Biochem. J. (1992) 282, 589–593 (Printed in Great Britain)

Spectrofluorimetric assessment of the surface hydrophobicity of proteins

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The equilibrium binding of the apolar fluorescent dye 1-anilinonaphthalene-8-sulphonate (ANS) to bacteriorhodopsin, BSA, chicken egg lysozyme, ovalbumin, porcine somatotrophin (PST) and bovine pancreatic ribonuclease (RNAase) was quantitatively evaluated using Scatchard- and Klotz-plot analyses. On the basis of the average association constant for ANS binding sites (K_a), the proteins could be ranked in order of surface hydrophobicity as:

Bacteriorhodopsin > BSA > ovalbumin > PST > lysozyme > RNAase

The number of protein–ANS binding sites was determined as 54, 10, 3, 1, 2 and 1 respectively. The ANS-based assessment of the surface hydrophobicity of these proteins was generally in agreement with the average hydrophobicity based on amino acid sequence [Bigelow (1967) J. Theor. Biol. **16**, 187–211], except for results with PST and ovalbumin. The proteins were also analysed by reversed-phase h.p.l.c. using C_1 and C_8 columns. There was no significant correlation between ANS and reversed-phase-h.p.l.c. assessment of hydrophobicity, with the results obtained by h.p.l.c. being dependent upon the column used. ANS-based measurement of surface hydrophobicity appears to be the most appropriate means for assessing proteins such as to reflect their overall three-dimensional structure in solution.

INTRODUCTION

Hydrophobicity is the tendency of non-polar solutes to adhere to one another in aqueous environments [1]. Hydrophobic interactions in proteins have a major role in defining conformation and mediating protein–protein interactions [2]. In addition, the number and the relative size of hydrophobic sites on a protein's surface usually dictates its solubility and propensity to aggregate under physiological conditions of pH, temperature and ionic strength [3]. The ability to measure the hydrophobicity of a protein may prove useful in understanding and predicting the effects of manipulation of the sequence of structural or functional domains, for example after site-directed mutagenesis. The present paper describes a novel method of evaluating the surface hydrophobicity of a protein in its native state, using the fluorescent dye 1-anilinonaphthalene-8-sulphonate (ANS). This allows a relatively rapid, non-destructive and simple means of quantitatively assessing the apolar nature of a series of different proteins.

Stryer [4] first reported that the quantum yield of ANS increased significantly after binding to the hydrophobic portions of proteins and suggested the use of ANS as a hydrophobic probe for the study of conformational changes in a given protein. However, ANS binding has not been previously used to assess the hydrophobicity of different proteins. We have established a method for quantitatively assessing the average surface hydrophobicity of proteins by estimating the association constant (K_a) of ANS–protein binding. These K_a values differ between known hydrophobic and hydrophilic proteins. We have also compared the results obtained using ANS with those derived by other common experimental means of assessing the hydrophobicity of proteins, such as reversed-phase h.p.l.c. Unlike previous methods, the ANS procedure allows an assessment of surface hydrophobicity.

EXPERIMENTAL

Chemicals

Purified proteins in freeze-dried form and all other analytical-grade reagents used were obtained from Sigma. The solutions were prepared using MilliQ RO water. Purified recombinant porcine somatotropin (PST) was produced as described in [4a]. The buffer used for binding studies was 0.1 M-Tris/HCl, pH 8.0, containing 0.1 M-NaCl.

Protein and ANS estimations

The concentration of protein and ANS solutions was calculated by using molar absorption coefficients (ϵ). The ANS concentration was determined by assuming an ϵ_{350} value of 5000 $M^{-1} \cdot cm^{-1}$ [4]. The concentration of the protein solutions was estimated spectrophotometrically by using an ϵ_{280} value of 43600 $M^{-1} \cdot cm^{-1}$ for BSA [5], 36000 $M^{-1} \cdot cm^{-1}$ for lysozyme [6], 29400 $M^{-1} \cdot cm^{-1}$ for ovalbumin [7] and 9630 $M^{-1} \cdot cm^{-1}$ for RNAase [8]. The ϵ_{280} for recombinant PST at 280 nm was determined to be 20500 $M^{-1} \cdot cm^{-1}$. The concentration of bacteriorhodopsin was determined by using an ϵ_{560} value of 54000 $M^{-1} \cdot cm^{-1}$ at 560 nm [9]. All photometric measurements were performed using a Varian DMS-90 spectrophotometer.

Fluorescence measurements

The interaction of the fluorescent dye ANS with proteins was analysed with a Hitachi F-2000 fluorescence spectrophotometer. The sample temperatures were equilibrated at 25 °C by a turretted four-cell thermostatically controlled holder connected to a circulating water bath, and fluorescence measurements were conducted in quartz optical cells of 10 mm pathlength. Quantum yields were calculated by the method of Parker & Rees [10], with β-carboline as the standard [11].

Abbreviations used: ANS, 1-anilinonaphthalene-8-sulphonate; PST, porcine somatotropin; RNAase, (bovine pancreatic) ribonuclease.

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Equilibrium binding of ANS and protein

Freeze-dried proteins were resuspended in buffer at a concentration of 3–5 mg/ml. Duplicate samples of ANS solutions ranging from 0 to 500 μM (final concn.) were prepared in 5 ml tubes. A 50 μl portion of a 3 mg/ml protein solution was added to one set of tubes and 50 μl of buffer to the other. The samples were vortex-mixed and equilibrated at 25 °C for 2 h before analysis, and fluorescence readings were made for triplicate samples. Measurements were taken at equilibrium, which in these studies was taken to be a fluorescence measurement unchanged over time. The ANS was excited at 350 nm, and 10 nm excitation and emission slitwidths were used. Fluorescence emission was monitored at the wavelength of maximum intensity, which was evaluated in each case from a variable-wavelength scan of the protein/ANS mixture. The fluorescence measurements were corrected for primary absorbance effects by using the procedure of Birdsall *et al.* [12].

Reversed-phase h.p.l.c. analysis

Proteins were analysed on a h.p.l.c. system (Beckman) using data-handling software (Varian). Proteins were separated using a C_1-alkyl-bonded silica column (LKB Ultropac; TSK TMS-250) with dimensions of 10 μm particle size, 25 nm mean pore diameter and 4.6 mm × 75 mm, and a C_8-alkyl-bonded silica column (Beckman Ultrasphere Octyl column) with dimensions of 5 μm particle size, 30 nm mean pore diameter and 4.6 mm × 250 mm. Proteins were loaded on to the two columns equilibrated with 15% acetonitrile/0.1% trifluoroacetic acid and eluted with a linear gradient of 30–70% (v/v) acetonitrile over 20 min.

RESULTS

Change in ANS fluorescence after binding of ANS to bacteriorhodopsin, lysozyme, ovalbumin, PST and RNAase

The binding of the apolar dye ANS to protein is associated with an enhanced fluorescence and a blue shift in the wavelength of peak emission ($\lambda_{max.}$), as illustrated by the binding of ANS to PST and RNAase in Fig. 1. The changes in $\lambda_{max.}$ and quantum yield after ANS binding to the studied proteins are summarized in Table 1. The change in ANS fluorescence is of sufficient magnitude to evaluate the average equilibrium constant, K_a, for each protein–ANS interaction. The analysis of ANS–PST equilibrium binding is described in detail as an example of the approach used to derive the respective K_a values for the various ANS–protein interactions.

For each given ANS–protein interaction a calibration factor was determined in order to relate the change in fluorescence to the amount of ANS bound to protein. For example, for PST, a plot of $1/\Delta F$ versus 1/[protein] was extrapolated to infinite protein concentration at 1/[protein] = 0 and $1/\Delta F = 1/\Delta F_{max.}$, as shown in Fig. 2. The ordinate intercept from Fig. 2 indicates a maximum fluorescence value of 155 arbitrary units/3.31 μM-

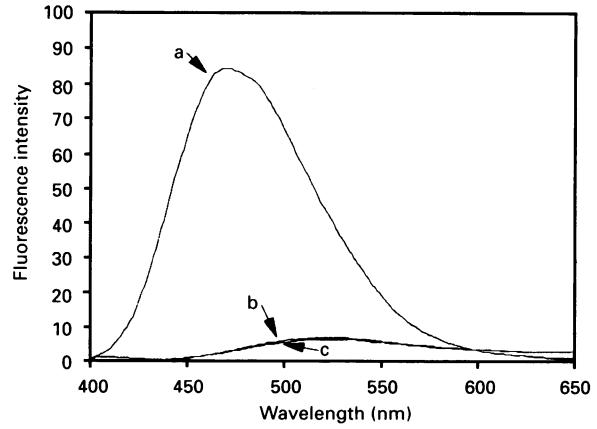

Fig. 1. Enhancement of ANS fluorescence

The fluorescence emission spectra of: (a) 50 μM-ANS added to PST (0.1 mg/ml); (b) 50 μM-ANS added to RNAase (0.1 mg/ml); (c) 50 μM-ANS in 0.1 M-Tris/HCl (pH 8.8)/0.1 M-NaCl. ANS in aqueous solution has a quantum yield of 0.004 and an emission maximum at 519 nm. After binding to PST there is an approx. 20-fold increase in quantum yield and a blue shift in maximum emission wavelength for ANS from 519 to 480 nm. By contrast, the change in the fluorescence of ANS after binding to ribonuclease is less pronounced. The excitation wavelength was 350 nm.

Table 1. Fluorescence characteristics of ANS binding to bacteriorhodopsin, BSA, lysozyme, ovalbumin, PST and RNAase

Protein	Emission maximum (nm)*	ANS quantum yield†
Bacteriorhodopsin	466	0.56
BSA	471	0.12
PST	480	0.09
Ovalbumin	485	0.008
Lysozyme	480	0.008
RNAase	519	0.005

* Emission wavelength of maximum fluorescence intensity and excitation wavelength of 350 nm.

† Quantum yield relative to β-carboline as standard [11].

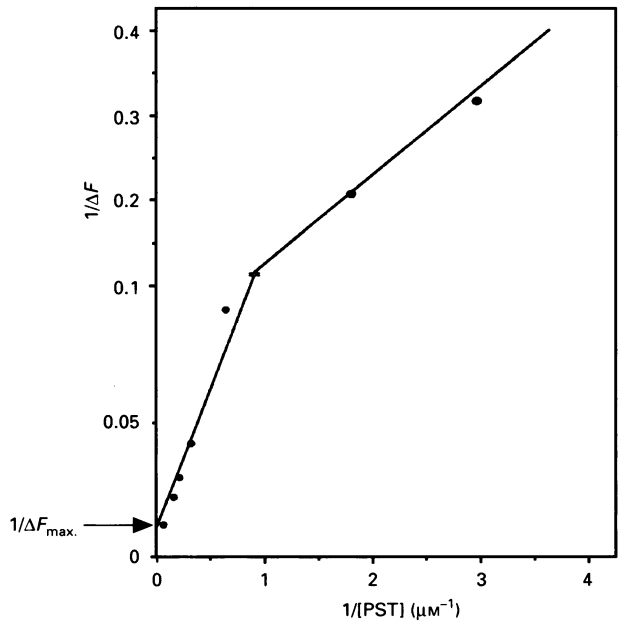

Fig. 2. Estimation of the fluorescence of ANS bound to PST

A double-reciprocal plot of 1/fluorescence intensity versus 1/protein concentration was used to determine the maximum increase in fluorescence per μM-ANS bound. For this, the ANS concentration was kept fixed (at approx. 5 μM) and the protein concentration varied between 0 and 140 μM. At the highest concentration of PST (140 μM) there was essentially no free ANS left in solution. The ordinate axis has been artificially expanded to show the ordinate intercept after extrapolation to infinite protein concentration. In this case, the maximum change in ANS fluorescence intensity per μM-ANS bound ($\Delta F_{max.}$) is 46.8 arbitrary units/μM.

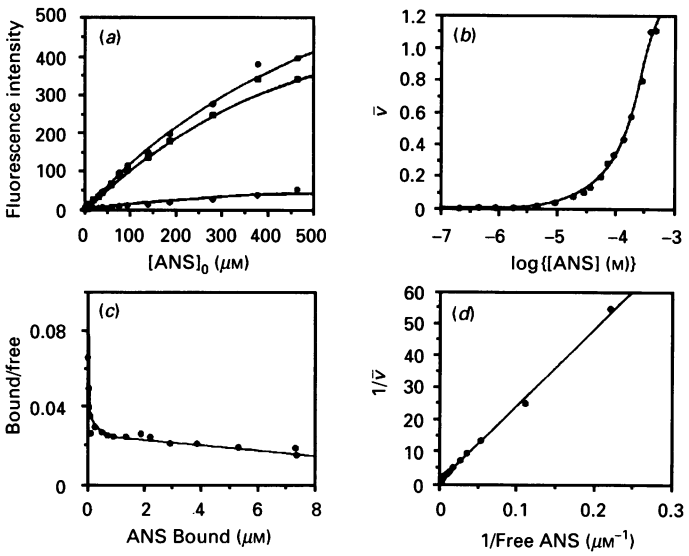

Fig. 3. Binding of ANS to PST

(*a*) Fluorescence titration of PST and ANS. The first curve measures the fluorescence of mixtures containing 0–500 μM-ANS and 6.63 μM-PST (●) and the second measures the fluorescence of solutions containing 0–500 μM-ANS alone (◆). The difference between these curves yields ΔF, the increase in fluorescence due to bound ANS (■). (*b*) Binding isotherm representing the number of mol of ANS bound per mol of protein ($\bar{v}$) versus the log of the concentration of ANS at equilibrium, that is, free [ANS]. The calibration factor of 46.8 arbitrary units per μM-ANS obtained from Fig. 2 allowed fluorescence changes shown in (*a*) to be translated into mol of bound ANS. (*c*) Scatchard treatment of the ANS binding data of (*a*). (*d*) Binding of ANS to PST was co-operative (non-linear Scatchard plot) and binding data was therefore obtained from a Klotz plot of the data. This gives an average equilibrium constant of 1.78×10^5 M^{-1} and a total of two ANS binding sites per PST molecule.

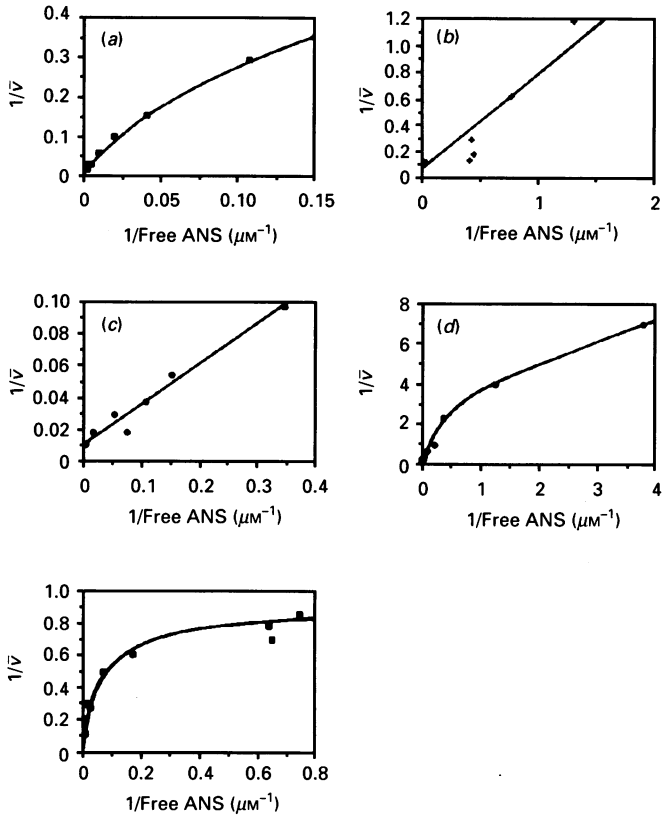

Fig. 4. Klotz-plot analyses of ANS binding to proteins

The Klotz plots of ANS binding to (*a*) bacteriorhodopsin, (*b*) BSA, (*c*) lysozyme, (*d*) ovalbumin and (*e*) RNAase. Least-squares regression was used to calculate the number of ANS binding sites and equilibrium binding constants.

Table 2. Comparison of the ANS equilibrium binding capacities and average hydrophobicities of bacteriorhodopsin, BSA, lysozyme, ovalbumin, PST and RNAase

Protein	*n**	K_a (M^{-1})†	Average hydrophobicity‡
Bacteriorhodopsin	54	9.21×10^5	1120
BSA	10	8.20×10^5	1000
PST	2	1.78×10^5	994
Ovalbumin	1	7.57×10^5	980
Lysozyme	3	7.69×10^4	890
RNAase	1	1.25×10^4	780

* Total number of ANS binding sites per protein molecule.
† Association constant derived from Klotz plots.
‡ Average hydrophobicities as defined by Bigelow & Channon [22].

ANS bound, i.e. 46.8 arbitrary units/μM-ANS bound. Subsequently PST was titrated with increasing amounts of ANS (Fig. 3*a*). The measured increase in fluorescence intensity was translated into mol of bound ANS using the calibration factor obtained from Fig. 2. Binding isotherms were subsequently derived in which mol of ANS bound were plotted against the concentration of free ANS (Fig. 3*b*). The data were then plotted according to Scatchard's equation [13], as shown in Fig. 3(*c*). The non-linear Scatchard plot suggested co-operative binding [14] of ANS to PST, and consequently a more accurate evaluation of the binding parameters using the graphical representation of Klotz & Hunston [1/$\bar{v}$ (mol of ANS bound/mol of protein) versus 1/[free ANS]] [15] was used to calculate equilibrium constants (Fig. 3*d*).

Equilibrium binding of ANS to bacteriorhodopsin, BSA, lysozyme, ovalbumin, PST and RNAase

The equilibrium binding constant at 25 °C (K_a) of the ANS–protein complexes were calculated for the various proteins using Klotz plots (Fig. 4). The Klotz graphs were necessary for the evaluation of binding constants, since non-linear Scatchard plots were obtained for all the proteins tested (results not shown). The average K_a constants and number of ANS-binding sites established from this study are summarized in Table 2. Bacteriorhodopsin and BSA appear to be the most 'hydrophobic' proteins, with 54 and 10 ANS-binding sites respectively, followed by PST and ovalbumin, which have a total of two and one ANS-binding sites respectively. Lysozyme and RNAase, the least 'hydrophobic' proteins, have lower binding affinities for ANS having respectively three and one sites for ANS.

Reversed-phase h.p.l.c. of Bacteriorhodopsin, BSA, lysozyme, ovalbumin, PST and RNAase

Reversed-phase high-performance chromatography is commonly used to determine the relative hydrophobicity of a mixture of proteins [3]. Two different column systems, namely a trimethylsilane C_1 reversed-phase column, and an octyl (C_8) column, were used in the present study. The results of h.p.l.c. analyses are shown in Fig. 5. There was inconsistency in the order of elution of the studied proteins between the C_1 and C_8 columns. In

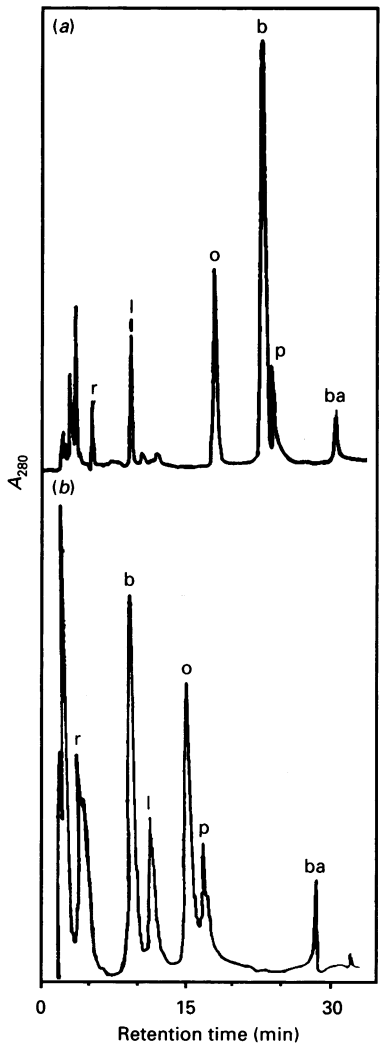

Fig. 5. Reversed-phase h.p.l.c. assessment of hydrophobicity

Reversed-phase h.p.l.c. separation of a mixture of bacteriorhodopsin (ba), BSA (b), lysozyme (l), ovalbumin (o), PST (p) and RNAase (r) proteins using a Beckman Ultrasphere Octyl (5 μm particle size; 4.6 mm × 250 mm) column (*a*) and a LKB Ultropac TSK TMS-250 (10 μm particle size; 4.6 × 75 mm) column (*b*). The column was maintained at ambient temperature. The mobile phase consisted of acetonitrile/trifluoroacetic acid (999:1, v/v). Note that the elution profile is dependent upon the type of column used.

particular, BSA eluted much earlier with the C_1 column compared with the octyl (C_8) column. The relative order of elution of RNAase, PST and bacteriorhodopsin was, however, identical on both column systems.

DISCUSSION

The use of ANS as a suitable fluorescent probe for non-polar sites on proteins has been previously established [4,16]. ANS is essentially non-fluorescent in aqueous solutions and becomes appreciably fluorescent in apolar environments. We observed a marked enhancement in ANS fluorescence after binding to bacteriorhodopsin and BSA; however, there was no significant increase in ANS fluorescence after interaction with lysozyme and RNAase, as suggested by the change in quantum yields and the respective K_a values. This difference in behaviour of ANS presumably reflects differences in surface hydrophobicity of these proteins, particularly the lack of suitable hydrophobic 'clefts' for ANS binding, as reflected by the lack of ANS-binding sites. The binding of ANS to the proteins studied generally followed a co-operative mode of interaction, as suggested by non-linear Scatchard-plot results. Consequently Klotz plots were used to derive equilibrium association constants (K_a) for various ANS–protein interactions.

By using the ANS-binding data as summarized in Tables 1 and 2, the proteins we studied could be arranged in order of increasing hydrophobicity according to quantum yield and, more precisely, according to the average association constant for the ANS–protein complex. Bacteriorhodopsin appeared to have the greatest degree of surface hydrophobicity and the largest number of ANS-binding sites. This would appear to be consistent with the location of this protein *in vivo*, that is, embedded in the non-polar environment of the purple membrane of *Halobacterium halobium* [9]. BSA also had an appreciable degree of surface hydrophobicity, which may reflect its *in vivo* function in fatty-acid transport [17]. RNAase, on the other hand, appeared to have a very hydrophilic surface, consistent with its function in interacting with highly negatively charged RNA molecules [18]. Similarly, the relative hydrophobic nature of the surface of PST may help explain why this protein exists as a non-covalent dimer under physiological conditions of ionic strength and pH [19].

There have been a number of attempts to establish a scale of hydrophobicity for proteins. Notably, Bigelow [20], Nozaki & Tanford [21] and Bigelow & Channon [22] compiled hydrophobicity indices for a large number of proteins on the basis of the sum of each amino acid residue's free energy of transfer from aqueous to organic solvent. In general we could correlate our experimentally determined binding constants of the protein–ANS complex, K_a, with the protein's average hydrophobic index as defined by Bigelow [20]. However, there were notable exceptions, as illustrated by our results with PST and ovalbumin, where the surface hydrophobicity based on ANS binding did not correlate with the average hydrophobicity defined by amino acid composition. Bigelow's [20] method would therefore appear to be most suitable for providing an overall average value for the hydrophobic/hydrophilic nature of a protein rather than specific information about the surface of a protein, such as the presence of localized non-polar regions on the surface. A further limitation of Bigelow's [20] approach is that the index is dependent upon the availability of at least the amino acid composition of the protein. The ANS-binding method does not have this constraint. It is therefore suggested that the usefulness of Bigelow's [20] reference tables may be significantly increased by incorporating ANS-based results, as the ANS-binding method provides a more appropriate means of assessing the surface hydrophobicity of proteins.

The separation of proteins by reversed-phase h.p.l.c. is thought to be based on the interaction between the hydrophobic groups of the protein and insoluble hydrophobic groups immobilized on the matrix [23,24]. Separation is commonly carried out under denaturing conditions using organic solvents, which may result, in an unpredictable fashion, in the exposure of the internal residues of the protein to the matrix. According to theoretical predictions, proteins will be eluted in order of hydrophobicity [23,25]. We observed differences in the order of elution of the same proteins depending upon the hydrophobicity of the reversed-phase column used, with the results suggesting that RNAase was hydrophilic and bacteriorhodopsin and PST relatively hydrophobic. There were also discrepancies between the ANS results and the results obtained using the C_1 and C_8 columns in assessing the hydrophobicity of BSA. The differences in behaviour of proteins depending on the hydrophobicity of the h.p.l.c. column used have been reported previously [3]. It is suggested that reversed-phase h.p.l.c. is not appropriate for

comparing the surface hydrophobicity of proteins in their physiological state.

In summary, the results presented here demonstrate that ANS binding and the resultant K_a constants and, to a lesser extent, ANS quantum yield, may be used as a measure of the relative surface hydrophobicity of proteins. The ANS results differ from those based on reversed-phase h.p.l.c. or using the method of Bigelow [20], where no allowances are made for the tertiary structure of a given protein. Although there is general agreement between our results and the average hydrophobicity indices for proteins quoted by Bigelow [20], unlike the limitations with Bigelow's [20] approach, the ANS method may be applied to proteins of unknown primary structure. Furthermore, the ANS-binding method is rapid, simple, and uses microgram quantities of purified protein for analysis.

We thank Dr. J. Bertolini, Dr. M. R. Brandon and Dr. K. Ashman for their critical comments. M.C. is the recipient of an Australian Postgraduate Industry Research award (P.I.R.A.).

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Received 15 April 1991/24 July 1991; accepted 31 July 1991