Fluorine Magnetic Resonance Study of the Binding of Long-Chain Trifluoroalkyl Sulfate Ions by Bovine Serum Albumin†

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ABSTRACT: Binding isotherms have been determined for bovine serum albumin with sodium 12,12,12-trifluorododecyl sulfate (NaF_3DS) and sodium 13,13,13-trifluorotridecyl sulfate (NaF_3TS). The latter compound, previously unknown, has a critical micelle concentration of 7.91 mM in water at 41°. Fluorine magnetic resonance spectra were used to find the variation of the fluorine chemical shifts and line widths with $\bar{n}$, the average number of ions bound per molecule of protein. At pH 6.8 the protein appears to bind 15 F_3DS^- ions at magnetically equivalent initial sites which persist even when the total number of bound ions is over 100. F_3TS^- ions are bound at 14 magnetically equivalent initial sites, but these sites are destroyed by a conformational transition which is essentially complete when $\bar{n} = 84$. Results of specific viscosity determinations confirm that F_3TS^- causes a major conformational change when $45 < \bar{n} < 85$, while F_3DS^- does not. The effect of binding at initial sites upon the fluorine chemical shifts is opposite to that caused by micelle formation, but for each detergent the shift at high binding ratios is close to the micelle shift. In each solution the detergent ions are exchanging rapidly among all bound sites and the free state.

Johnson and Muller (1970) used equilibrium dialysis and fluorine nuclear magnetic resonance to study the binding of 8,8,8-trifluorooctylbenzene-*p*-sulfonate ions (F_3OBS^-) by bovine serum albumin.[1] They found that up to 17 of these amphiphilic ions can be bound at initial binding sites where they give a fluorine chemical shift lower than that for aqueous, monomeric F_3OBS^- and where their motion is sufficiently restricted to cause marked broadening of the nmr signal. At higher concentrations of free detergent (D_f) many additional ions are bound in quite a different fashion, giving a chemical shift and line width about equal to those of micellized F_3OBS^-. This apparently occurs without loss of the original binding sites, *i.e.* when the number of bound ions (r) is larger than 17 the observed properties seem to represent a weighted average for 17 ions at initial sites and $r - 17$ at "micelle-like" sites.

Other investigators have explored the interactions between serum albumin and a variety of anionic detergents and have reported that at low detergent concentrations the protein binds from 10 to 16 anions at equivalent, noninteracting sites, many more ions being bound cooperatively when D_f increases (Pallansch and Briggs, 1954; Decker and Foster, 1966; Reynolds *et al.*, 1967; Hagenmaier and Foster, 1971). This cooperative binding is associated with a major conformational change of the protein with destruction of the initial binding sites if the alkyl chain of the detergent ions is long, but not otherwise. In practice, what constitutes a "long" chain appears to depend somewhat on the nature of the ionic group. Thus, dodecyl and tetradecyl sulfate are long-chain, or unfolding ligands, while octyl and decyl sulfate and also octyl-, decyl-, and dodecylsulfonate are nonunfolding. Data for octyl- and dodecylbenzenesulfonate were interpreted by Decker and Foster (1966) on the assumption that the initial binding sites for these ions disappear at high binding ratios. Since detergents with terminal trifluoromethyl groups resemble their unfluorinated analogs rather closely (Muller and Platko, 1971) it was somewhat surprising that the nmr results showed F_3OBS^- to be a nonunfolding ligand.

The present work was undertaken with the aim of proving that the nmr method permits one to distinguish between the two kinds of binding behavior and with the expectation that trifluorododecyl sulfate (F_3DS^-) would act as an unfolding ligand. When it was found instead that this detergent is again a nonunfolding species, the work was extended to include the previously unknown 13,13,13-trifluorotridecyl sulfate ion (F_3TS^-). Binding isotherms, nuclear magnetic resonance (nmr) data, and results of specific viscosity measurements, presented below, show for the first time that the addition of only one methylene group can convert a nonunfolding ligand into an unfolding one.

Experimental Section

Materials. The preparation of NaF_3DS using a modification of the method reported by Muller and Johnson (1969) is described in detail elsewhere (Mead, 1972). 1,10-Dibromodecane was converted to 12-bromododecanoic acid using malonic ester, and the reaction of this acid with sulfur tetrafluoride yielded 12,12,12-trifluoro-1-bromododecane. This was treated with potassium acetate, giving 12,12,12-trifluorododecyl acetate which was converted to 12,12,12-trifluorododecanol using methanol and a little sodium methoxide. The alcohol was treated with chlorosulfonic acid in chloroform and then with sodium hydroxide in 90% ethanol to give NaF_3DS, which was then purified by extraction with petroleum ether and recrystallized four times from water. *Anal.* Calcd: C, 42.07; H, 6.48. Found: C, 41.78; H, 6.49.

To obtain NAF_3TS, a Grignard reagent was prepared from

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[1] Abbreviations used are: F_3OBS^-, 8,8,8-trifluorooctylbenzene-*p*-sulfonate; F_3DS^-, 12,12,12-trifluorododecyl sulfate; F_3TS^-, 13,13,13-trifluorotridecyl sulfate; cmc, critical micelle concentration; AD_r, a complex consisting of one molecule of albumin and r detergent ions.

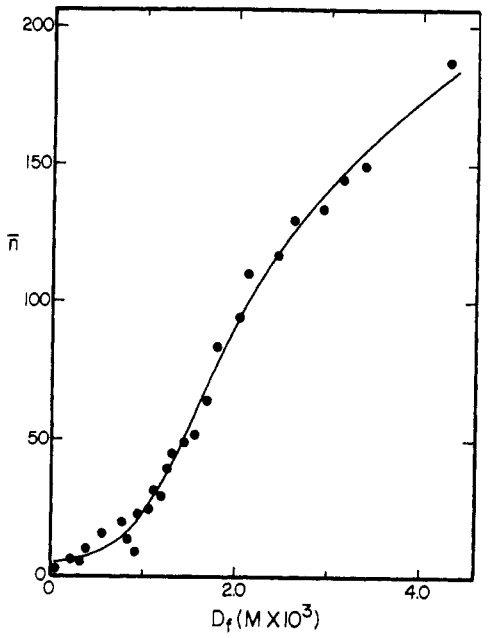

FIGURE 1: Binding of F_3DS^- by bovine serum albumin at pH 6.8 and 34° shown as a plot of $\bar{n}$, the average number of detergent ions bound per molecule of protein, against free detergent concentration, D_f.

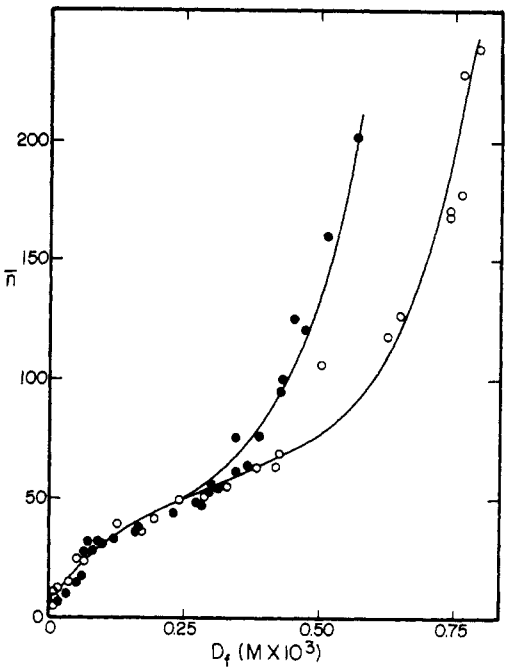

FIGURE 2: Binding of F_3TS^- by bovine serum albumin at pH 6.8 (open circles) and pH 6.1 (filled circles) and 36° shown as a plot of $\bar{n}$ against D_f.

12,12,12-trifluoro-1-bromododecane and magnesium, and this was treated with carbon dioxide to produce 13,13,13-trifluorotridecanoic acid. The acid was reduced to the corresponding alcohol with lithium aluminum hydride, and the alcohol converted to the alkyl sulfate and purified as described for NaF_3DS above. *Anal.* Calcd: C, 43.81; H, 6.79. Found: C, 43.60; H, 6.99.

Crystalline bovine serum albumin (Nutritional Biochemicals Corporation, control no. 5612) was used without further purification. Buffer salts were reagent grade. Twice distilled water, deionized on a Barnstead still using a standard (0802) column, was used in preparing solutions.

Methods. Equilbrium dialysis experiments were performed as described by Johnson and Muller (1970). Two buffer solutions were used, one containing 0.048 M NaH_2PO_4 and 0.021 M Na_2HPO_4 with a nominal pH of 6.8, and the other containing 0.0725 M NaH_2PO_4 and 0.0093 M Na_2HPO_4 with a nominal pH of 6.1. Protein concentrations were determined spectrophotometrically as before, and solutions from the protein-free compartments were analyzed for free detergent by the following procedure, based on the work of Mukerjee (1956). A small quantity, typically 100 μl, of the detergent solution is diluted to 25.0 ml with a solution containing 21 ppm of Methylene Blue in 0.01 M HCl. A 10.0-ml aliquot is pipeted into a 12-dram vial with 5.00 ml of chloroform, the tightly capped vial is shaken to extract the detergent–dye complex into the organic layer, and the latter is analyzed spectrophotometrically at 655 nm in a 1-cm silica cell using a Beckman DU-2. Reference solutions were prepared by the same procedure beginning with a small sample of buffer solution instead of the detergent solution. Determinations were made in duplicate, and standard detergent samples were analyzed with each set of unknowns to recalibrate the procedure.

Nuclear magnetic resonance measurements were carried out essentially as described earlier (Johnson and Muller, 1970) except that most of the data were obtained with a Varian XL-100 spectrometer operated at 94.077 MHz with a Nicolet 1080 computer for signal enhancement by multiple scanning. All chemical shifts are reported in parts per million upfield from the external reference, 1,1,2-trichlorotrifluoropropene. To obtain adequate signal strength solutions with protein concentrations between about 0.2 and 6% by weight were used for the nmr measurements.

To avoid ambiguities arising from the puzzling dependence of the reduced viscosity of bovine serum albumin on the protein concentration (Reynolds *et al.*, 1967) the variation of viscosity with binding number was studied using separately prepared solution with a constant protein concentration of 0.1%. As before, solutions of protein and detergent were dialyzed against protein-free detergent solutions, and after equilibrium was attained the protein-free solutions were analyzed to find D_f and allow the binding number to be calculated. The solutions from the protein-containing compartment were then filtered through Millipore paper, and their viscosities were measured at 34.5 ± 0.005° with a Cannon-Ubbelohde L605 viscometer which gave flow times ranging from 230 to 250 sec, reproducible to within 0.2 sec.

Results and Discussion

Behavior of the Detergents in Water and Aqueous Buffer. Fluorine magnetic resonance data of Muller and Johnson (1969) show that the critical micelle concentration (cmc) of NaF_3DS in water is 14.6 mM and that the chemical shifts are 3.70 ppm for the monomeric form and 4.87 ppm for the micellar form at 35°. By the same method we found that in the buffer of pH 6.85 and at 34° the cmc is 6.07 mM and the monomer and micelle shifts are 3.65 and 4.89 ppm, respectively. The change in cmc is about as expected (Corron, 1948) for an anionic detergent in the presence of added salt.

NaF_3TS is very similar to NaF_3DS except in that its solubility is quite low below 31°. Spectra obtained at 41° gave a monomer shift of 3.59 ppm, a micelle shift of 4.77 ppm, and a cmc of 7.91 mM in water. In the buffer solution at pH 6.8 the

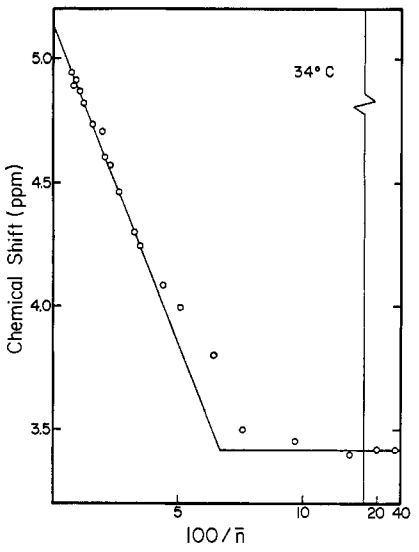

FIGURE 3: Fluorine chemical shift (ppm to higher field from the external reference 1,1,2-trichlorotrifluoropropene) for bound F_3DS^- as a function of $100/\bar{n}$.

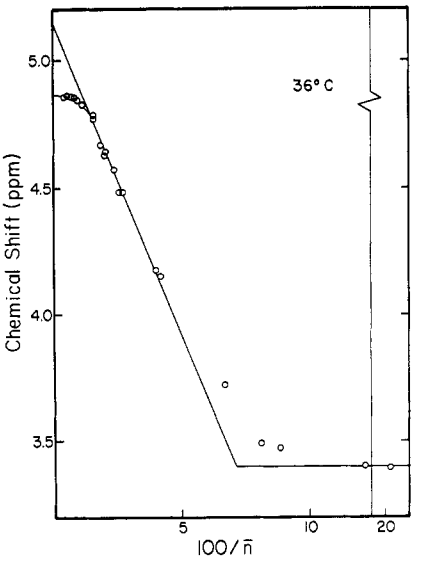

FIGURE 4: Fluorine chemical shift for bound F_3TS^- as a function of $100/\bar{n}$.

corresponding values were 3.60 ppm, 4.77 ppm, and 2.09 mM, respectively. The chemical shifts differ from those reported for NaF_3DS mainly because of the temperature difference. Data of Johnson (1970) and Muller and Birkhahn (1968) indicate that NaF_3DS should give a monomer shift of 3.57 ppm and micelle shift of 4.81 ppm at 41°. Unlike the shifts, the cmc values should be nearly independent of temperature in the interval 30–40°. The ratio of the cmc values is nearly 2:1 as expected for detergents having hydrocarbon chains differing in length by one methylene group.

All equilibrium dialysis experiments were conducted at D_f values well below the cmc to eliminate anomalies caused by micelle formation.

Binding Isotherms. The average number of bound detergent ions per mole of protein ($\bar{n}$) as found from the equilibrium dialysis measurements, plotted as a function of D_f, is presented in Figure 1 for NaF_3DS at pH 6.8 and in Figure 2 for NaF_3TS at pH 6.1 and 6.8. The smooth curves drawn through the experimental points have no theoretical significance. The observed scattering of the data points is not unusual for studies of this kind; much of it arises from uncertainties in the detergent analysis which become increasingly severe at low values of D_f, *i.e.*, in the region of small binding numbers and especially for NaF_3TS. Since the primary aim was to ascertain whether or not the protein conformation is drastically modified when $\bar{n}$ is large, some inaccuracies in the low-$\bar{n}$ region of the isotherms can be tolerated. Scatchard plots made using selected data points which in our judgment represent the most reliable measurements suggest that for both detergents the number of thermodynamically equivalent noninteracting initial sites is 11 and that the corresponding binding constants are 6×10^5 M^{-1} for F_3DS^- and 4×10^6 M^{-1} for F_3TS^- (Mead, 1972).

The present results are qualitatively similar to those for other alkyl sulfates (Ray *et al.*, 1966; Reynolds *et al.*, 1967) with F_3DS^- being appreciably less tightly bound that ordinary dodecyl sulfate, while F_3TS^- is slightly more tightly bound. This trend is parallel to the sequence of cmc values, which is 14.6, 8.1, and 7.9 mM for NaF_3DS, sodium dodecyl sulfate, and NaF_3TS in water. By either criterion, NaF_3TS is more closely similar to sodium dodecyl sulfate than NaF_3DS. The data do not show a systematic variation of $\bar{n}$ with protein concentration at constant D_f, as reported by Ray *et al.* (1966).

Chemical Shifts. For each protein-containing solution, the analytical results furnish values of D_f, and the bound detergent concentration (D_b) is readily calculated. The chemical shift of the single observed fluorine nmr signal (δ_{obsd}) is then the weighted average of the shifts for the free and bound detergent ions (δ_f and δ_b), that is

$$\delta_{obsd} = (D_f\delta_f + D_b\delta_b)/(D_f + D_b) \quad (1)$$

Since δ_f was determined independently for each detergent in the buffer solutions, δ_b is easily evaluated. The results are presented as plots of δ_b as a function of the quantity $100/\bar{n}$ in Figure 3 for NaF_3DS at 34° and pH 6.8 and in Figure 4 for NaF_3TS at 36° and the same pH. Data obtained with NaF_3TS at pH 6.1 yielded a plot which is identical within experimental error with that in Figure 4 (Mead, 1972).

The results shown in Figure 3 are very similar to those of Johnson and Muller (1970) for the binding of F_3OBS^-. The value of δ_b is essentially constant at 3.42 ppm when $\bar{n} < 15$. At higher binding numbers, the data points fall near the straight line (eq 2), up to the maximum value of $\bar{n}$ for which

$$\delta_b = 5.11 - 25.4/\bar{n} \quad (2)$$

measurements were made, which was 144. Following the discussion of Johnson and Muller, such behavior is most simply interpreted using the following model. Up to 15 F_3DS^- ions can be bound at magnetically equivalent initial binding sites with a chemical shift of 3.42 ppm. When $\bar{n} > 15$, these sites remain occupied and the shift is unaltered while additional ions are bound with a shift of 5.11 ppm or slightly less. Exchange of ions between alternative sites is rapid so that the bound shift is given by an average (eq 3) which is another form of eq 2.

$$\delta_b = (15/\bar{n})(3.42) + [(\bar{n} - 15)/\bar{n}](5.11) \quad (3)$$

Since the extrapolated shift of 5.11 ppm is only a little different from the value for micellized F_3DS^- we call the environment of the more loosely bound ions micelle-like although we are

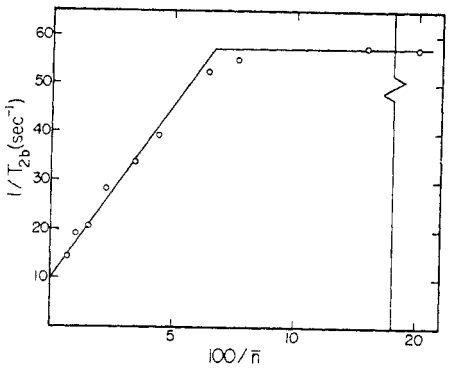

FIGURE 5: Fluorine nuclear relaxation times for bound F_3DS^- shown as a plot of $1/T_{2b}$ against $100/\bar{n}$.

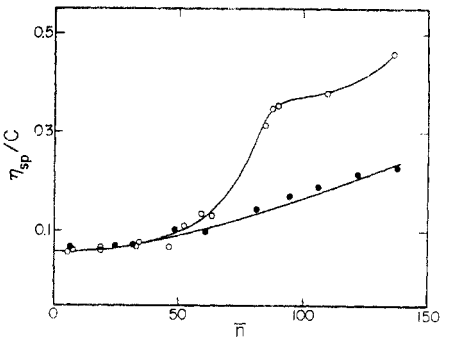

FIGURE 7: Reduced viscosities of bovine serum albumin in the presence of F_3DS^- (filled circles) and F_3TS^- (open circles) at pH 6.8, shown as a function of $\bar{n}$.

unable to propose a detailed geometrical model for the protein–detergent complex.

Turning to the data for NaF_3TS, it appears again that δ_b is independent of $\bar{n}$ for the lowest binding numbers, this time for $\bar{n} < 14$. The situation at high binding numbers is strikingly different; up to about $\bar{n} = 80$, δ_b obeys eq 4 but soon there-

$$\delta_b = 5.15 - 24.5/\bar{n} \quad (4)$$

after δ_b reaches a plateau at 4.86 ppm, almost exactly the micelle shift expected at 36°. The straight line of eq 4 and a horizontal one at 4.86 ppm intersect at $\bar{n} = 84$.

Again the data suggest the existence of a set of magnetically equivalent initial binding sites, with a shift of 3.40 ppm at 36°. When $14 < \bar{n} < 84$, species AD_{14} may be in equilibrium with one or more AD_r species having $r \leq 84$, and somewhere in this region the initial sites are destroyed, so that when $\bar{n} \geq 84$ only micelle-like sites with $\delta_b = 4.86$ ppm are in use. At higher values of $\bar{n}$ (the data in this case extend to $\bar{n} = 239$) many additional micelle-like sites are available, and since the initial sites have now disappeared there is no further change in δ_b. Information presented below provides some additional clues but does not make it possible to deduce rigorously what specific complexes are formed. However, there is little doubt that the addition of one methylene group to the alkyl chain in passing from F_3DS^- to F_4TS^- produces a drastic change in the binding phenomenon, the initial sites being preserved throughout the entire range of binding numbers for the former but not for the latter.

Nuclear Magnetic Resonance Line Widths. As observed earlier by Johnson and Muller (1970) the fluorine magnetic resonance signals are rather broad at the lowest $\bar{n}$ values,

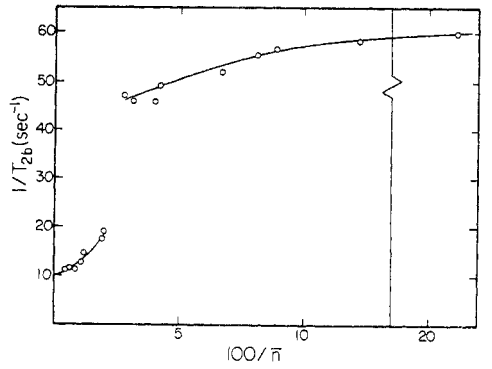

FIGURE 6: Fluorine nuclear relaxation times for bound F_3TS^- shown as a plot of $1/T_{2b}$ against $100/\bar{n}$.

becoming progressively sharper as the binding number increases. When the procedures described there are used to evaluate the relaxation times (T_{2b}) for bound F_3DS^- and F_3TS^- the results presented in Figures 5 and 6 are obtained. In each case, $1/T_{2b}$ is plotted against $100/\bar{n}$. Again, the data for F_3DS^- closely resemble those for F_3OBS^-. For $\bar{n} < 15$, where only the initial binding sites are apparently occupied, $T_{2b} = T_{2i} = 0.021$ sec. For $\bar{n} > 15$, T_{2b} is represented to a good approximation by

$$1/T_{2b} = 1/T_{2a} + (15/\bar{n})(1/T_{2i} - 1/T_{2a}) \quad (5)$$

with $T_{2a} = 0.102$ sec. This is in accord with the supposition that 15 tight binding sites with a T_2 of 0.021 sec are occupied together with a variable number of micelle-like sites with a T_2 of 0.102 sec, the overall T_{2b} then being given by an appropriate average.

Once more the results for F_3TS^- are qualitatively different. $1/T_{2b}$ falls only slowly with increasing $\bar{n}$ up to about $\bar{n} = 40$. Then there is an abrupt drop followed for $\bar{n} > 50$ by another region of rather gradual decrease. These findings are difficult to interpret quantitatively, but they suggest that a major part of the conformational change which presumably destroys tight binding sites may occur in the domain $40 < \bar{n} < 50$. The limiting values or T_2 at the highest and lowest binding numbers are quite similar for the two detergents.

Viscosities. Viscosity determinations provided the most readily accessible nonspectroscopic method for verifying that the conformational change suggested by the magnetic resonance data was actually occurring. Figure 7 shows the variation of the reduced viscosity with binding number for serum albumin in the presence of the two ligands. It is at once apparent that the longer chain detergent causes the sort of viscosity change associated by Reynolds *et al.* (1967) with a major conformational transition, while the shorter chain detergent does not. It also appears that the transition does not begin as soon as $\bar{n}$ exceeds the number of initial binding sites, but that it occurs predominantly in the region $45 < \bar{n} < 85$.

A further effort to visualize more explicitly the nature of solutions of serum albumin and F_3TS^- may be made in light of the work of Decker and Foster (1966) and others cited there, who found that several unfolding ligands tend to form three discrete complexes with the protein, AD_m, AD_n, and AD_{2n}, where m is not greatly different from 12 and n is near 40. For F_3TS^- in the region $14 < \bar{n} < 84$ this would suggest postulating three species, AD_{14}, AD_{42}, and AD_{84}. The first of these would obviously be a protein molecule with only the initial set of binding sites in use, and the last would be an un-

folded species with a much larger reduced viscosity and only micelle-like binding sites. A simple calculation shows that the existence of the species AD_{42} would lead to a bend in the plot of δ_b against $100/\bar{n}$ *unless* its chemical shift is (14/42)(3.40) + (28/42)(4.86). Since no such change of slope is found, one might tentatively conclude that this species still has the 14 initial sites and binds 28 ions at micelle-like sites. Such a conjecture is consistent with the observation that $\bar{n}$ can be as large as 42 before any pronounced rise in the reduced viscosity is found. Only the line-widths results fail to be entirely consistent with such a model, inasmuch as it does not account for the abrupt change in T_{2b} in the range $40 < \bar{n} < 50$.

Conclusions

On the basis of the fluorine chemical shifts, line widths, and viscosity data it seems well established that bovine serum albumin binds F_3DS^- as a nonunfolding ligand while F_3TS^- is an unfolding ligand. As the binding number is gradually increased, unfolding commences only at about $\bar{n} = 45$ and is complete when $\bar{n} \sim 85$. The bound-detergent shift at the highest values of $\bar{n}$ depends somewhat on whether the protein is unfolded or not, but in either case is not very different from the shift for micellized detergent.

For the three materials, NaF_3DS, NaF_3TS, and NaF_3OBS (Johnson and Muller, 1970), the number of magnetically equivalent initial binding sites decreases as the length of the detergent ion increases. The chemical shift associated with these sites continues to present an intriguing puzzle. When the results are corrected to 35°, all three detergents give the same value, 3.41 ppm. Thus, in contrast with micellization, which increases the shift by about 1.25 ppm, binding at the initial sites decreases the shift by about 0.25 ppm. If this is caused by an interaction between the trifluoromethyl groups and some specific functional groups, perhaps ionic groups, of the protein, it is remarkable that it should be so nearly independent of the size and type of detergent ion. These observations do not suffice to define the nature of the initial binding sites, but they would seem to rule out any model which provides a fixed point of attachment for the ionic head group of the detergent, with the hydrocarbon tail falling into a preexisting hydrophobic pocket or crevice which holds the terminal trifluoromethyl group rather firmly in place.

Among the three fluorine-labeled detergents, the one which most closely resembles sodium dodecyl sulfate as a ligand is not the one which most closely resembles it geometrically but rather is the compound which, although the chain is slightly longer, has nearly the same cmc.

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