formation of the nucleosome.

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# Ligand-Induced Transfer of Proteins between Phases: Dependence upon the Strength of Ion Pair Interactions†

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ABSTRACT: Chemical modifications of ionizable groups of bovine serum albumin and lysozyme are described which lessen both pH and solvent polarity restrictions to the transfer of the proteins from water to alcohol phases on addition of suitable anions. Esterification of one-third of the carboxyl groups of albumin with triethyloxonium fluoroborate yielded protein fractions which could be transferred, by addition of *p*-toluenesulfonate or octylsulfonate, into butanol at pH 4.8 but not pH 7.1, while the intact protein could only be transferred at pH 2.4. Further, guanidation of 30% of the $\epsilon$-amino groups yielded an ethylated-guanidated albumin which could be transferred into 1-butanol at pH 7.1 or into 1-octanol at pH 2.4. This notable increase in the ease of partition upon guanidation is directly traceable to the higher stability of the neutral ion pairs formed by guanidine ($pK \sim 13$) above those formed by the $\epsilon$-amino groups of lysine ($pK \sim 10$). Differences in the partition of intact albumin and lysozyme at pH 2.4 are explained by their different arginine/lysine ratios. The rotational relaxation times of modified dansylated protein–ligand complexes in the alkanols were measured by combining fluorescence polarization, fluorescence lifetime, and alcohol viscosity data, over the range of 2–38 °C. The times observed (10–25 ns) are much shorter than those in water, indicating a large increase in the internal motions of the protein in the less polar solvent in the nanosecond range.

The ability to alter the partition equilibrium of a protein between an aqueous phase and an organic phase by the binding of ligands to the protein is a fundamental concept in understanding the interaction of proteins with membranes. Further, gating phenomena may achieve their sensitivity by combining ligand-induced partition changes of the receptor protein with electrophoresis in the presence of a transmembrane potential. Ligand-stabilized interfacial electrophoresis is a sensitive, on–off switchable function of ligand concentration even in the presence of electric fields weak relative to the electric fields of biological membranes. This has been demonstrated with serum albumin and lysozyme at pH 2.4 in a saturated water–butanol system with *p*-toluenesulfonate as the ligand (Mustacich & Weber, 1978). High reaction orders of ligand

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binding were characteristic of both proteins in the equilibrium binding/partitioning studies.

Two experimental limitations were evident in our early modeling of protein partition between phases. First, acidic pH was required to protonate the carboxyl groups to increase the nonaqueous solubility. No partitioning was observed at pH ≥5 for both proteins, even when anionic ligands having much greater solubilities in the nonaqueous phases were added. Moreover, no partition was observed at pH 2.4 into alcohols of longer carbon chain than 1-pentanol. The decreased solubility of *p*-toluenesulfonic acid in higher alcohols was partly responsible, but use of octylsulfonate as ligand would still not achieve partitioning of the proteins into 1-hexanol or higher alcohols.

This paper describes protein modifications which overcome both limitations of the earlier model system. By a combination of esterification of the carboxyls, guanidation of lysine groups, and a suitable choice of ligand, partitioning of the modified protein by ligand binding can be achieved into 1-hexanol at neutral pH and into 1-octanol at pH 2.5. By use of dansyl[1] conjugates of the modified proteins, the rotational motions of the protein–ligand complexes are further characterized by fluorescence polarization measurements.

## Materials and Methods

Reagent grade chemicals and distilled solvents were used in making all solutions. Bovine serum albumin (BSA) was purchased from Reheis; egg white lysozyme, 5-(dimethylamino)napthalene-1-sulfonyl chloride (dansyl-Cl), 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride, and 2,4,6-trinitrobenzenesulfonic acid were from Sigma; sodium *p*-toluenesulfonate (pTS) was from Eastman; sodium octylsulfonate was from Research Plus; *O*-methylisourea was from Fischer.

Fluorescence emission spectra were recorded with an emission spectrometer equipped with a bipolar averaging circuit and a digital integrator (Wehrly et al., 1976; Jameson et al., 1977). Fluorescence polarization measurements were obtained by using a photon-counting polarization photometer (Jameson et al., 1978). Fluorescence lifetime measurements were made with a Model 480 SLM subnanosecond phase fluorometer (SLM Instruments, Champaign, IL). Excitation of 360 nm was used for fluorescence experiments with dansyl-conjugated proteins.

Dansyl derivatives of BSA were prepared by reaction of a 1% BSA solution in 1% sodium bicarbonate with dansyl-Cl in acetone. Lysozyme was dansylated by reaction in pH 9 Tris-HCl with dansyl-Cl adsorbed onto Celite (10% dansyl-Cl by weight). Gel filtration with Sephadex G-25 removed the free dye from the dansylated proteins. The label ratios ranged from 0.6 to 1.0 for dansyl-lysozyme and from 1 to 2 for dansyl-BSA. The dansyl conjugates were dialyzed to pH 2.4 with 55 mM phosphate buffer that had been saturated with 1-butanol for partition experiments at pH 2.4.

Equilibrium partitioning experiments with the ligand pTS and 1-butanol as the nonaqueous phase were conducted by mixing a series of test tubes in which only the concentration of pTS was varied according to the method described previously (Mustacich & Weber, 1978). The concentration-dependent distribution coefficients of the ligand in the water–butanol system were measured separately at different pH values. The partition coefficient, $\beta$, of the polyvalent protein–ligand complexes was calculated from the relationship $\beta = F/(F_\infty - F)$, where $F$ is the integrated fluorescence emission spectrum in the upper (alcohol) phase and $F_\infty$ is the integrated fluorescence emission spectrum observed when all of the protein is in the upper phase.

Ethyl esterification of dansyl-BSA was achieved by reaction with triethyloxonium fluoroborate, which was synthesized by the method given by Meerwein (1966) and reacted similarly to procedures developed for esterification of lysozyme and trypsin (Parson et al., 1967; Nakayama et al., 1970). The triethyloxonium fluoroborate was added in a 10–20-fold excess as a solid rather than as an acetonitrile solution. After reaction at pH 4.5 in a pH stat at room temperature, the mixture was dialyzed against pH 4.8 acetate buffer to remove the ether and fluoroborate. Following dialysis, the solution together with any precipitate was equilibrated with a 1-butanol phase in the presence of a 50-fold excess of pTS. The fluorescent upper phase was then dialyzed against pH 4.8 acetate buffer. A two-phase system gradually formed in the dialysis bag. The fluorescence was observed to migrate to the lower phase followed by the eventual disappearance of the butanol phase. A moderate amount of precipitate formed and was discarded after separation by low-speed centrifugation. This preparation was used in subsequent experiments requiring ethylated dansyl-BSA.

In this way an esterified albumin fraction was selected which could be reversibly transferred between the two phases. The chemical modification procedures, esterification or guanidation, result in evidently heterogeneous populations as regards the fraction of modified groups and therefore the transfer properties. Our aim was to demonstrate changes in partition with pH or second phase after chemical modification, under conditions of reversibility, and therefore we used in this and other instances this method of selection of the transferable protein fraction.

Ethylation of dansyl-lysozyme was accomplished by an identical procedure to that described for dansyl-BSA. After dialysis of the reaction product, the mixture was equilibrated with 1-butanol and the pH adjusted to pH 7. Addition of pTS partitioned most of the modified protein into the butanol. The remaining reaction product precipitated at the interface between the two phases. The centrifuged upper phase was dialyzed against pH 7.1 phosphate buffer at 4 °C. A small amount of precipitate formed in the transfer of the ethylated dansyl-lysozyme to the aqueous phase during dialysis. The precipitate was separated by low-speed centrifugation and discarded.

The $\epsilon$-amino groups of dansyl-BSA were guanidated by the method of Klee & Richards (1957) using *O*-methylisourea. BSA was precipitated with acetone following dansylation and dissolved in 0.5 M *O*-methylisourea to give a final protein concentration of 0.5%. The pH was adjusted to pH 10.25 with 2.5 M NaOH, and the mixture was stirred for 4 days at 4 °C. The reaction mixture was then dialyzed against phosphate buffers to the pH desired for subsequent experimentation.

Ethylation of the guanidated dansyl-BSA was achieved by the ethylation procedure described for dansyl-BSA above. The reaction produced a fluorescent precipitate which remained insoluble after the dialysis with pH 7.1 phosphate buffer. This insoluble reaction product was used directly after dialysis in subsequent partition experiments.

For an estimate of the extent of the ethyl esterification of dansyl-BSA, the ethylated protein was reacted with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride and

[1] Abbreviations used: BSA, bovine serum albumin; pTS, *p*-toluenesulfonic acid; dansyl-Cl, 5-(dimethylamino)naphthalene-1-sulfonyl chloride.

excess glycine methyl ester hydrochloride (Carraway & Koshland, 1972). Both dansyl-BSA and ethylated dansyl-BSA were glycinated by this procedure. After dialysis both were prepared for amino acid analysis by standard procedures (Spackman et al., 1958) on a Beckman Model 120C amino acid analyzer. The amino acid determinations for excess glycine were analyzed by using glutamic acid, alanine, and leucine as standards.

The extent of guanidation of dansyl-BSA by *O*-methylisourea was estimated by reaction of the guanidated dansyl-BSA with 2,4,6-trinitrobenzenesulfonic acid (Snyder & Sobocinski, 1975). A linear standardization curve was obtained by reaction with BSA solutions in 0.1 M pH 9.3 borate buffer for 30 min at 25 °C.

For construction of the Perrin plots, the viscosities of the water-saturated 1-alkanols (butanol through octanol) were determined in the temperature range 2–38 °C by using a calibrated no. 200 Ostwald viscometer. Water-saturated alkanol densities were simultaneously measured with a pycnometer over the temperature range.

## Results

Reaction of dansyl-BSA with triethyloxonium fluoroborate gave a product which was water soluble at pH 4.5 after removal of the ether and fluoroborate by dialysis. Electrophoresis of the ethylated dansyl-BSA at pH 4.8 showed migration to the cathode rather than migration to the anode as observed with unreacted dansyl-BSA. The ratio of the fraction of carboxyl sites protected from glycination by carbodiimide to the total number of sites reactive to glycination was ~28:80. This provides an estimated average ethylation of approximately one-third of the available carboxyl groups in dansyl-BSA.

The fluorescence emission maximum of ethylated dansyl-BSA at pH 4.5 was 502 nm compared to an emission maximum of 497 nm for dansyl-BSA. Rotational relaxation times were calculated from Perrin plots constructed from fluorescence polarization values over the temperature range 2–38 °C. $T/\eta$ values were multiplied by fluorescence lifetimes measured by modulation at each temperature at 10 MHz for lifetime correction of the Perrin plot. The rotational relaxation times calculated for dansyl-BSA and ethylated dansyl-BSA at 25 °C are 123 and 105 ns, respectively, indicating a retention of the globular native structure.

The fluorescence emission maximum of the ethylated protein–ligand complex in 1-butanol was 521 nm at pH 4.8 and 517 nm at pH 2.4. The rotational relaxation time of the protein–ligand complex in 1-butanol at pH 2.4 was calculated by a Perrin plot to be 17 ns at 25 °C.

Equilibrium partitioning of the ethylated dansyl-BSA into 1-butanol was obtained at pH 4.8 by binding with the ligand pTS at 25 °C. The Hill plot of ethylated protein transfer to 1-butanol had a slope of ~5 and a critical partition concentration (aqueous ligand concentration at $\beta = 0.5$) equal to 3.8 mM pTS. In contrast, dansyl-BSA will not partition into 1-butanol at pH 4.8 even in the presence of very large ligand concentrations.

Efforts to partition this preparation of ethylated dansyl-BSA with pTS into 1-butanol at pH 7.1 resulted in complete precipitation at the interface. Dansyl-BSA in an identical experiment will neither partition to 1-butanol or precipitate at the interface. Both ethylated and unmodified protein partition into 1-butanol at pH 2.4 with pTS, but both completely precipitate at the interface upon neutralizing the pH of the system. At pH 7.1 the use of sodium octylsulfonate instead of pTS as the ligand with the ethylated dansyl-BSA gave some partitioning in addition to precipitation at the interface.

Attempts to further react the ethylated dansyl-BSA with triethyloxonium fluoroborate were made by first partitioning the reaction product into a 1-butanol phase by addition of pTS. However, further addition of triethyloxonium fluoroborate precipitated the upper phase to a completely insoluble material which could not be partitioned into nonaqueous phases.

Unlike the dansyl-BSA reaction product with triethyloxonium fluoroborate, dansyl-lysozyme reacted to give a product which mostly partitioned into 1-butanol at pH 7.1 with the addition of pTS. This product was separated and dialyzed back into the aqueous phase. The fluorescence emission maximum in water was 520 nm for the ethylated dansyl-lysozyme at pH 7.1. The fluorescence polarization was 0.15 compared to a 0.17 for unethylated dansyl-lysozyme.

The emission maximum of the ethylated dansyl-lyzosyme–pTS complex in 1-butanol at pH 7.1 was 514 nm. The Hill plot for transfer of the ethylated dansyl-lysozyme at pH 7.1 into 1-butanol showed curvature at low saturation approaching a critical ligand concentration greater than 10 mM. This curvature likely reflects heterogeneity resulting from differing extents of ethylation.

A Hill plot of the transfer of ethylated dansyl-lysozyme into 1-butanol with pTS at pH 2.4 gave a slope of 7.6 and a critical partition concentration of 5.5 mM pTS. For comparison, the Hill plot for unethylated dansyl-lysozyme transfer to 1-butanol gives a slope of 10 and a critical partition concentration of 7.1 mM pTS at pH 2.4 (Mustacich & Weber, 1978).

Dansyl-BSA modification by guanidation with *O*-methylisourea gave ~30% guanidation as estimated by reaction with trinitrobenzenesulfonic acid. The emission maximum of the guanidated dansyl-BSA was 502 nm at pH 2.4. The rotational relaxation time calculated from a Perrin plot was 107 ns at 25 °C compared to 23 ns for dansyl-BSA (Mustacich & Weber, 1978), indicating failure of the acid expansion for the guanidated protein.

The effect of guanidation of dansyl-BSA on the partitioning into 1-butanol by binding with pTS at pH 2.4 was determined by a Hill plot. The Hill coefficient was 6.2, and the critical partition concentration was 4.8 mM pTS. Upon transfer to 1-butanol at pH 2.4 the emission maximum was 522 nm. The rotational relaxation time determined for the complex in the upper phase by a Perrin plot was 13 ns at 25 °C. This is comparable to the rotational relaxation time of 10 ns for dansyl-BSA when partitioned into 1-butanol at pH 2.4 (Mustacich & Weber, 1978).

Ethylation of the guanidated dansyl-BSA produced a precipitate which remained insoluble after removal of the ether and fluoroborate by dialysis. However, this doubly modified dansyl-BSA partitioned into 1-butanol at pH 7.1 with the addition of pTS. Further, this material also partitioned into 1-pentanol and 1-hexanol with the ligand octylsulfonate at pH 7.1. (The very small partition coefficient of pTS into 1-pentanol and higher alcohols necessitates the use of an alternative ligand.) All previous efforts to partition dansyl-BSA, dansyl-lysozyme, ethylated dansyl-BSA, ethylated dansyl-lysozyme, and guanidated dansyl-BSA with octylsulfonate into alcohols of chain length greater than 1-pentanol had failed at neutral or acidic pH.

Only octylsulfonate concentrations comparable to the previously used pTS concentrations were employed in order to avoid emulsification. Partitioning of the guanidated and ethylated dansyl-BSA into 1-butanol at pH 2.4 with pTS and with octylsulfonate in the same total concentrations (10 mM in the aqueous phase before adding equal volumes of the 1-butanol phase) gives upper phase complexes with comparable

Table I: Observed Effects in the Partitioning of Modified Proteins into 1-Butanol by Binding with the Ligand *p*-Toluenesulfonate[a]

| | BuOH | | |
|---|---|---|---|
| | pH 2.4 | pH 4.8 | pH 7.1 |
| dansyl-BSA | $C_0$ = 7.5 mM<br>$H$ = 25 | – | – |
| ethylated dansyl-BSA | + | $C_0$ = 3.8 mM<br>$H$ = 5.0 | – |
| dansyl-lysozyme | $C_0$ = 7.1 mM<br>$H$ = 10 | – | – |
| ethylated dansyl-lysozyme | $C_0$ = 5.5 mM<br>$H$ = 7.6 | + | $C_0$ > 10 mM |
| guanidated dansyl-BSA | $C_0$ = 4.8 mM<br>$H$ = 6.2 | – | – |
| guanidated, ethylated dansyl-BSA | + | + | + |

[a] Critical partition concentrations are given where available; otherwise, "+" and "–" indicate partitioning and the absence of partitioning, respectively. $C_0$, ligand concentration in aqueous phase required for partition coefficient $\beta$ = 0.5. $H$, slope in the plot of log (ligand concentration) vs. log $[\beta/(1-\beta)]$.

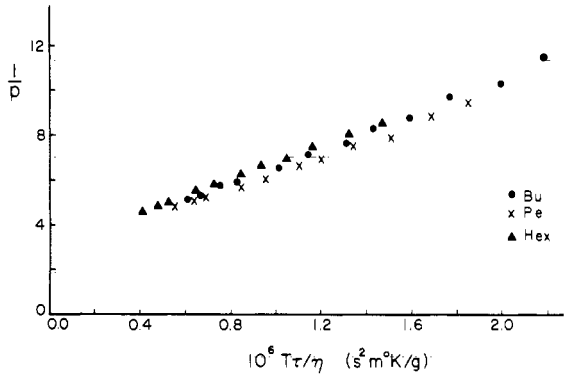


FIGURE 1: Perrin plots of 1/(fluorescence polarization) vs. temperature over the range 2–38 °C for complexes of guanidated and ethylated dansyl-BSA with octylsulfonate at pH 7.1 in 1-butanol, 1-pentanol, and 1-hexanol. The Perrin plots are corrected for the temperature dependence of fluorescence lifetime and the viscosity of the water-saturated alcohols (Table I).

rotational relaxation times of 15 and 16 ns, respectively. The fluorescence emission maxima of both protein–ligand complexes in 1-butanol at pH 2.4 were 522 nm. The partition characteristics of all of the modified proteins are summarized in Table I.

Perrin plots of fluorescence polarization vs. temperature are shown in Figure 1 for ethylated and guanidated dansyl-BSA partitioned at pH 7.1 with octylsulfonate into 1-butanol, 1-pentanol, and 1-hexanol. The plots are also corrected for the temperature variation of the fluorescence lifetime. The lifetimes of the dansyl-BSA complexes in 1-butanol through 1-octanol varied from 14 to 16.5 ns over the temperature range 2–38 °C. Viscosities for the water-saturated alcohols calculated from flow viscometry measurements are tabulated in Table II. These measurements agree, with the exception of saturated 1-hexanol, with values reported by Krasnov at 12, 25, and 40 °C (Krasnov & Gartseva, 1970). Krasnov's viscosity values for saturated hexanol are uniformly 0.25 cP greater than those measured in our laboratory. Reasonable fits to the data were obtainable with single exponentials of the form $\eta$ (cP) = $a + be^{-cT\,(K)} \pm \epsilon$ (cP), where $\epsilon$ is the standard error. Values for $a$, $b$, $c$, and $\epsilon$ are included in Table I.

At pH 2.4, partitioning of the guanidated and ethylated dansyl-BSA with octylsulfonate can also be obtained into 1-heptanol and 1-octanol. Perrin plots for the protein–ligand complexes in 1-butanol through 1-octanol are shown in Figure 2. Also included in Figure 2 is a Perrin plot for the pTS complex in 1-butanol. Approximate collinearity of the Perrin plots for the higher alcohols is observed.

Table II[a]

| | $\eta$ (cP) | | | | |
|---|---|---|---|---|---|
| °C | 1-butanol | 1-pentanol | 1-hexanol | 1-heptanol | 1-octanol |
| 2 | 6.47 | 7.90 | 9.96 | 14.14 | 17.73 |
| 4 | 5.98 | 7.28 | 9.16 | 12.96 | 16.17 |
| 6 | 5.52 | 6.85 | 8.43 | 11.89 | 14.77 |
| 8 | 5.11 | 6.21 | 7.77 | 10.92 | 13.51 |
| 10 | 4.74 | 5.75 | 7.17 | 10.04 | 12.37 |
| 12 | 4.40 | 5.33 | 6.63 | 9.24 | 11.35 |
| 14 | 4.09 | 4.95 | 6.13 | 8.52 | 10.42 |
| 16 | 3.80 | 4.60 | 5.69 | 7.86 | 9.59 |
| 18 | 3.55 | 4.28 | 5.28 | 7.26 | 8.84 |
| 20 | 3.31 | 3.99 | 4.91 | 6.72 | 8.16 |
| 22 | 3.10 | 3.73 | 4.57 | 6.23 | 7.55 |
| 24 | 2.90 | 3.49 | 4.27 | 5.78 | 7.00 |
| 26 | 2.72 | 3.28 | 3.99 | 5.38 | 6.51 |
| 28 | 2.56 | 3.08 | 3.74 | 5.01 | 6.06 |
| 30 | 2.41 | 2.90 | 3.52 | 4.68 | 5.66 |
| 32 | 2.28 | 2.74 | 3.31 | 4.38 | 5.29 |
| 34 | 2.16 | 2.59 | 3.12 | 4.10 | 4.97 |
| 36 | 2.04 | 2.45 | 2.95 | 3.86 | 4.67 |
| 38 | 1.94 | 2.33 | 2.80 | 3.63 | 4.41 |
| $a$ | 0.89850 | 1.0991 | 1.2845 | 1.4239 | 1.9883 |
| $b \times 10^{-6}$ | 2.0261 | 3.1491 | 5.3841 | 8.2860 | 25.655 |
| $c$ | 0.046534 | 0.047415 | 0.048477 | 0.048654 | 0.051985 |
| $\epsilon$ | 0.030 | 0.033 | 0.031 | 0.044 | 0.085 |
| wt % $H_2O$, 25 °C | 20.27[c] | 9.66[b,c] | 7.16,[b] 7.38[d] | 5.82[b] | 4.87[e] |

[a] The values for fits to single exponentials of the form $\eta$ (cP) = $a + b \exp[-cT\,(K)] \pm \epsilon$ (cP) are given for the water-saturated alcohols 1-butanol through 1-octanol. A reference table of viscosities calculated from these parameters is listed above. The weight percent water content of the saturated alkanols at 25 °C is also given. [b] Krasnov & Gartseva (1970). [c] *Solubilities of Inorganic and Organic Compounds* (1963). [d] Mindowicz & Biallozor (1962). [e] Leo & Hansch (1971).

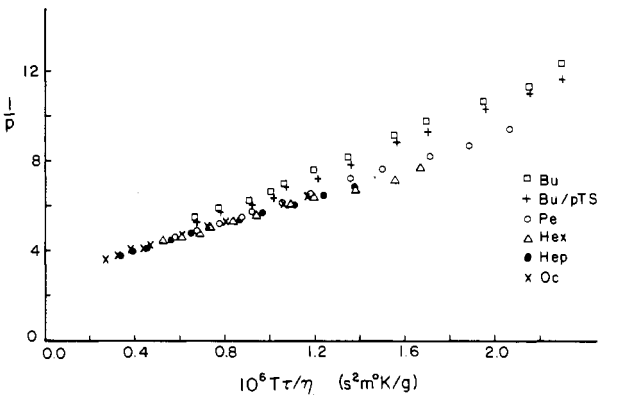


FIGURE 2: Perrin plots at pH 2.4 of 1/(fluorescence polarization) vs. temperature over the range 2–38 °C for the guanidated and ethylated dansyl-BSA complexes with octylsulfonate in 1-butanol through 1-octanol. Corrections for the temperature variation of the fluorescence lifetime and the viscosity are included. The plots in the higher alcohols are collinear.

The ratio of rotational relaxation times to viscosities at 25 °C vs. alcohol chain length is plotted in Figure 3a. This ratio, $\rho/\eta$, should scale as the molecular volume. The more extensive pH 2 data set shows a maximum in the ratio occurring for complexes in 1-hexanol. The $\rho/\eta$ value for the dansyl-BSA complex with pTS in 1-butanol is included on the graph for reference.

The fluorescence emission maxima of the ethylated and guanidated dansyl-BSA complexes with octylsulfonate in the

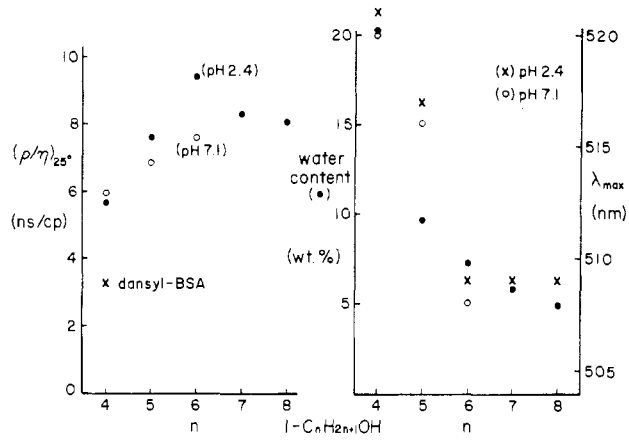


FIGURE 3: (a) Ratios of rotational relaxation time to viscosity at 25 °C for the guanidated and ethylated dansyl-BSA in different alcohols calculated from the Perrin plots in Figures 1 and 2. Unmodified dansyl-BSA partitioned into 1-butanol is included in the graph. The ratios are proportional to apparent molecular volumes. The maximum ratio is observed on 1-hexanol while the minimum ratio occurs for the unmodified dansyl-BSA in 1-butanol. (b) Water content of the water-saturated 1-alkanols together with the emission maxima of the guanidated and ethylated dansyl-BSA complexes in the alkanols at pH 7.1 and pH 2.4. The emission maxima of the complexes follow the changing water content of the saturated alcohols.

different alcohols are plotted in Figure 3b. Included in this figure are the water contents of the different water-saturated alcohols (Krasnov & Gartseva, 1970; *Solubilities of Inorganic and Organic Compounds*, 1963; Mindowicz & Biallozor, 1962; Leo & Hansch, 1971). At both neutral and acidic pH, the emission maxima of the modified dansyl–protein–ligand complexes follow the polarity of the alcohol phases (Lippert, 1957).

## Discussion

The ability to significantly alter the ligand-induced partitioning of proteins by chemical modification of the ionizable residues, carboxyls and $\epsilon$-amino group of lysine, is clearly demonstrated by these experiments with bovine serum albumin and lysozyme. A decrease in the number of carboxylate ions on the protein by ethylation lessens the hydrogen ion concentration requirement for stabilization in the nonaqueous environments. This is directly observed in the increased pH at which the ethylated proteins will partition into butanol. Also, this is reflected by the reduction of the critical partition concentration of pTS required for partitioning of the ethylated proteins. As a further example, the guanidated and ethylated dansyl-BSA was probably insufficiently ethylated because of the acid requirement for partitioning into heptanol and octanol. The uniform observation of smaller Hill coefficients in the partition of the modified proteins reflects the heterogeneity of the chemically modified populations. Even a small amount of heterogeneity can drastically reduce the originally large values of this quantity observed with intact albumin (25) and lysozyme (10).

The motivation for the guanidation modifications originated from the observed difference in pH limitations for partitioning between ethylated dansyl-BSA and ethylated dansyl-lysozyme. The total fractions of carboxyl groups in both proteins only differ slightly, as well as the overall ratio of negatively charged groups to positively charged groups in the two proteins at neutral pH. However, the balances of arginine to lysine in the two proteins are reversed. The arginine/lysine ratio for BSA is only 0.39 compared to 1.8 for lysozyme.

Partition of the charged protein into a nonaqueous phase is only possible because of the neutralization of the charge of the ionizable groups either by proton association (for $COO^-$) or by association of the basic groups of lysine and guanidine with an appropriate counterion (pTS or octylsulfonate). The partition coefficient is uniquely determined by the free energies of formation of the protein–counterion complexes in the two media (Mustacich & Weber, 1978) and, other things being equal, this difference in free energy is proportional to the difference in p$K$ values of the protein interacting groups. Conversion of the $\epsilon$-amino group of lysine into guanidine groups increases the free energy of formation of the ion pair in the nonpolar medium by the amount of $-1.38$ (p$K_{\text{arginine}}$ − p$K_{\text{lysine}}$) kcal/mol. Consequently, conversion of a fraction of the lysines into homoarginines ought to facilitate the transfer to a less polar medium at equal pH or to the same medium at a higher pH in comparison with the unmodified protein. These expectations are confirmed by experiment. Guanidation of dansyl-BSA was found to reduce the critical concentration of pTS required for transfer of the protein to 1-butanol at pH 2.4. Moreover, guanidation of ethylated dansyl-BSA made possible not only the transfer into 1-butanol at pH 7.1 but also the transfer at acid pH into 1-heptanol and 1-octanol (Figure 2). Lysines and arginines are virtually 100% charged at acid or neutral pH, and a simple computation of the charge interactions with the same counterion, without reference to the difference in free energy of ionization of the two groups, would not explain the remarkably different transfer properties of the guanidated protein.

The structural counterpart of the greater interaction free energy of the guanidino groups with carboxyl, sulfonic, and phosphate groups can be found in the ability of this planar group to form multiple hydrogen bonds (Cotton et al., 1973) and is no doubt responsible for the presence of arginine in many anion-binding active centers of enzymes (Riordan et al., 1977) and in the binding cavity of phosphorylcholine binding antibodies (Padlan et al., 1976). Proteins do not possess acid binding groups of strength beyond carboxyls, with a p$K$ of the order of 3.5. The presence of the lower p$K$ sulfonic groups in peptides (gastrone and fibrinopeptides) and polysaccharides (heparin) suggests a possible physicochemical function: the formation of stronger ion pairs capable of augmenting the partition into membranes.

The rotational relaxation times calculated from Perrin plots for the different modified proteins show low degrees of rotational freedom. Modification of dansyl-BSA by either ethylation or guanidation results in loss of the acid expansion normally observed with BSA at pH 2.4. The rotational relaxation times of the ethylated dansyl-BSA and the guanidated dansyl-BSA in butanol-saturated water at 25 °C were 105 and 107 ns, respectively. Dansyl-BSA under the same experimental conditions has a rotational relaxation time of 23 ns. The mechanism of the acid expansion is thought to involve the disruption of internal ionic bonds as well as the general electrostatic repulsion on acquiring a charge like in a flexible polyelectrolyte (Tanford, 1961). Guanidation of lysines in BSA, possibly involved in this process, could strengthen the internal interactions and shift the acid expansion to higher hydrogen ion concentrations. The mechanism by which ethylation hinders the acid expansion is not clear.

The rotational relaxation times of the modified dansyl-BSA–ligand complexes in the alcohols indicate large increases in rotational freedom, confirming previous studies (Mustacich & Weber, 1978). The rotational relaxation times of the pTS complexes in 1-butanol at pH 2.4 for dansyl-BSA (10 ns), guanidated dansyl-BSA (13 ns), ethylated dansyl-BSA (17 ns), and guanidated and ethylated dansyl-BSA (15 ns; 16 ns with the ligand octylsulfonate) all indicate considerable

freedom of rotation in the protein complexes in the upper phase. The relaxation time of dansyl-BSA in 1-butanol was calculated previously to be only 13% of that predicted from the viscosity change in transfer from the aqueous phase to the alcohol phase. The absence of appreciable aggregation of the protein–ligand complexes in the alcohols is indicated by the short relaxation times and the linearity of the Perrin plots.

A maximum in the apparent molecular volume of the complexes in different alcohols occurs in hexanol. Hexanol may provide a relative minimum between more extreme hydrophilic and lipophilic interactions of the protein–ligand complexes with the solvent phase. In 1-butanol, the water content is 20.3% by weight compared to 7.2% for 1-hexanol. The larger water content in butanol can provide extension through hydrophilic interactions, resulting in the increased degree of rotational freedom. The small $\rho/\eta$ ratios in 1-butanol provide the maximum rotational freedoms for proteins observed in these experiments. Increasing chain lengths beyond hexanol result in a similar increase in rotational freedom presumably through increased lipophilic and internal ionic interactions.

The ligand-induced partitioning of proteins from an aqueous phase entirely into an organic phase is an extreme model of the processes felt to be important in the recognition of molecules by membrane proteins. Rather than the transfer of entire macromolecules by ligand binding across the water–phospholipid interface, conformational changes of membrane proteins, either peripheral or integral, could result by partial ligand-induced partitioning into or out of the membrane phase. Partitioning can then be limited to protein domains having suitable balances of hydrophilic and hydrophobic residues, and in this respect the accumulation at the interface observed in our modified proteins provides an evident example. Indeed, the masking of various groups by chemical modification is one approach to the modeling of smaller systems once the directional effects of modifications on partitioning are understood.

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