., and Warner, R. C. (1948), *J. Amer. Chem. Soc. 70*, 3318.

Timasheff, S. N., and Townend, R. (1961), *J. Amer. Chem. Soc. 83*, 470.

Townend, R., Weinberger, L., and Timasheff, S. N. (1960), *J. Amer. Chem. Soc. 82*, 3175.

Wishnia, A. (1963), *J. Phys. Chem. 67*, 2079.

Wishnia, A. (1969a), *Biochemistry 8*, 5070.

Wishnia, A. (1969b), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 28*, 910.

Wishnia, A., and Pinder, T. W. (1966), *Biochemistry 5*, 1534.