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Hydrophobicity of Bovine Serum Albumin and Ovalbumin Determined Using Uncharged (PRODAN) and Anionic (ANS^-) Fluorescent Probes

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The influence of ionic interactions on quantitation of protein surface hydrophobicity was assessed by comparing the protein binding of an uncharged fluorescent probe, 6-propionyl-2-(*N*,*N*-dimethylamino)naphthalene (PRODAN), with that of an anionic probe, 1-(anilino)naphthalene-8-sulfonate (ANS^-). Binding constants for the protein–probe complexes involving bovine serum albumin (BSA) and ovalbumin (OVA) in phosphate buffer (pH 7.0, I = 0.01 M) at 30 °C were fluorometrically determined to be $K_{P-BSA} = (1.00 \pm 0.01) \times 10^6\ M^{-1}$ and $K_{P-OVA} = (4.2 \pm 0.1) \times 10^3\ M^{-1}$, respectively, for PRODAN, compared to $K_{A-BSA} = (6.21 \pm 0.04) \times 10^6\ M^{-1}$ and $K_{A-OVA} = (1.97 \pm 0.09) \times 10^3\ M^{-1}$, respectively, for ANS^-. A procedure was established using PRODAN to determine protein surface hydrophobicity (S_0) values from the initial slope of relative fluorescence intensity versus protein concentration plots, and the results were compared to S_0 values measured using ANS^-. Increasing ionic strength up to 1.0 M decreased the S_0 values of BSA measured by ANS^-, increased S_0 of BSA measured by PRODAN and of OVA measured by ANS^-, and had no significant effect on the S_0 of OVA measured by PRODAN. These results demonstrate the importance of considering charge effects when determining protein surface hydrophobicity.

Keywords: *Protein hydrophobicity; fluorescent probe; electrostatic interactions; PRODAN; ANS*

INTRODUCTION

Protein structure is dependent on hydrophobic, electronic, and steric parameters, which must all be taken into consideration to explain not only the biological properties of protein molecules, such as enzymatic activity, but also the functional properties that give food its characteristic texture and form (Li-Chan, 1991). By quantitatively establishing the relationship between protein structure and function, systematic and predictable enzyme and protein engineering for tailoring of specific biological and functional properties should become a reality.

The importance of hydrophobic interactions for the stability, conformation, and function of proteins is well recognized. Due to the macromolecular structure of proteins, surface (or effective) hydrophobicity is more influential for functionality than total hydrophobicity. Surface hydrophobicity influences intermolecular interactions, such as binding of small ligands or association with other macromolecules, including protein–protein or protein–lipid interactions.

Much effort has been made to quantify protein surface hydrophobicity; however, no consensus has been reached on a standard method for its measurement (Nakai et al., 1996). Methods using fluorescent probes have proved most popular due to their simplicity, speed, ability to predict functionality, and use of small quantities of purified protein for analysis. Fluorescence probe methods (Kato and Nakai, 1980; Hayakawa and Nakai, 1985) measure hydrophobic groups on the protein surface that are able to bind the probe; the probe's quantum yield of fluorescence and wavelength of maximal emission depend on the polarity of its environment. Under conditions with excess probe, the initial slope (S_0) of the fluorescence intensity versus protein concentration plot has been shown to be correlated to the effective hydrophobicity determined by a hydrophobic partition method and to protein surface properties such as interfacial tension and emulsifying activity (Kato and Nakai, 1980). Thus, the S_0 value has been proposed as an index of protein surface hydrophobicity. Fluorescence probe methods are accepted as a means for assessing hydrophobic sites available on the surface of protein molecules, such as to reflect their overall three-dimensional structure in solution. Another advantage of these methods is their applicability to complex systems composed of several interacting molecular species, giving average surface hydrophobicity of a protein mixture.

The most popular types of probes used for spectrofluorometric measurement include the anionic probes of the aromatic sulfonic acid class, such as 1-(anilino)-naphthalene-8-sulfonate (ANS^-), its dimeric form (bis-ANS^{2-}), and 6-(*p*-toluidinyl)naphthalene-2-sulfonate (TNS^-). These probes have been used extensively to quantify protein hydrophobicity, to monitor conformational changes in biological macromolecules, and to study protein binding sites. Another group of anionic fluorescence probes is the fatty acid analogue type, including *cis*-parinaric acid (CPA), which dissociates in solution to form *cis*-parinarate (CPA^-) and has been used as a probe for proteins and biological membranes.

Limitations in using ionic probes, such as ANS^- and

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CPA^-, to determine protein hydrophobicity include the possibility that electrostatic as well as hydrophobic interactions may contribute to the probe−protein interaction. Greene (1984) assessed the influence of electrostatic forces between proteins and charged probes. Although the major contribution to the energy of binding is derived from hydrophobic interactions, significant ionic influences are also recognized. Cations and anions do not appear to have equal access to the same binding sites, and it has been suggested that, in general, electrostatic effects are more likely to enhance the protein binding of anions rather than cations. Hence, the use of anionic probes is more likely to result in an overestimation of protein hydrophobicity. The use of neutral or uncharged probes may circumvent this problem.

More recently, the nonpolar nondissociable fluorescent probe, diphenylhexatriene (DPH), which clearly labels the interior of membranes and has been used to determine the fat binding capacity of proteins, has been suggested as an appropriate fluorescence probe to quantitate protein surface hydrophobicity (Tsutsui et al., 1986). Unfortunately, the nonpolar nature of DPH restricts its solubility in aqueous systems and thus limits its use as a probe for protein solutions. This problem has been overcome by dissolving DPH in corn oil prior to interaction with aqueous protein solution. However, the rather tedious and time-consuming nature of the procedure has resulted in a lack of wide use. Furthermore, the resulting parameter is correlated to fat binding capacity rather than hydrophobicity per se.

The fluorescent probe 6-propionyl-2-(*N,N*-dimethylamino)naphthalene (PRODAN) offers outstanding sensitivity to environmental polarity, and the absence of a permanent charge (Weber and Farris, 1979) will eliminate potential inclusion of electrostatic interaction contributions in the measurement of protein hydrophobicity. This electrically neutral fluorescent probe exhibits shifts in both excitation and emission spectra with changes in solvent polarity (Slavik, 1994). It has already received photobiological use in determining microscopic polarity in biological systems such as lipid bilayers, membranes, and protein interiors (Bunker et al., 1993). It has also been used to probe the interaction of alcohols with biological membranes (Rottenberg, 1992) and may be useful to show exposure of hydrophobic sites on enzymes or to detect aggregation of hydrophobic moieties (Bruins and Epand, 1995). Although low, the solubility of PRODAN in aqueous solution (5×10^{-6} M) (Mazumdar et al., 1992) gives this probe an advantage over DPH.

The objective of the work described in this paper was to compare PRODAN and ANS^- as fluorescent probes for measuring protein hydrophobicity. A new procedure was established using the uncharged fluorescent probe PRODAN to determine S_0 as an index of protein surface hydrophobicity. The influence of electrostatic interactions on hydrophobicity measurement was monitored by determining the binding constants of PRODAN and ANS^- with bovine serum albumin (BSA) and ovalbumin (OVA) and by comparing S_0 values measured for BSA and OVA under conditions of varying ionic strength.

EXPERIMENTAL PROCEDURES

Materials. The magnesium salt of ANS^- was prepared according to the method of Weber and Young (1964) and stored in the dark. PRODAN (Molecular Probes, Inc., Junction City, OR) was used without further purification as no major impurities were detected upon excitation at 280 nm. Bunker et al. (1993) reported a fluorescent contaminant in samples of PRODAN from Molecular Probes, which was revealed by the appearance of an emission near 430 nm upon 280 nm excitation. If present, this contaminant can be extracted from a saturated water solution with *n*-hexane. The PRODAN stock solution was prepared by stirring a suspension for 2 days, followed by filtering. BSA (product no. A-4503) and OVA (product no. A-5503) from Sigma-Aldrich Canada (Oakville, ON) were used without further purification. Phosphate buffer (pH 7.0, $I = 0.01$ M) was prepared (Dawson et al., 1969) using analytical reagent grade chemicals. Sodium azide (0.02%) was added as an antimicrobial agent to all buffer solutions. Distilled deionized water was used in the preparation of all solutions. Fresh solutions were used for all measurements, and exposure to light was kept to a minimum by wrapping containers in aluminum foil. No change in spectral properties of the PRODAN solution was detected over 60 days when stored at 7 °C.

Concentrations of the ANS^- and PRODAN stock solutions were determined spectrophotometrically at 350 and 365 nm, respectively, using molar absorption coefficients of ϵ_{350}(ANS) $= 4.95 \times 10^3$ M^{-1} cm^{-1} (Weber and Young, 1964) and ϵ_{365}(PRODAN) $= 1.45 \times 10^4$ M^{-1} cm^{-1} (Weber and Farris, 1979). Concentrations of the BSA and OVA stock solutions were determined by measuring the light absorption at 280 nm and using extinction coefficients for the absorption of a 1% solution in a 1 cm path length cuvette ($E^{1\%}_{1cm}$) of 6.61 (Fasman, 1992) and 7.12 (Glazer et al., 1963), respectively. When these were converted to molar values, molecular weights of 66 000 (Fasman, 1992) and 42 699 (Nisbet et al., 1981) were assumed for BSA and OVA, respectively.

Binding Constant Determinations. Buffered (pH 7.0, $I = 0.01$ M) stock solutions, 1.11×10^{-5} M and 7.47×10^{-6} M in ANS^- and 1.31×10^{-6} M and 2.76×10^{-6} M in PRODAN, were used in determining the BSA and OVA binding constants, respectively. Stock BSA and OVA solutions were prepared in the same buffer at final concentrations of 3.3 and 8.8% (w/v), respectively.

Fluorescence spectra were recorded in a 1 cm path length quartz cuvette on a Perkin-Elmer LS 50B fluorometer in which the samples were thermostated at 30 ± 0.5 °C. Excitation and emission slit widths of 5 nm were used for all measurements. An excitation wavelength of 365 nm was selected, and emission spectra were collected between 380 and 620 nm for all systems. The molar fluorescences of ANS^-, PRODAN, BSA, and OVA were each determined from spectra run at six different concentrations. Background fluorescence attributed to all sample components except the probe was subtracted from the spectra.

Fluorescence spectra were recorded for at least 20 protein concentrations at a constant probe concentration. The range of protein concentrations and the probe concentration used for each protein−probe system were as follows: 2.10×10^{-7} to 1.49×10^{-5} M BSA and 5.20×10^{-7} M ANS^-; 6.84×10^{-6} to 1.50×10^{-4} M OVA and 2.50×10^{-6} M ANS^-; 6.32×10^{-7} to 2.52×10^{-5} M BSA and 9.83×10^{-7} M PRODAN; and 7.06×10^{-6} to 1.43×10^{-4} M OVA and 9.21×10^{-7} M PRODAN. At the probe concentrations used, a linear dependence of fluorescence on probe concentration exists in the absence of protein.

Binding constants were determined for the strongest binding sites from a simultaneous fit of the fluorescence data (Haskard et al., 1996) at 0.5 nm intervals over the protein ranges and wavelength ranges where greatest spectral change was observed upon addition of protein. The wavelength range selected had no significant effect on the binding constants obtained. Thus, a minimum of 4600 data points were simultaneously used in the derivation of each stability constant. All data fitting was carried out on a Pentium (75 MHz, 8 MB RAM) computer using a nonlinear least-squares regression routine based on method 5 of Pitha and Jones (1966) through the program DATAFIT/SPECFIT (T. Kurucsev, The University

of Adelaide, South Australia, personal communication), which outputs best-fit parameters and their standard deviations.

Effect of Excitation Wavelength. Fluorescence spectra were also collected for one protein concentration in each system using a range of excitation wavelengths from 320 to 420 nm. The contributions of free and bound probe were separated by fitting two symmetric Gaussians in the wavenumber (cm^{-1}) domain using GRAMS/386 (version 3.02, level 1, Galactic Industries Corp.), fixing only the peak center for free probe. The emission peak center and width (full width at half-maximum height or fwhm) of the bound probe were determined and reported in wavenumber (cm^{-1}) units, for comparison to previous studies on the spectral properties of PRODAN (Weber and Farris, 1979; MacGregor and Weber, 1986; Baasov and Sheves, 1987; Bunker et al., 1993; Lasagna et al., 1996).

Measurement of Surface Hydrophobicity. Stock solutions of 8×10^{-3} M ANS^-, 3×10^{-6} M PRODAN, and 1.5 or 0.2% (w/v) protein were prepared in phosphate buffer (pH 7.0, I = 0.01 M). Measurements were performed essentially according to the method of Kato and Nakai (1980), with modifications as described below.

To successive samples containing 4 mL of buffer and 20 μL of ANS^- stock solution were added 10, 20, 30, 40, and 50 μL of 1.5% protein solution. To successive 8 mL samples of PRODAN stock solution were added 10, 20, 30, 40, and 50 μL of 0.2% protein solution. (The low water solubility of PRODAN results in larger volumes of probe stock solution being required to ensure sufficient excess PRODAN to saturate all binding sites on the protein.) Samples were mixed by inversion.

Relative fluorescence intensity (RFI) was measured at 30 ± 0.5 °C with either a Perkin-Elmer LS 50B fluorometer or a Shimadzu RF-540 spectrofluorophotometer. Excitation and emission wavelengths of 390 and 470 nm for ANS^- and 365 and 465 nm for PRODAN, respectively, and slit widths of 5 nm were used. Day-to-day fluctuations in the RFI values were monitored by measuring the reading of the fluorometer for PRODAN or ANS^- stock diluted in methanol. A solution containing 1.5 g of stock PRODAN made up to 3.0 g with methanol had an RFI of 3.5, and a solution containing 0.01 g of stock ANS^- made up to 10.0 g with methanol had an RFI of 7.0, under the excitation and emission conditions stated above.

The RFI of each protein in buffer alone (no probe) was also measured at five concentrations. The net RFI for each sample with probe was then computed by subtracting the RFI attributed to protein in buffer. The initial slope (S_0) of the net RFI versus protein concentration (percent) plot was calculated by linear regression analysis and used as an index of the protein surface hydrophobicity as suggested by Kato and Nakai (1980).

No time dependence of the fluorescence intensity was observed in the systems studied within 5–15 min after mixing. Each system was run at least in duplicate.

Effect of Ionic Strength. To the phosphate buffer (pH 7.0, I = 0.01 M) was added NaCl, producing 50 mL buffer solutions with final ionic strengths of 0.15, 0.3, 0.5, 0.75, and 1.0 M. Similarly, NaCl was added to the PRODAN stock solution, producing 50 mL stock solutions with these five ionic strengths. These new stocks were used in addition to the original stock with ionic strength of 0.01 M, in determining the protein surface hydrophobicity at increasing ionic strength.

RESULTS

The fluorometrically determined binding constants (K) for the complexation of the probes, ANS^- and PRODAN, by BSA and OVA and the wavelengths of maximal emission (λ_{max}) for the complexes formed are shown in Table 1. In each case the probe's λ_{max} was blue shifted upon complexation with protein, and a greater shift was observed for the complexes formed with BSA than with OVA. The best-fit parameters obtained by data fitting, using a nonlinear least-squares regression routine, indicated that no complex species besides the 1:1 complexes listed in Table 1 were detected in significant amounts.

Table 1. Binding Constants (K) and Wavelengths of Maximal Emission (λ_{max}) of the Complexes Formed by ANS^- and PRODAN with BSA and OVA in Phosphate Buffer at pH 7.0, I = 0.01 M, and 30 °C (This Work, Shown in Bold Type) and Reported Values for Protein·PRODAN Complexes

species	K^a (M^{-1})	λ_{max} (nm)
ANS^-		**509.0**
OVA·ANS^- [b]	**(1.97 ± 0.09) × 10^3**	**476.5**
BSA·ANS^- [c]	**(6.21 ± 0.04) × 10^6**	**471.0**
PRODAN		**511.0**
OVA·PRODAN[d]	**(4.2 ± 0.1 × 10^3**	**466.0**
BSA·PRODAN[e]	**(1.00 ± 0.01) × 10^6**	**461.5**
BSA·PRODAN[f]	1 × 10^5	469
tubulin·PRODAN[g]	5 × 10^4	450
spectrin·PRODAN[h]	2 × 10^6	433
apohorseradish peroxidase·PRODAN[i]	1.15 × 10^4	478

[a] Errors represent one standard deviation. [b] Fitted over 425–550 nm. [c] Fitted over 420–535 nm. [d] Fitted over 400–515 nm. [e] Fitted over 415–480, 500–570 nm. [f] Weber and Farris (1979). [g] Mazumdar et al. (1992). [h] Chakrabarti (1996). [i] Lasagna et al. (1996).

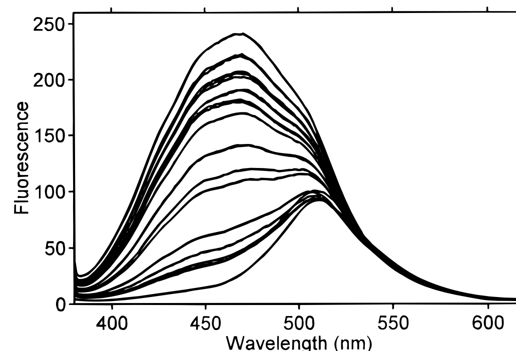

Figure 1. Variation in fluorescence emission spectrum of PRODAN (9.21×10^{-7} M) with concentration of OVA in the range 7.06×10^{-6} to 1.43×10^{-4} M in aqueous phosphate buffer at pH 7.0, I = 0.01 M, and 30 °C. Increasing OVA concentration corresponds to a blue shift and increase in intensity. Excitation wavelength was 365 nm, and slit widths were 5 nm.

The fluorescence spectra that were used to derive the binding constant for OVA·PRODAN are shown in Figure 1. The best fit of the data at 466 nm is shown in Figure 2. The inability to obtain a better visual fit to the curve may be an indication that either the fluorophore or the protein sample consisted of two components. However, it may also be attributed to heterogeneous binding of PRODAN, as reported by Lasagna et al. (1996). Similar spectral montages were obtained for the other systems studied. Due to the stronger fluorescence of PRODAN in aqueous solution, by comparison with ANS^-, the shift in λ_{max} upon protein binding was more readily seen in the PRODAN systems.

The effect of excitation wavelength on the bound probes' emission peak center and width is shown in Figure 3. The emission peak center of bound PRODAN was significantly red shifted, with an increase in excitation wavelength from 320 to 420 nm, whereas no substantial shift was observed for bound ANS^- over this excitation wavelength range. An increase in excitation wavelength resulted in narrowing of the emission peak width.

The effect of increasing ionic strength on measured protein surface hydrophobicity (S_0) values is shown in

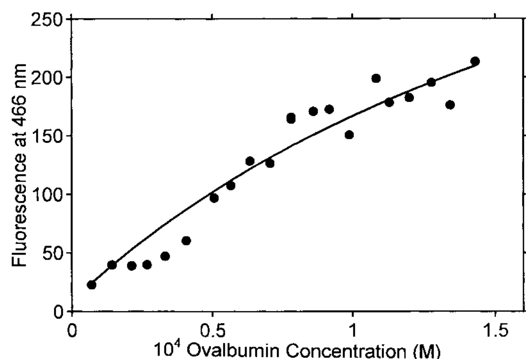

Figure 2. Variation in relative fluorescence intensity of PRODAN (9.21×10^{-7} M) at 466 nm with concentration of OVA in the range 7.06×10^{-6} to 1.43×10^{-4} M in aqueous phosphate buffer at pH 7.0, $I = 0.01$ M, and 30 °C. Excitation wavelength was 365 nm, and slit widths were 5 nm. The solid curve represents the best fit of the data, collected over the range 400−515 nm, to the model for formation of OVA·PRODAN.

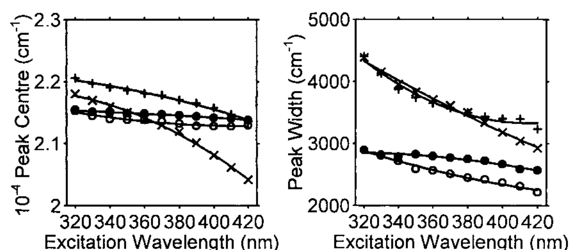

Figure 3. Variation in the bound probes emission peak center and width with excitation wavelength, for solutions containing ANS⁻ and BSA (●), ANS⁻ and OVA (○), PRODAN and BSA (+), and PRODAN and OVA (×), in aqueous phosphate buffer at pH 7.0, $I = 0.01$ M and 30 °C.

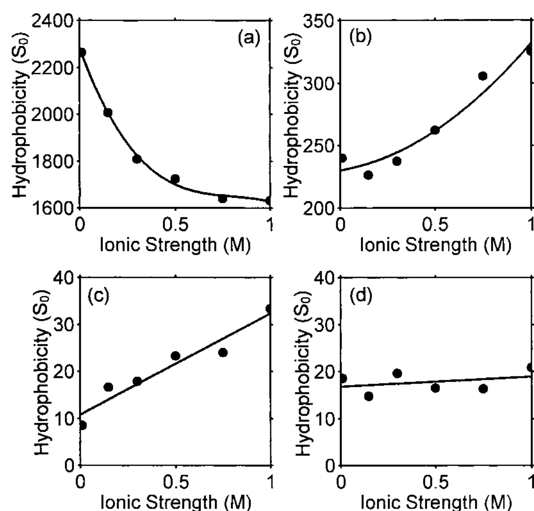

Figure 4. Protein surface hydrophobicity (S_0) variation with ionic strength in the range 0.01−1.0 M in aqueous phosphate buffer at pH 7.0 and 30 °C: (a) BSA hydrophobicity measured with ANS^-; (b) BSA hydrophobicity measured with PRODAN; (c) OVA hydrophobicity measured with ANS^-; (d) OVA hydrophobicity measured with PRODAN.

Figure 4. An increase in ionic strength resulted in a decrease in S_0 of BSA measured by ANS^- and an increase in its S_0 measured by PRODAN. For OVA, an increase in S_0 was observed with increasing ionic strength when measured by ANS^-, but no difference in S_0 was observed when measured by PRODAN. The values of S_0 for BSA and OVA at the lowest and highest ionic strengths studied in this work are listed along with related literature data in Table 2.

Table 2. Hydrophobicity (S_0) Values for BSA and OVA Determined by ANS⁻ and PRODAN (This Work, Shown in Bold Type) and Other Fluorescent Probe Methods

probe	S_0 (%$^{-1}$) BSA	S_0 (%$^{-1}$) OVA
ANS⁻	**2263**,[a] **1630**,[b] **941**,[c] 1600,[d] 124[e,f]	**8**,[a] **33**,[b] **0.5**,[c] 10,[d] 177[e]
CPA⁻	2750[d]	40[d]
DPH	92[g]	14[g]
PRODAN	**240**,[a] **325**,[b] **152**[c]	**18**,[a] **21**,[b] **1**[c]

[a] This work (pH 7, $I = 0.01$ M, 30 °C). [b] This work (pH 7.0, $I = 1.0$ M, 30 °C). [c] This work (from binding constants determined in phosphate buffer at pH 7, $I = 0.01$ M, 30 °C). [d] Li-Chan (1991). [e] Cardamone and Puri (1992) (from binding constants). [f] Average binding constant for 10 binding sites. [g] Tsutsui et al. (1986).

DISCUSSION

Hydrophobic interactions between ANS^- or PRODAN and a protein are expected to be similar. Both ANS^- and PRODAN contain a naphthyl moiety, and competition experiments with tubulin indicate that they probably bind to a common region (Mazumdar et al., 1992).

Comparison of the binding constants (Table 1) indicates a 6-fold greater value for the BSA·ANS^- complex compared to the BSA·PRODAN complex, suggesting that BSA may possess a positively charged residue adjacent to the hydrophobic binding site, which results in enhanced binding of the ANS^- anion by comparison with uncharged PRODAN. These results are in agreement with reports that binding of hydrophobic anions to BSA requires a hydrophobic environment and cationic charges on the protein (Weber and Laurence, 1954). BSA binds anions more tightly than cations or neutral molecules with similar hydrophobic groups, even when the overall charge on the protein is negative (Jonas and Weber, 1971), as it is at pH 7. An anion was also found to bind a higher number of discrete sites on BSA than did a related cation (Greene, 1984).

A strong ANS^- binding site of BSA is located near the negatively charged N-terminal end of the protein, subdomain 1-C, which has a high relative content of basic residues (mainly arginine) and hydrophobic amino acid residues, including one tryptophan (Jonas and Weber, 1971; Brown and Shockley, 1982; Era et al., 1985). Although there is no direct evidence that positively charged arginine residues contribute to the binding of hydrophobic anions by BSA, they are strongly implicated by their high relative concentration in the region and by the effect that their chemical modification has on the binding of anions such as ANS^- (Jonas and Weber, 1971). Era et al. (1985) speculated that a hydrophobic cluster with two arginines and one glutamate on the C-terminal side of Trp-134 in subdomain 1-C may be a hydrophobic anion binding site. This supports the possibility of a positively charged residue being involved in the binding of ANS^- to BSA. The additional aromatic ring of ANS^-, by comparison with PRODAN, may also enhance its hydrophobic interactions with the protein and overcome any additional steric hindrance.

Conversely, OVA may possess a slight negative charge adjacent to the hydrophobic binding site, resulting in inhibited binding of the ANS^- anion by comparison with neutral PRODAN. The binding constant of OVA·ANS^- was approximately half that of the OVA·PRODAN

complex (Table 1). From crystallographic analysis, there are four OVA molecules in the unit cell along with a single metal ion, which is proposed to be coordinated by four oxygen atoms from the side chains of two adjacent molecules: phosphoserine-350 (molecule A), Glu-281b (molecule D), and Glu-201 and water (molecule A) (Stein et al., 1991). Probe binding in this region of the protein would be consistent with repulsive electrostatic interactions between ANS^- and OVA. Steric factors may also inhibit binding of the larger ANS^- molecule relative to PRODAN, swamping any increase in hydrophobic interactions.

The binding constant determined for BSA·PRODAN in the current study is 10-fold greater than that approximated by Weber and Farris (1979). This difference may arise from variations in experimental conditions [solutions in water reported by Weber and Farris (1979) versus in buffers with controlled pH, ionic strength, and temperature in this study] and also in the fatty acid content of BSA, since both ANS^- and fatty acids are known to bind the 1-C subdomain of BSA.

The observed blue shift in λ_{max} of both probes upon complexation by BSA or OVA (Table 1; Figure 1) is indicative of an increase in hydrophobicity of their environment upon protein binding. For each probe, the greater extent of blue shift observed for the probe bound to BSA than that bound to OVA is consistent with the higher hydrophobicity of the strongest binding sites of BSA. However, despite the contrast in hydrophobicity of BSA (high) and OVA (low), when excited at 365 nm the difference between the two proteins in λ_{max} of bound ANS^- was only 5.5 nm and the difference in λ_{max} of bound PRODAN was only 4.5 nm (Table 1). This may be due, in part, to the presence of one or more water molecules in the vicinity of the bound probe.

The red shift in emission maximum and decrease in peak width of PRODAN bound to BSA or OVA with increasing excitation wavelength in the range 320−420 nm (Figure 3) has also been observed for PRODAN bound to apohorseradish peroxidase, while no variation was found for PRODAN in solvent alone (Lasagna et al., 1996). Such spectral changes are characteristic of relaxation processes in the solvent surrounding the fluorophore and may be indicative of a heterogeneous population of protein·PRODAN complexes (Lasagna et al., 1996). It has been suggested that, in most cases, the presence of more than one fluorescent compound should be suspected if the shape of a fluorescence emission spectrum is altered by varying the wavelength of the exciting light (Slavik, 1994). However, in the case of PRODAN, the variation in fluorescence emission spectrum could also be attributed to the spectral contributions of free and bound probe.

For each probe, the relative order of λ_{max} (OVA > BSA) was independent of excitation wavelength over the range 320−420 nm (Figure 3). No significant increase in the difference between λ_{max} of OVA·ANS^- and BSA·ANS^- could be achieved by changing the excitation wavelength. The difference in λ_{max} between OVA·PRODAN and BSA·PRODAN could be doubled by excitation at 420 nm rather than at 365 nm, thereby expanding the range of measurements to increase distinction between proteins of various hydrophobicities. However, there are other limitations to the use of λ_{max} as a hydrophobicity scale.

The λ_{max} values of 466.0 and 461.5 nm for OVA- and BSA-bound PRODAN, respectively, are intermediate on the PRODAN scale, which is based on spectral properties in solvents of different polarities and ranges from 401 nm for cyclohexane to 505 nm for methanol (Weber and Farris, 1979). The binding constants shown in Table 1 imply increasing hydrophobicity in the order OVA < apohorseradish peroxidase < tubulin < BSA < spectrin, whereas the λ_{max} values are consistent with the order apohorseradish peroxidase < OVA < BSA < tubulin < spectrin. Several investigators (Ainsworth and Flanagan, 1969; Penzer, 1972; Dodiuk et al., 1979) have indicated that the spectroscopic properties of fluorescence probes in the binding site of a protein should be interpreted carefully. The spectroscopic properties depend not only on the polarity of the environment but on its rigidity and ability to solvate the chromophore. For example, the binding site of PRODAN on tubulin is known to be shielded from solvent (Mazumdar et al., 1992), and ANS^- molecules bound to rigid hydrophilic binding sites in chymotrypsin and apomyoglobin have been incorrectly interpreted as being hydrophobic sites (Slavik, 1994). Hence, binding constants give a more reliable measure of the relative surface hydrophobicity of proteins than does λ_{max}.

The variation with increasing ionic strength of BSA and OVA surface hydrophobicity index values measured by ANS^- or PRODAN (Figure 4) is consistent with electrostatic interactions interfering in the measurement of protein surface hydrophobicity when ANS^- is used. However, there is also a possibility that the probes are bound to different sites on the proteins and that these sites are affected differently by the changes in ionic strength.

The decrease in ANS^--measured surface hydrophobicity of BSA with increasing ionic strength contrasts the increase in that measured with PRODAN under the same conditions. This implies that factors besides the effect of increasing ionic strength on BSA surface hydrophobicity should be considered. If the anionic probe, ANS^-, binds BSA at a site that is adjacent to a positively charged residue, as the ionic strength is increased the positive charge on the protein is likely to be neutralized by the smaller Cl^- ions. The decreased electrostatic interactions between ANS^- and BSA with increasing ionic strength would thus contribute to the observed decrease in binding of ANS^- to BSA. Daniel and Weber (1966) found that addition of NaCl weakens the affinity of BSA for ANS^- but that this effect is reversed at high concentration (3 M) where the curve takes on some cooperative character. It is possible that as ionic strength is increased, the anion binding, which can stabilize BSA against denaturation (Putnam, 1960), is reduced to the point at which the protein becomes denatured.

The binding of uncharged PRODAN to BSA is enhanced by an increase in ionic strength, which may strengthen hydrophobic interactions or denature the protein, exposing hydrophobic sites that were previously buried in the protein interior. The effects of various conditions (e.g., ionic strength, pH, temperature, denaturing agents) on the free probes as well as proteins need to be considered when one is interpreting hydrophobicity measurements. No substantial change in the fluorescence of ANS^-, BSA, or OVA was observed over the ionic strength range, but increasing the ionic strength to 1.0 M resulted in an exponential decrease in the fluorescence of the PRODAN stock solution to 44% of that at $I = 0.01$ M. These results suggest that

PRODAN may form micellar aggregates under high ionic strength conditions. However, this possibility was not pursued in the current work since the fluorescence of the PRODAN stock should have no effect on the protein hydrophobicity index (S_0) values, which are determined from the initial slope of RFI versus protein concentration plots rather than from absolute values. Despite the decreasing fluorescence of the free PRODAN stock solution with increasing ionic strength, a net increase in fluorescence was observed for BSA-bound PRODAN under conditions of increasing ionic strength.

The enhanced binding of ANS^- to OVA with an increase in ionic strength is consistent with Na^+ neutralization of the proposed negative charge adjacent to the binding site, reducing repulsion between the probe and protein, thus enhancing binding. Increasing ionic strength had no significant effect on the binding of PRODAN to OVA. The fact that the hydrophobicity values did not significantly change even at the high ionic strength of unity supports the concept that the affinity of PRODAN for OVA is hydrophobic.

As can be seen in Table 2, considerable variations are apparent in the literature values for the fluorescent probe measured hydrophobicity of BSA and OVA. These variations may arise from differences in ionic strength, pH, temperature, buffer salts, and source and purity of the protein used in the measurements. In the ionic strength range studied in this work, ANS^- hydrophobicity of BSA was at least 49-fold that of OVA, whereas PRODAN hydrophobicity of BSA was at least 13-fold that of OVA.

Much work on protein binding properties has been carried out with ionic ligands having hydrophobic side chains. The affinity of the protein for such ligands depends on both hydrophobic and electrostatic interactions (Pederson et al., 1995). If hydrophobic regions of a protein are adjacent to charged side-chain residues, the binding interaction of ionic probes with the protein may be perturbed by electrostatic effects operating in addition to hydrophobic effects (Greene, 1984). The significance of electrostatic interactions will vary from one protein to another. Few, if any, proteins exhibit such strong affinity for anions as does serum albumin (Putnam, 1960). Hence, overestimation of hydrophobicity is probably greatest with BSA.

Further work is in progress to evaluate the potential advantages of the electrically neutral fluorescence probe PRODAN over anionic probes such as ANS^- and CPA^- by determining the surface hydrophobicity index values for a wide spectrum of food proteins. The nondissociable nature of PRODAN may be of particular value in investigating the effects of changes in broad ranges of pH and ionic strength on protein surface hydrophobicity. It may also be useful to resolve the reported inconsistencies between hydrophobicities measured by ANS^- and CPA^- probes for some proteins, for example, β-lactoglobulin (Hayakawa and Nakai, 1985).

ABBREVIATIONS USED

ANS^-, 1-(anilino)naphthalene-8-sulfonate; PRODAN, 6-propionyl-2-(*N,N*-dimethylamino)naphthalene; BSA, bovine serum albumin; OVA, ovalbumin.

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