Aromatic Hydrophobes and β-Lactoglobulin A. Kinetics of Binding by Nuclear Magnetic Resonance†

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ABSTRACT: α,α,α-Trifluorotoluene and hexafluorobenzene complex hydrophobically with β-lactoglobulin A. Two distinct forms of binding occur: a strong association and a weak residual association. ^{19}F nuclear magnetic resonance studies show that the magnetic environments of the strong binding site and weaker binding site(s) are different. For α,α,α-trifluorotoluene, the strong binding site is 0.54 ppm upfield relative to water; the weaker binding site has a chemical shift of 0.20 ppm downfield relative to water. The corresponding values for hexafluorobenzene are 2.04 and 1.43 ppm downfield. These chemical shifts are compared to the chemical shifts of the two fluoro compounds in various solvents. For both ligands the association reactions are very fast. The influence of chemical exchange on the ^{19}F line shape of hexafluorobenzene binding at the strong binding site has been calculated for the fast-exchange limit and conditions of initial line broadening. The rate constant for the association reaction is 1.6×10^7 M^{-1} sec^{-1} at 27°; an energy of activation only 1 ± 1 kcal in excess of the diffusion-controlled limit is estimated.

In the previous paper (Robillard and Wishnia, 1972) we showed that the specific hydrophobic region of β-lactoglobulin which binds alkanes and dodecyl sulfate also binds one molecule of hexafluorobenzene, toluene, or α,α,α-trifluorotoluene very tightly, and a second molecule of toluene or $PhCF_3$[1] less readily. A class of weaker, "residual" sites of indeterminate number binds lesser amounts of toluene or $PhCF_3$, and still smaller amounts of PhF_6.

The aromatic ligands also show the same interesting enthalpy-derived "hyperstrong" binding to the specific site previously noted for alkanes (Wishnia, 1969); their static and dynamic behavior may be taken as representative of the entire class. This is fortunate, since our choice of nuclear magnetic resonance (nmr) probes was dictated in part by necessity. In typical enzyme studies (*e.g.*, Gerig, 1968; Sykes *et al.*, 1970; Taylor *et al.*, 1971), substrate concentrations on the order of 0.05–0.50 M are used; we were perforce limited to the solubility of hydrophobic ligands, the order of 10^{-3} M, as well as to comparable protein concentrations. ^1H nmr spectra of ligands would not be observable; even ligands with strong single-line ^{19}F spectra severely taxed the sensitivity of the apparatus.

We determined the ^{19}F chemical shifts of PhF_6 and $PhCF_3$ in water, bound to the strong and residual sites (in any case needed for the kinetic studies), and in a number of solvents. Since ^{19}F chemical shifts are sensitive to a variety of nonbonded interactions (*vide*, *e.g.*, Emsley and Phillips, 1966), it was hoped that some insight into the nature of the binding sites might be gained.

The kinetic analysis of PhF_6 binding to βLG-A was made using classical theory (Gutowsky *et al.*, 1953) on the measured line widths of the ^{19}F nmr spectra obtained at low, increasing, concentrations of protein (initial broadening conditions). For slow H_0 field sweep and low H_1 radiofrequency field intensity the time derivatives of the transverse ($M_+ = M_x + iM_y$) and longitudinal (M_z) components of the magnetization vanish, and the Bloch differential equations reduce to a set of equations linear in the $M_+^i(\omega)$, the contributions of each species i to the total transverse magnetization at the experimental frequency ω (*cf.* Johnson (1965), eq 48 and 2-8)

$$-M_+^i(1/T_{2i} + 1/\tau_i + i(\omega_i - \omega)) + \Sigma_{j\neq i} M_+^j p_{ji}/\tau_j = -i\omega_r M_0 f_i \quad i = 1, \ldots, n \quad (1)$$

$\omega_r = \gamma H_1$ and $M_0 = \chi_0 H_0$ are, in effect, arbitrary scale factors; i is, of course, $(-1)^{1/2}$. The T_{2i}, ω_i, and f_i are the intrinsic transverse relaxation times, natural angular resonant frequencies, and fractions, respectively, of nuclei in each state i. Equation 1 is the prescription for constructing a spectrum: given the other parameters, one determines the imaginary part of ΣM_+^i for all ω. For the case where one species, say 1, predominates, and other species (2, 3) exchange only with 1, a simpler equation may be used (Swift and Connick (1962), eq 7)

$$1/T_{2,obsd} = 1/T_{21} + \Sigma_{j\neq 1}\tau_{1j}^{-1}\left[\frac{1/T_{2j}^2 + 1/(T_{2j}\tau_{j1}) + (\omega_j - \omega_1)^2}{(1/T_{2j} + 1/\tau_{j1})^2 + (\omega_j - \omega_1)^2}\right] \quad (2)$$

$1/T_{2,obsd}$ is the experimental half-width (in radians per second) at half-height.

The τ_i are mean residence times, *i.e.*, the inverses of pseudo-first-order rate constants for the exit of nuclei with the magnetization of state i to all other states; τ_{ij} is the inverse constant for exit to state j; p_{ji} is a relative fractional exit rate, the probability that transfers out of state j will be to state i. They are all formally related to the equivalent irreversible chemical reactions. If a nucleus in L is involved in two equilib-

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[1] Abbreviations used are: βLG-A, β-lactoglobulin A; $PhCF_3$, α,α,α-trifluorotoluene; PhF_6, hexafluorobenzene.

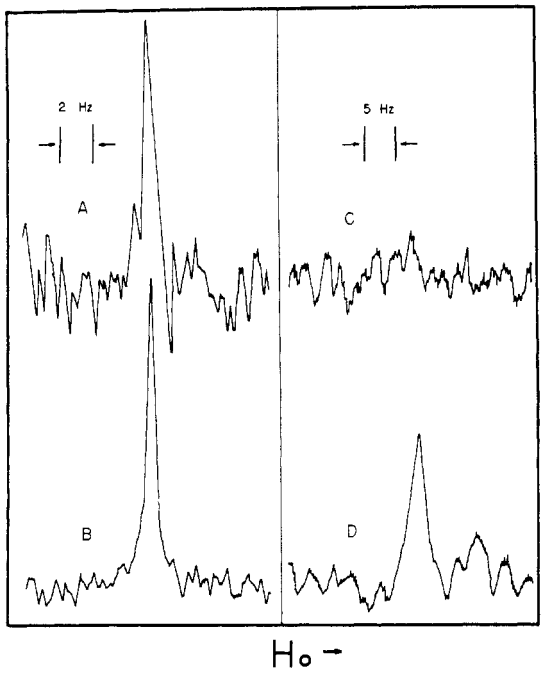

FIGURE 1: The ^{19}F resonances of $PhCF_3$ and PhF_6 in aqueous solution. (A) PhF_6, 3.4 mM, a single scan; (B) an average of ten scans; (C) $PhCF_3$, 2.5 mM, a single scan; (D) an average of 100 scans.

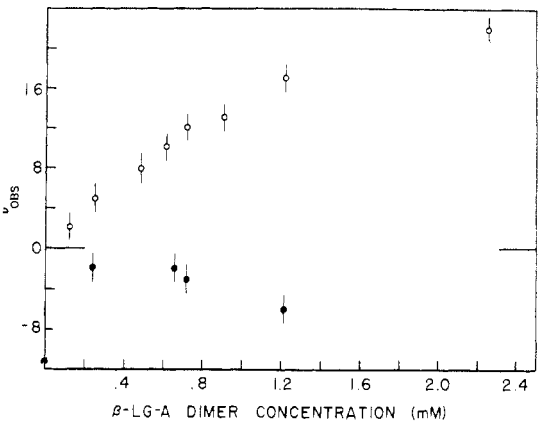

FIGURE 2: The ^{19}F chemical shift of $PhCF_3$ as a function of protein concentration. (○) Native βLG-A; (●) βLG-A–dodecyl sulfate. Chemical shifts are expressed relative to $PhCF_3$ in water. Positive shifts are at higher applied field.

ria, L + A = AL, L + B = BL, then, labeling states L, AL, and BL by 1, 2, and 3, $1/\tau_1 \equiv d[L]/dt/[L] \equiv k_{12}[A] + k_{13}[B]$, $1/\tau_{12} = k_{12}[A]$, $p_{12} = k_{12}[A]/(k_{12}[A] + k_{13}[B])$, $1/\tau_{21} \equiv k_{21}$, and so forth.

We have used both nonlinear least-squares methods on eq 1, and graphical methods on eq 2, to obtain the four rate constants k_{12}, k_{13}, k_{21}, and k_{31}, of the βLG-A–PhF_6 system.

Experimental Section

The provenance and purification of the compounds used in this work, β-lactoglobulin A, [3-^{3}H]α,α,α-trifluorotoluene, hexafluorobenzene, and sodium dodecyl sulfate, are given in the previous paper (Robillard and Wishnia, 1972). Solvents were high-quality commercial products.

The $PhCF_3$–βLG-A solutions were prepared either by external equilibration with saturated [^{3}H]$PhCF_3$ vapor, transfer to nmr tubes, and subsequent analysis by tritium counting, or simply by adding liquid $PhCF_3$ directly to the nmr tubes, as was done with PhF_6. Both methods gave the same results. In the presence of liquid, the free ligand concentration is its solubility; in any case, the concentrations of free and bound ligand, free and occupied binding sites, are readily computed (Robillard and Wishnia, 1972).

All ^{19}F nmr spectra were obtained with a Varian HR-100 nmr spectrometer operating at 94.1 MHz (Varian 4311 radio frequency unit) in the center-band detection mode (V3521 Integrator). Chemical shifts were determined with respect to the positions of modulated reference signals (either internal solute or capillary) using a Hewlett-Packard Model 200AB audiooscillator. All spectra were obtained at ambient temperature, 27.5 ± 2°.

For the aqueous solutions of $PhCF_3$ and PhF_6, where the signal-to-noise ratio is very low, we employed a time-averaging technique devised by Lauterbur and his students (Runde, 1970; Hutton, 1969; Ramirez, 1970), which uses an IBM 1800 computer to operate the slow sweep controls of the nmr spectrometer and to store the individual scans. Before summing, a field-drift correction, necessary with Varian HR spectrometers, was applied by aligning all scans with respect to a reference signal (in our case, the modulation side band of the 0.1 M sodium trifluoroacetate internal standard). Sweep rates, the order of 1 Hz/sec or less, were determined after each run from the observed separation of two peaks of a reference standard, whose separation had been precisely determined directly.

Results

Chemical Shifts. The spectra of α,α,α-trifluorotoluene and hexafluorobenzene in aqueous solution are shown in Figure 1. For $PhCF_3$ at its limiting solubility (2.5 mM in 0.1 M sodium acetate–0.1 M sodium trifluoroacetate, pH 5.8) the line, presumably the envelope of unresolved ^1H–^{19}F splittings, is too broad to be observed in a single scan; several hundred scans were necessary to enhance the signal:noise ratio to approximately 5:1. For hexafluorobenzene, with twice the number of equivalent nuclei and a limiting solubility of 3.4 mM, it was possible to observe the spectrum on a single scan; however, all reported chemical shift and line-width values are averages of at least 10 scans.

The binding of $PhCF_3$ and PhF_6 was studied by observing the effects of protein concentration on their ^{19}F spectra. Figure 2 shows the results for $PhCF_3$ for the accessible concentration range of βLG-A, 0–8 mM. The fraction of ^{19}F nuclei in free ligand molecules (f_1), occupying residual sites (f_2), or strong sites (f_3), may be calculated from the appropriate dissociation constants (Robillard and Wishnia, 1972). At the highest concentrations of βLG-A–dodecyl sulfate complex, f_2 was 0.26 for $PhCF_3$ and 0.15 for PhF_6. For βLG-A itself the maximum values of f_3 were 0.5 for $PhCF_3$ and 0.4 for PhF_6.

For both ligands all observed spectra consisted of a single resonance line at frequencies (ν_{obsd}) which are simple linear functions of the f_i, as in eq 3 (Figure 3). This behavior is characteristic of the fast-exchange limit; we conclude, therefore, that for both ligands the mean residence time in any state is short compared to the difference in chemical shifts between two exchanging states.

$$\nu_{(obsd)} = f_1\nu_1 + f_2\nu_2 + f_3\nu_3 \qquad (3)$$

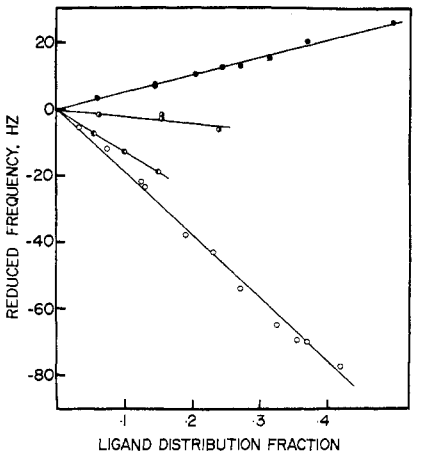

FIGURE 3: Protein concentration dependence of ligand ^{19}F chemical shifts. Ordinate, βLG-A–dodecyl sulfate: ν_{obsd}, hertz, relative to ligand in water. Abscissa, f_2. (◐) PhF_6 and (◑) $PhCF_3$. Ordinate, βLG-A: $\nu_{obsd} - \nu_2 f_2$. Abscissa, f_3. (○) PhF_6 and (●) $PhCF_3$.

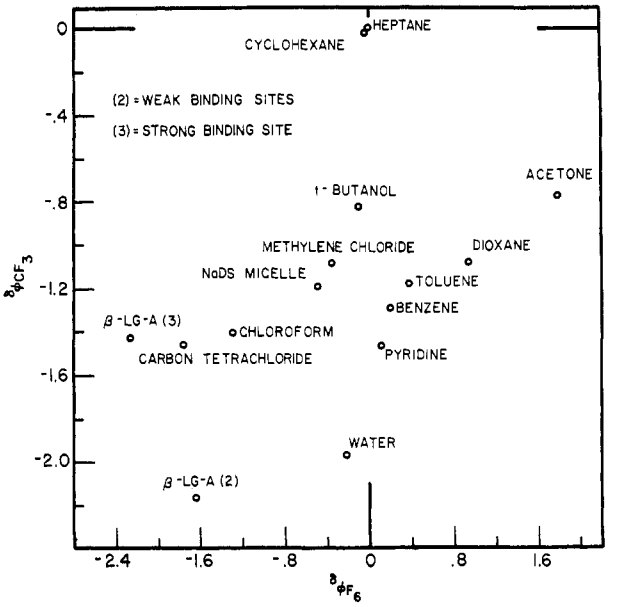

FIGURE 4: The chemical shifts of PhF_6 and $PhCF_3$ in various solvents. Chemical shifts values are relative to a 1% solution in heptane; all values of the chemical shifts have been corrected for bulk diamagnetic shielding; all nonaqueous solutions are 1% (mole/mole) in concentration; positive values for the chemical shifts are at higher applied field.

In the 1:1 (molecule–subunit) complex of dodecyl sulfate with β-lactoglobulin the strong site is unavailable for further binding (Robillard and Wishnia, 1972). With f_3 equal to zero, the chemical shift for ligand in residual sites, ν_2, may be determined from the βLG-A–dodecyl sulfate data directly. The chemical shift in strong sites, ν_3, may then be determined from the βLG-A data. For $PhCF_3$, $\nu_2 - \nu_1 = -19 \pm 5$ Hz (-0.20 ∓ 0.05 ppm); $\nu_3 - \nu_1 = 51 \pm 2$ Hz (0.54 ± 0.02 ppm). The large uncertainty for ν_2 contributes only a few per cent uncertainty to the results for strong binding. For PhF_6 both shifts are downfield: $\nu_2 - \nu_1 = -135 \pm 2$ Hz (-1.43 ± 0.02 ppm); $\nu_3 - \nu_1 = -191 \pm 2$ Hz (-2.04 ± 0.02 ppm).

TABLE I: Chemical Shifts of $PhCF_3$ and PhF_6 in Various Solvents, at 27°.

Solvent	Solvent Shift in Ppm Rel to Heptane Solvent[a,b]			
	$PhCF_3$		PhF_6	
	Obsd	Cor[c]	Obsd	Cor[c]
Heptane	0.0	0.0	0.0	0.0
Water	−2.26	−1.97	−0.51	−0.22
βLG-A				
Strong binding site	−1.72	−1.43	−2.55	−2.27
Weak binding site(s)	−2.46	−2.17	−1.94	−1.65
1.80% (w/w) sodium dodecyl sulfate micelle solution[d]	−1.48	−1.19	−0.78	−0.49
Acetone	−0.64	−0.77	1.90	1.78
Benzene	−1.35	−1.29	0.14	0.20
Carbon tetrachloride	−1.68	−1.46	−1.99	−1.76
Chloroform	−1.74	−1.41	−1.63	−1.30
Cyclohexane	−0.11	−0.012	−0.18	−0.087
Methylene chloride	−1.41	−1.09	−0.68	−0.36
Dioxane	−1.13	−1.08	0.89	0.94
Pyridine	−1.53	−1.47	0.046	0.11
tert-Butyl alcohol	−0.89	−0.83	−0.19	−0.13
Toluene	−1.29	−1.18	0.23	0.31

[a] Positive chemical shifts are at higher applied field. [b] All nonaqueous solutions are 1% (mole/mole) concentrations. [c] Corrected for bulk diamagnetic shielding. [d] Chemical shift is for micelle interior.

These data, and the chemical shifts of the two ligands in a number of solvents, are collected in Table I and compared in Figure 4. All solvent shifts have been corrected for differences due to bulk diamagnetic shielding using the factor $\Delta H/H = (2\pi/3)\Delta K$, where ΔK is the difference in volume susceptibilities of the solvents (Evans, 1960).

Kinetics of Association. The PhF_6 line-width data are shown in Table II and Figure 5. We consider that exchange occurs only between sites and solution, not between two sites on the same protein molecule. That is, ligand does not tunnel from one site to another *through* the protein, and there are no

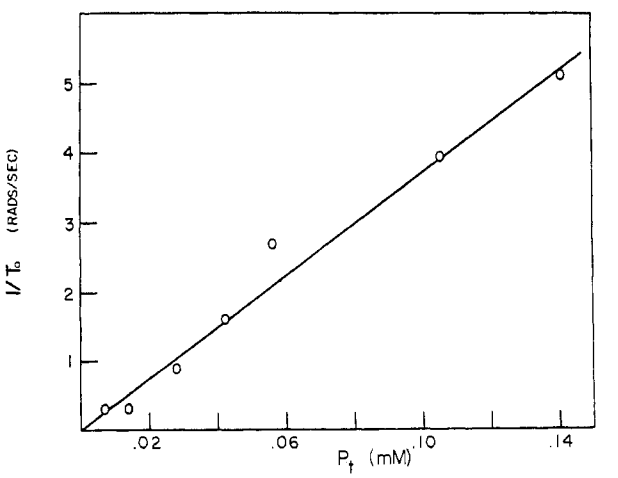

FIGURE 5: The effect of βLG-A on the ^{19}F line width of PhF_6, at 27°.

TABLE II: Line Widths and Chemical Shifts of PhF_6 in Aqueous Solutions of βLG-A–Sodium Dodecyl Sulfate at pH 5.8, μ = 0.2, and 27°.

Total Protein Concn (mM) Monomer	Chemical Shift[a] (Hz)	Half-Width[b] (Rads/sec)
0.0	0	2.5 ± 1
0.81	−7 ± 1	7.5 ± 2
1.61	−13 ± 1	12.6 ± 3

[a] Relative to the resonance of hexafluorobenzene in water. [b] The half-width is one-half the resonance width at half-height.

"local concentration" effects. Ligand dissociated from one site is considered more likely to wander into the bulk solution than to wander over to another site, or to wait for the protein to rotate another site over to it (see below). Equation 2 may be used; in eq 1 we set $p_{23} = p_{32} = 0$.

The intrinsic chemical shifts ω_1, ω_2, and ω_3, were given in the previous section. We now seek values of T_{22} and T_{23}, either to use them explicitly or to dismiss them. (The experimental line width for free ligand is proper for calculating $1/T_{2,\mathrm{obsd}} - 1/T_{21,\mathrm{obsd}}$, although the real $1/T_{21}$ is buried by instrument inhomogeneities.) Relaxation in PhF_6 is governed primarily by dipole–dipole interactions.[2] For the extreme case that PhF_6 is bound rigidly to the site, and rotates only with the protein as a whole, the dipolar part of $1/T_{22}$ or $1/T_{23}$ is 18 radians/sec (see Carrington and McLachlan, 1967, eq 11-69. The F–F distance is 2.72 Å (Almenningen *et al.*, 1964). The nmr rotational correlation time $\tau_c = 4\pi\eta a^3/3kT$ is 3.6×10^{-8} sec at 28°, using the experimental value of 35 Å as the radius of the equivalent hydrodynamic sphere. This quantity, obtained from fluorescence depolarization studies (Wahl and Timasheff, 1969) is free of assumptions regarding "microviscosity," *etc.*). We provisionally assign an upper limit of 31 radians/sec to $1/T_{2i}$ and a lower limit of 1.2 radians/sec (further reduction to the free ligand value of perhaps 0.1 radian/sec would produce minimal changes in the calculations).

[2] The literature is mostly concerned with T_1. ^{19}F chemical shifts in the fluorobenzenes are anisotropic (Nehring and Saupe, 1970), and it has been reported that this anisotropy contributes a substantial, but not the major, term to $1/T_{1F}$ for the fluorines, compared to $1/T_{1H}$ for the protons, in 1,3,5-trifluorobenzene (Gutowsky and Woessner, 1954). However, the contribution calculated from T_{1H}/T_{1F} is much larger than that calculated from the theoretical H_0^2 field dependence, $1/T_{1F}(\omega) = 1/T_{1F}(0) + \omega^2/k$. Moreover, Green and Powles (1965), examining the T_1 data for benzene, chlorobenzene, hexafluorobenzene, and fluorobenzene for the spin–rotation interaction contribution (negligible for PhF_6 at room temperature), report values at a higher frequency inconsistent with the earlier conclusions, and conclude that PhF_6 relaxes almost exclusively by the dipolar mechanism at low temperatures. The same would then be true of T_2.

Compound	Frequency (MHz)	Proton T_1 (sec)	Fluorine T_1 (sec)
$C_6H_3F_3$[a]	20	26.0	16.7
$C_6H_3F_3$[a]	26.5	26.7	15.6
C_6H_6, C_6H_5Cl[b]	60	21.6–22.6	
C_6F_6[b]	56		18.9
C_6H_5F[b]	56–60	16.7	13.7

[a] Data of Gutowsky and Woessner (1954) at room temperature. [b] Data of Green and Powles (1965) at 30°, taken from the graphs, so ±0.5 sec.

Next, we recall that $1/\tau_{i1} = k_{i1}$, $K_i \equiv k_{i1}/k_{1i}$, and $1/\tau_{1i} = k_{1i}P_i$, where P_i is the concentration of unoccupied type i sites. Further, $P_2 = NK_2P_t/(K_2 + c_L)$ and $P_3 = K_3P_t/(K_3 + c_L)$; P_t is the total βLG-A monomer concentration, c_L the free PhF_6 concentration, and N the number of type 2 ("residual") binding sites per monomer.

Now, approximations may be made in eq 2. As usual, these are judged *a posteriori*. If $A \gg B$ is hypothesized, and, in the end, indeed $A \gg B$, the approximation is valid. The denominator in the bracketed term in (2) reduces to k_{i1}^2 if $[k_{i1}/(\omega_i - \omega_1)]^2 \gg 1$. The ratios as finally calculated are 62 and 84 for $i = 2, 3$. The numerator reduces to $(\omega_i - \omega_1)^2$ if $k_{i1}/T_{2i}(\omega_i - \omega_1)^2 \ll 1$. For the allowed range of T_{2i} the ratio is 0.01–0.29 for residual sites and 0.01–0.24 for the strong site. Neglecting the term k_{i1}/T_{2i} can produce an error in k_{i1} of at most 22%. Finally, only $K_{2,\mathrm{obsd}} = K_2/N = 17$ mM is known, because at all values of c_L, $K_2 \gg c_L$. Making all these substitutions, eq 2 becomes

$$1/T_0 = 1/T_{2,\mathrm{obsd}} - 1/T_{21,\mathrm{obsd}} = [(\omega_2 - \omega_1)^2/k_{21}K_{2,\mathrm{obsd}} + (\omega_3 - \omega_1)^2/k_{31}(K_3 + c_L)]P_t \quad (4)$$

The predicted linear dependence of $1/T_0$ upon P_t, at constant c_L, is observed (Figure 5).

For the βLG-A–dodecyl sulfate complex, where the second term in brackets is zero, we obtain $k_{21} = 6.7 \times 10^3$ sec^{-1} (range, 4.3–11 × 10^3). The rate constant for association is determined only within a factor of $1/N$: $k_{12} = k_{21}/K_2 = (4 \times 10^5)/N$ M^{-1} sec^{-1}.

Our primary concern is with the strong site. From the slope in Figure 5, we obtain $k_{31} = 1.1 \times 10^4$ sec^{-1}, $k_{13} = 1.6 \times 10^7$ M^{-1} sec^{-1}. Since the residual binding term in eq 4 is small, the uncertainty in k_{21} does not produce serious uncertainty in k_{31}.

We have also obtained values of the rate constants using nonlinear least-squares methods on eq 1 directly. If we study a range of T_{2i} values between 1.2 and 31 radians per sec, it turns out that the k_{ij} are not entirely insensitive to the T_{2i}, but the variation is close to the experimental error. Thus, $k_{21} = 6.8 \times 10^3$ sec^{-1} (range, 5.1–8.6 × 10^3 sec^{-1}), and $k_{12} = 4 \times 10^5/N$ M^{-1} sec^{-1}. The value of k_{31} is independent of consistent (T_{22}, k_{21}) pairs. $k_{31} = (1.24 \pm 0.20) \times 10^4$ sec^{-1} and $k_{13} = 1.8 \times 10^7$ M^{-1} sec^{-1}, at 28°.

Discussion

The chemical shift changes observed for both $PhCF_3$ and PhF_6 upon association with βLG-A demonstrate the "solvent" sensitivity of the ^{19}F nucleus and its usefulness in detecting environmental changes within proteins. Both probes clearly showed a difference between the magnetic environments of the strong binding site and the residual binding sites, which would be expected from the thermodynamics of binding (Figure 4). At first glance, the trend in solvent shifts demonstrated by $PhCF_3$ seems more reasonable than that displayed by PhF_6. For the former, cyclohexane and heptane are at one end of the scale (at high field) and water at the other. The strong hydrophobic site appears to have a net magnetic environment midway between water and heptane, like dodecyl sulfate micelles, while the residual binding sites have an environment more closely resembling bulk water, which is consistent with the observation that residual binding increases as the aqueous solubility of the ligand increases (Robillard and Wishnia, 1972). Even so, a scale which puts acetone and *tert*-butyl

alcohol closer to heptane, and CCl_4, benzene, and toluene closer to water should give one pause. For hexafluorobenzene, the shifts in most solvents, including heptane and water (which is interesting if not yet understood), cluster near the middle, the shifts in dioxane and in acetone lie at very high field, while the shifts in the chloromethanes progress downfield toward that of the residual and then of the strong binding site of βLG-A, which is completely outside the range of all other chemical shifts observed.

However, the fact that there is no correlation between the solvent-induced changes in chemical shifts for the two ligands means that *no* attempt to rationalize the results on the basis of a one-parameter description of the solvent can succeed. Neither refractive index (Evans, 1960), nor a modified polarizability (Emsley and Phillips, 1966), nor solvent Z values (Kosower, 1968), which have been used to relate optical to other molecular properties, produces any obvious order in either set of chemical shift data. There are clearly several kinds of ligand–solvent interaction for which there is as yet no adequate theory, and others (*e.g.*, ring-current effects) for which the required geometric data are unknown. In particular, the nature of an unknown environment cannot safely be assessed from the observation that the chemical shift of a probe falls within the range produced by mixtures of two solvents.

The lifetime of the βLG-A–hexafluorobenzene complex is very short (8.5×10^{-5} sec), and the reaction between the ligand and the strong binding site is very fast ($k_{31} = 1.6 \times 10^7$ M^{-1} sec^{-1}). For comparison we calculated the rate constant, k_D, for an ideal diffusion-controlled reaction between two different spheres of radii r_H and r_P with no interaction potential. Then, the upper limit for the association reaction is given by (Caldin, 1964, p 11)

$$k_D = \frac{2RT}{3\eta}[2 + (r_H/r_P) + (r_P/r_H)] \text{ ml mole}^{-1} \text{ sec}^{-1} \quad (5)$$

If we take 35 Å for r_P and 3.6 Å for r_H, k_D is 2×10^{10} M^{-1} sec^{-1}. This model assumes that contact at any surface point results in binding. To approximate a realistic steric factor, the hydrophobic site was considered to be a sphere of volume $230/6 \times 10^{23}$ ml half-embedded in a protein monomer sphere of radius 17.5 Å. The area into which the center of the ligand must strike is thus about 1% of the total surface area of the monomer, reducing k_D to 2×10^8. (For such a value, when c_L is 3.4 mM, there are 7×10^5 collisions/sec per site, or 60–100 during the time in which a (possibly) nearby occupied site would dissociate once.) The steric requirements of the association may be greater, or rotational diffusion of the protein may make them somewhat less. In any case, the association reaction is close to diffusion controlled; the excess energy of activation for specific binding is small, 1 ± 1 kcal.

Other ligand-protein associations have much larger activation energies. For example, succinate binds to the carbamyl phosphate complex of aspartyl transcarbamylase or its catalytic subunit with about the same affinity as PhF_6 for βLG-A, but the rates are 100 times slower (Sykes *et al.*, 1970). It is presumed that large ligand-induced conformational changes are responsible. The hexafluorobenzene–βLG-A data are consistent with the model proposed from thermodynamic studies (Wishnia, 1969; Robillard and Wishnia, 1972), in which no gross rearrangements occur, and where the strong specific site is accessible, able to expand the required amount without strain, and even poised and waiting for a suitable ligand.

The dissociation rate of the βLG-A–dodecyl sulfate–hexafluorobenzene complex (that is, of a residual binding complex), $k_{21} = 7 \times 10^3$ sec^{-1}, is comparable to the rate of dissociation from the strong site, $k_{31} = 11 \times 10^3$ sec^{-1}. The weakness of binding arises from the association reaction, $k_{12} = 4 \times 10^5/N$ M^{-1} sec^{-1}, which, since N is not less than two, and probably much greater, is at least 100 times slower than the rate of binding at the specific site. Presumably some 3-kcal worth of surface structures must be destroyed for binding to occur.

References

Almenningen, A., Bastiansen, O., Seip, R., and Seip, H. M. (1964), *Acta Chem. Scand. 8*, 2115.

Caldin, E. F. (1964), Fast Reactions in Solution, Oxford, Blackwell Scientific Publications, p 11.

Carrington, A., and McLachlan, A. D. (1969), Introduction to Magnetic Resonance, New York, N. Y., Harper & Row, Chapter 11.

Emsley, J. W., and Phillips, L. (1966), *Mol. Phys. 11*, 437.

Evans, D. F. (1960), *J. Chem. Soc.*, 877.

Gerig, J. T. (1968), *J. Amer. Chem. Soc. 90*, 2681.

Green, D. K., and Powles, J. G. (1965), *Proc. Phys. Soc. (London) 85*, 87.

Gutowsky, H. S., McCall, D. W., and Slichter, C. P. (1953), *J. Chem. Phys. 21*, 279.

Gutowsky, H. S., and Woessner, D. E. (1954), *Phys. Rev. 104*, 843.

Hutton, R. S. (1969), M.S. Thesis, State University of New York at Stony Brook, Stony Brook, N. Y. 11790.

Johnson, C. S. (1965), *Advan. Magn. Resonance 1*, 33.

Kosower, E. M. (1968), Introduction to Physical Organic Chemistry, New York, N. Y., John Wiley and Sons, Inc., p 293.

Nehring, J., and Saupe, A. (1970), *J. Chem. Phys. 52*, 1307.

Ramirez, J. (1970), Ph.D. Thesis, State University of New York at Stony Brook, Stony Brook, N. Y. 11790.

Robillard, K. A., Jr., and Wishnia, A. (1972), *Biochemistry 11*, 3835.

Runde, E. J. (1970), M.S. Thesis, State University of New York at Stony Brook, Stony Brook, N. Y. 11790.

Swift, T. J., and Connick, R. E. (1962), *J. Chem. Phys. 37*, 307.

Sykes, B. D., Schmidt, P. G., and Stark, G. R. (1970), *J. Biol. Chem. 245*, 1180.

Taylor, P. W., Feeney, J., and Burgen, A. S. V. (1971), *Biochemistry 10*, 3866.

Wahl, P., and Timasheff, S. N. (1969), *Biochemistry 8*, 2945.

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