Quantitative Estimation of Protein Binding Site Polarity. Fluorescence of *N*-Arylaminonaphthalenesulfonates*

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ABSTRACT: The fluorescence characteristics of *N*-arylaminonaphthalenesulfonates make these dyes useful probes of the polarity at protein binding sites. The fluorescence yield, emission maximum, and band width of 1-anilinonaphthalene-7-sulfonate in a variety of solvents vary as a function of *Z*, an empirical solvent polarity scale suggested by Kosower (Kosower, E. M. (1958), *J. Am. Chem. Soc. 80*, 3253). The effect of other environmental factors such as pH, viscosity, and solvent deuteration have been examined and a plausible mechanism for the general polarity effect is suggested. It is concluded that the emission maximum of adsorbed probes gives the most reliable estimate of binding site polarity.

1-Anilinonaphthalene-7-sulfonate and two other compounds, for which emission maximum is a function of *Z*, were adsorbed to 20 proteins. Estimated *Z* values of the protein sites ranged from 80 to 88. Enzymes of the same functional class appeared to have sites of similar polarity.

Spectroscopic changes observed when dyes are adsorbed or covalently bound to proteins have been used to evaluate the chemical and physical properties of binding sites. Studies of this type have been reviewed by Horton and Koshland (1967) and by Edelman and McClure (1968). Of special interest as protein probes have been those molecules which fluoresce intensely when bound to proteins or when dissolved in nonpolar solvents, but which have extremely low fluorescence in aqueous solution. Studies of 1,8-ANS[1] (Stryer, 1965) and 2,6-TNS (McClure and Edelman, 1966) in a variety of solvents have supported the concept that observed changes in the fluorescence quantum yield, emission maximum, and emission band width are related to the polarity of the environment around the fluorescent species and that these molecules can serve as probes for hydrophobic sites on proteins. However, it has also been reported that viscosity, hydrogen bonding, and other specific chemical interactions can affect the fluorescence properties of 2,6-TNS (McClure and Edelman, 1966).

Our purpose has been to study the fluorescence of a dye of the *N*-arylaminonaphthalenesulfonate class, 1,7-ANS, in a variety of solvents in order to quantitate the relationship between polarity and fluorescence properties and to estimate the effects of viscosity and of specific interactions. In the past, changes in the fluorescence properties of protein probes have been correlated with such physical characteristics of the solvent as dielectric constant and refractive index. We have found that the effect of solvent on fluorescence parameters of 1,7-ANS cannot be described by any such macroscopic measure of solvent polarity. However we have been able to show close correlation of our data with the empirical *Z* solvent polarity scale of Kosower (1958), so that it is possible to estimate the polarity of the environment around 1,7-ANS without knowledge of the chemical composition of that environment.

Similar dependence of fluorescence properties upon the *Z* polarity scale was also established for 1,8-ANS and 1,5-ANS. The three isomeric ANS dyes were then used to probe the binding sites of twenty proteins.

Materials and Methods

1,5-AmNS and 1,7-AmNS were obtained from Matheson Coleman and Bell (East Rutherford, N. J.); 1,4-AmNS, 1,6-AmNS, and 2,6-AmNS were obtained from Aldrich Chemical Co. (Milwaukee, Wis.); and 1,8-AmNS was obtained from Distillation Products Industries (Rochester, N. Y.). All these compounds were recrystallized as the sodium salts from Norit-treated aqueous solutions of sodium bisulfite (1.4 M) and sodium sulfite (0.08 M). Samples of each were tested for homogeneity on Eastman silica gel, alumina, and cellulose thin-layer sheets (Distillation Products Industries, Rochester, N. Y.), using a solvent system of 1-butanol saturated with 20% aqueous acetic acid.

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: 1,8-ANS, 1-anilinonaphthalene-8-sulfonate, and similarly, 1,7-ANS, 1,5-ANS, 2,6-ANS; 2,6-TNS, 2-toluidinylnaphthalene-6-sulfonate; 2,6-MANS, 2-N-methyl-2-anilinonaphthalene-6-sulfonate; 1,5-DNS, 1-dimethylaminonaphthalene-5-sulfonate; 1,7-AmNS, 1-aminonaphthalene-7-sulfonate, and similarly, 1,8-AmNS, 1,6-AmNS, 1,5-AmNS, 1,4-AmNS, 2,6-AmNS; 1,5-DNS-glycine, 1-dimethylaminonaphthalene-5-sulfonylglycine.

Except for 1,8-AmNS, all AmNS isomers ran as one major fluorescent spot, with only traces of fluorescent impurities, if any.

1,5-DNS was obtained from J. Feitelson, Jerusalem, Israel. It ran as a single spot on silica gel and cellulose chromatogram sheets with acidified butanol as the solvent, but showed several small contaminating fluorescent spots on alumina in the same solvent. 1,5-DNS-glycine (95.7%) was obtained from Mann Research Laboratories (New York, N. Y.).

1,8-ANS (sodium salt), obtained from Distillation Products Industries, was converted into the magnesium salt and then recrystallized four times from hot water with Norit treatment. The resulting yellow crystals were protected from light, because they became green with prolonged exposure. 1,7-ANS was prepared as follows. Recrystallized 1,7-AmNS (1 mole) was refluxed with 1 mole of aniline hydrochloride and 4 moles of redistilled aniline for 16 hr. The product crystallized from the reaction mixture as the aniline salt. Heating in 1 N HCl converted this into the free acid, which crystallized upon cooling. After decolorization with Norit, the acid was twice recrystallized from dilute HCl and dried. The following analysis was obtained for this material from Galbraith Chemical Laboratories, Knoxville, Tenn. *Anal.* Calcd for $C_{16}H_{13}NO_3S$: C, 64.2; H, 4.35; N, 4.68; S, 10.7. Found: C, 63.90; H, 4.50; N, 4.67; S, 10.48. The sodium salt of 1,7-ANS was prepared by recrystallization from the bisulfite–sulfite solution followed by two recrystallizations from water. The molar extinction coefficient at 350 mμ for 1,7-ANS in water was found to be 6.0×10^3 cm^2/mmole. 1,7-ANS showed no deviation from the Beer–Lambert relationship in water, ethanol, dioxane, or methanol at concentrations below 1.2×10^{-4} M (A_{350} less than 0.7). 1,5-ANS was prepared in the same way as 1,7-ANS, using recrystallized 1,5-AmNS as starting material. All three ANS isomers were tested for purity on the three kinds of thin-layer sheets in the acidified butanol solvent system. These and other tests on silica gel plates in five other solvents revealed no fluorescent impurities in any of these ANS dyes.

2,6-ANS, 2,6-TNS, and 2,6-MANS were prepared according to Cory *et al.* (1968),[2] tested for homogeneity on silica gel, alumina, and cellulose thin-layer sheets in acidified butanol, and were found to be free of significant fluorescent contamination.

The following Matheson Coleman and Bell (East Rutherford, N. J.) spectroquality solvents were used without further purification: acetone, acetonitrile, *p*-dioxane, dimethylformamide, dimethyl sulfoxide, 2-propanol, methanol, and glycerol. The ethylene glycol was chromatoquality grade from Matheson Coleman and Bell. Pharmco ethanol (Publicker Industries, Philadelphia, Pa.) was used without further purification. The following reagent grade solvents were redistilled before use: 1-propanol, acetic acid, 1-butanol, and pyridine. Deuterium oxide (*ca.* 99.7 atom %) was obtained from Volk (Burbank, Calif.). Solvent mixtures were made up in per cent by volume. Absorbance measurements were made with a Cary Model 14 spectrophotometer (Applied Physics Corp., Monrovia, Calif.) or with a Beckman DU spectrophotometer (Beckman Instruments Inc., Fullerton, Calif.). For the studies with 1,7-ANS, 1,8-ANS, and 1,5-ANS, the absorption spectra were measured on the Cary instrument using the 0–2.0 slide wire and the samples were then diluted exactly 1:10 for fluorescence measurements.

The samples used for fluorescence studies had absorbances <0.1 at the exciting wavelength, and all solutions containing less than 50% H_2O were outgassed with dry nitrogen immediately before each fluorescence measurement.

Continuous corrected emission spectra were recorded with a spectrophotofluorometer described elsewhere (Witholt and Brand, 1968), using a Data-Trak curve follower (Research Incorporated, Minneapolis, Minn.). The correction curve was obtained by comparing the observed emission of a standard tungsten lamp (National Bureau of Standards, No. 8063) with that calculated from Wien's law. A corrected spectrum of quinine sulfate in 1 N H_2SO_4 obtained in this manner agreed with published spectra for that compound (Argauer and White, 1964). Fluorescence was measured at room temperature in quartz cuvets at right angles to the excitation beam. We estimate that the emission wavelength can be determined to ± 1 mμ. The areas of the corrected spectra were obtained by planimetry, by weighing the recorder paper under the curve, or by using the digital integration capability of the spectrophotofluorometer (Witholt and Brand, 1968). All three methods gave identical results.

Absolute quantum yields, ϕ, were calculated by the method of Parker and Rees (1960), with a correction for the refractive index of the solvent (Hermans and Levinson, 1951)

$$\phi_S = \phi_R \frac{(1 - T)_R}{(\text{area})_R} \frac{(\text{area})_S}{(1 - T)_S} \frac{n_R^2}{n_S^2}$$

where S and R refer to sample and reference, respectively, and where n is the refractive index (Handbook of Chemistry and Physics, 1961). Quinine sulfate in 1 N H_2SO_4 was the reference substance; ϕ_R was taken to be 0.55 (Melhuish, 1961). The quantum yields for several standard substances were determined and compared with literature values. For 1,5-DNS in water a ϕ of 0.39 was found (compared with 0.37 found previously by Chen, 1966), for 1,8-ANS in ethanol the ϕ was 0.40 (0.37; Stryer, 1965), and for 2,6-AmNS in water and ethanol the ϕ values were 0.47 and 0.42 (compared with 0.63 and 0.45, respectively, reported by McClure and Edelman, 1966).

As a measure of the emission transition energy, we have used the reciprocal of the wavelength of maximum fluorescence, which we call $\bar{\nu}_F$. This value differs very slightly from the maximum on a plot of fluorescence *vs.* wave number (Parker and Rees, 1960), and, over the range of wavelengths used, this difference is not greatly

[2] We thank Dr. Isenberg for making the manuscript available to us prior to publication.

affected by changes in wavelength. The effective half-band width of emission is calculated as

$$\Delta\bar{\nu}_F\ (\text{cm}^{-1}) = \frac{\text{area (cm}^2)}{\text{peak height (cm)}} \frac{K\ (\text{m}\mu/\text{cm})}{\lambda_F^2\ (\text{m}\mu)^2}\ 10^7\ \frac{\text{m}\mu}{\text{cm}}$$

where K is the factor relating millimicrons to centimeters on the wavelength axis of the recorder and where λ_F is the wavelength of maximal emission.

Fluorescence polarization spectra were automatically recorded on the instrument described by Witholt and Brand (1968). The samples were maintained at 3°.

The proteins were obtained from the following sources: from Boehringer Mannheim Corp., New York, N. Y.: α-chymotrypsin, crystallized, lot 647112; horse liver alcohol dehydrogenase, lot 6427321; rabbit muscle lactate dehydrogenase, lot 6017320; beef liver glutamate dehydrogenase, lot 06496449; rabbit muscle phosphofructokinase, lot 6087409; rabbit muscle myokinase, lot 6446412; rabbit muscle aldolase, lot 6286107; rabbit muscle enolase, lot 6486206; calf intestine adenosine deaminase, lot 06516406; calf intestine alkaline phosphatase, lot 606110; from Worthington Biochemical Corp., Freehold, N. J.: beef pancreas deoxyribonuclease, once crystallized, lot D-6HB; beef pancreas ribonuclease, lot R663; egg-white lysozyme, twice crystallized; bovine trypsin, twice crystallized; from the Sigma Chemical Co., St. Louis, Mo.: yeast alcohol dehydrogenase, lot 44B-1583; from P-L Biochemicals Inc., Milwaukee, Wis.: yeast hexokinase, lot X-1598-B; from Armour Pharmaceutical Co., Kankakee, Ill.: bovine plasma albumin, crystalline, lot D71209; from California Biochemical Corp., Los Angeles, Calif.: yeast phosphoglucose isomerase, lot 71412; from Mann Research Laboratories, New York, N. Y.: chymotrypsinogen, six-times crystallized, lot P1895; and a sample of firefly luciferase was kindly provided by Dr. Marlene DeLuca. Solutions of the crystalline proteins were made up by weight in 0.1 M sodium phosphate buffer (pH 7.4) or, for chymotrypsin, trypsin, and chymotrypsinogen, in 10^{-3} M HCl. Aliquots of these solutions, and of commercial protein solutions and suspensions, were added to solutions of 1,8-ANS, 1,7-ANS, 1,5-ANS, and 2,6-TNS in 0.1 M sodium phosphate buffer (pH 7.4). Emission spectra were recorded using an Aminco spectrophotofluorometer (American Instrument Co., Silver Spring, Md.) and corrected for nonlinear response of the detector with wavelength. The wavelength of fluorescence can be estimated to ±2 mμ. Absorbances at the excitation wavelength, 350 mμ, were kept below 0.06.

Results

Solvent Effects on the Absorption and Fluorescence of 1,7-ANS. The 1,7-ANS isomer was chosen for detailed study in order to avoid the intramolecular interactions possible in 1,8-ANS (Balasubramaniyan, 1966). The absorption spectrum of 1,7-ANS is shown in Figure 1. There are three strong absorption bands: one, with a shoulder, near 350 mμ and the others near 260 and 220 mμ. The difference in the degree of fluorescence polarization on the two sides of the lowest energy band suggests that this band is made up of two electronic transitions (Stryer, 1965). It would be of great interest to understand the nature of the excited state which results from the lowest energy transition since this is the one which fluoresces and is subject to quenching processes. Because the two transitions lie close together and because both are susceptible to solvent-induced changes in extinction coefficient and absorption maximum, the usual type of analysis based on the direction of wavelength shift in polar media cannot be readily undertaken. The transition of lower energy appears to be relatively more intense in nonpolar solvents than in water, accounting for most of the change in spectral shape seen in Figure 1. As the percentage of nonpolar solvent in water is increased, the net absorption maximum is first red shifted, then blue shifted, while the net extinction continues to increase (Table I). The lowest energy absorption transition, as for other aromatic amines, probably leads to an excited state with a large percentage of charge-transfer character (Kasha, 1967).

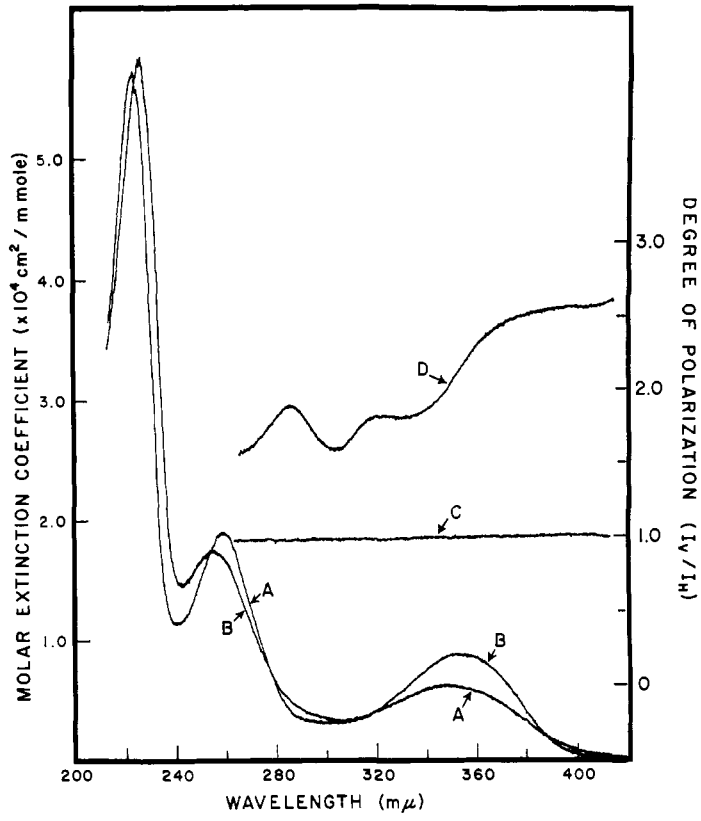

FIGURE 1: Absorption spectra of 1,7-ANS in water (A) and ethanol (B). Curve D is the principal polarization spectrum of 1,7-ANS in glycerol with an emission wavelength of 460 mμ. Curve C is the polarization base line.

Our main aim has been to measure the effects of the changes in the environment around the ANS molecule on its fluorescence properties, and in particular, to measure the effect of changes in solvent polarity. The fluorescence yield, wave number of maximum emission, and emission band width for 1,7-ANS in a number of solvents and solvent mixtures are listed in Table I. Many different scales of solvent polarity, both theoreti-

TABLE I: Absorption and Fluorescence Measurements of 1,7-ANS in Various Solvents and the Corresponding Values for Four Solvent Polarity Scales.

Solvent (%)	Absorption Max λ_A (mμ)	ϵ ($\times 10^3$ cm^2/mmole)	Absolute Quantum Yield (ϕ)	Emission Max $\bar{\nu}_F$ ($\times 10^4$ cm^{-1})	Emission Band Width $\Delta\nu_F$ ($\times 10^2$ cm^{-1})	Dielectric[a] Constant, D	$\frac{D-1}{2D+1}$[b]	Y[c]	Z[d]
H_2O	347	6.0	0.0091	1.940	5.57	80	0.491	3.493	94.6
Ethanol (10)	348	6.1	0.017	1.988	5.21	74	0.490	3.312	93.6
Ethanol (20)	350	6.1	0.027	2.016	5.14	69	0.489	3.051	92.6
Ethanol (30)	351	6.9	0.064	2.066	5.25	64	0.488	2.721	91.6
Ethanol (40)	352	7.2	0.11	2.096	4.87	58	0.487	2.196	90.5
Ethanol (50)	353	7.9	0.18	2.132	4.84	52	0.486	1.655	89.2
Ethanol (60)	354	8.2	0.26	2.150	4.70	47	0.485	1.124	87.9
Ethanol (70)	353	8.3	0.33	2.164	4.62	40	0.482	0.595	86.4
Ethanol (80)	353	8.3	0.43	2.179	4.17	35	0.479	0.000	84.8
Ethanol (90)	352	8.4	0.52	2.198	4.15	29	0.475	−0.747	82.5
Ethanol (100)	352	8.6	0.56	2.257	4.01	24	0.471	−2.033	79.6
Dioxane (10)	351	6.6	0.030	2.036	5.38	76	0.490	3.217	93.0
Dioxane (20)	352	7.1	0.062	2.079	5.27	64	0.488	2.877	91.4
Dioxane (30)	353	7.6	0.12	2.137	5.11	50	0.485	2.455	89.9
Dioxane (40)	354	7.9	0.21	2.155	4.95	42	0.482	1.945	88.4
Dioxane (50)	354	8.9	0.30	2.169	4.75	32	0.477	1.361	86.7
Dioxane (60)	353	9.0	0.42	2.188	4.67	25	0.471	0.715	85.0
Dioxane (70)	352	9.2	0.50	2.208	4.53	16	0.465	0.013	82.8
Dioxane (80)	352	9.2	0.57	2.222	4.38	10	0.428	−0.833	80.2
Dioxane (90)	353		0.68	2.227	4.33	5	0.364	−2.030	76.7
Dioxane (100)	352		0.68	2.315	4.33	2	0.200		
Methanol (50)	350	6.9	0.099	2.100	5.17	58	0.487	1.972	90.9
Methanol (70)	351	7.3	0.21	2.145	4.68	47	0.484	0.961	88.5
Methanol (80)	352	7.4	0.28	2.155	4.59	42	0.482	0.381	87.3
Methanol (90)	352	7.9	0.35	2.169	4.40	36	0.479	−0.301	85.2
Methanol (100)	352	8.0	0.39	2.198	4.40	33	0.477	−1.090	83.6
1-Propanol	353		0.70	2.278	4.02	20	0.463		78.3
2-Propanol	351		0.65	2.304	4.1	18	0.459	−2.73	76.3
Ethylene glycol	356		0.36	2.150	4.5	38	0.481		85.1
Glycerol	359		0.40	2.145	4.5				
Dimethyl sulfoxide	351		0.46	2.237	4.43	49	0.485		71.1
Dimethylformamide	348		0.67	2.294	4.24	37	0.480		68.5
Pyridine	350		0.0046	2.331	3.7	12	0.440		64.0
Acetic acid	358		0.025	2.203	4.3	6	0.385	−1.639	79.2
Acetonitrile	343		0.43	2.320	4.2	38	0.481		71.3
Acetone			0.30	2.410	3.8	21	0.465		65.7

[a] Landolt-Börnstein (1959). [b] Kirkwood (1934). [c] Grunwald and Winstein (1948). [d] Kosower (1958).

cal and empirical, have been used to correlate solvent effects on kinetics, equilibria, and spectra (Reichardt, 1966; Leffler and Grunwald, 1963).

We have examined the correlation of our data with the following solvent scales: dielectric constant, D; $(D - 1)/(2D + 1)$ (Kirkwood, 1934); Y (Grunwald and Winstein, 1948); Z (Kosower, 1958); E_T (Dimroth *et al.*, 1963); S (Brownstein, 1960); and χ_B and χ_R (Brooker *et al.*, 1965). Included in Table I are the solvent polarities in terms of four commonly used scales. The best correlation of our data was obtained with the Z scale suggested by Kosower (1958). Figure 2A shows that in three organic solvent–water binary mixtures, the quantum yield values have the same dependence upon Z. In contrast, when quantum yield is plotted against the other scales, a smooth curve results for any one solvent system, but the curves for different solvent systems are not superimposed. Certain solvents such as acetonitrile, pyridine, acetic acid, and dimethyl sulfoxide deviate markedly from the relationship established for the aqueous binary mixtures. Figure 2B shows the emission maximum, $\bar{\nu}_F$, plotted as a function of solvent Z value. Deviations of nonaqueous solvents from the relationship established with the binary solvent mixtures are far less severe than those observed with the quantum yield measurements. Figure 2C shows the half-band width as a function of Z. Although a larger scatter of the data is evident, deviations in the unusual solvents are again less severe than for the quantum yield data shown in Figure 2A.

It is of interest that the value for ethylene glycol (viscosity = 21 cP at 20°) shown in Figure 2A fits into the general quantum yield *vs.* Z relationship. This indicates that unlike TNS (McClure and Edelman, 1966), 1,7-ANS shows no viscosity effect on fluorescence yield. The emission maximum of 1,7-ANS is also in the region predicted by the Z value for this solvent. Studies of the fluorescence of 1,7-ANS bound to proteins (where the effective viscosity around the dye might be quite high) are thus not complicated by effects of viscosity in addition to those of polarity.

Solvent Effects on the Fluorescence of 1,5-ANS and 1,8-ANS. The emission transition energies, the quantum yields, and the emission band widths for these two dyes in a more restricted range of solvents are given in Table II. The variations observed in the absorption spectra in different solvents are similar to those found with 1,7-ANS. Here again, the three fluorescence parameters correlate best with the Z polarity scale. The plots of $\bar{\nu}_F$ *vs.* Z are shown in Figure 3A,B for 1,5-ANS and 1,8-ANS, respectively.

Use of the Dyes in Probing Protein Binding Sites. The emission transition energies of the three ANS isomers and of 2,6-TNS bound to twenty proteins are given in Table III. With the aid of Figures 2B and 3A,B and of a similar plot of the data of McClure and Edelman (1966) for 2,6-TNS. the measured values of $\bar{\nu}_F$ can be used to estimate the Z value of the environment around the bound dye. These estimates are included in Table III. Several conclusions can be drawn from the results: (1) the Z values estimated from measurements of 1,8-ANS fall consistently below the

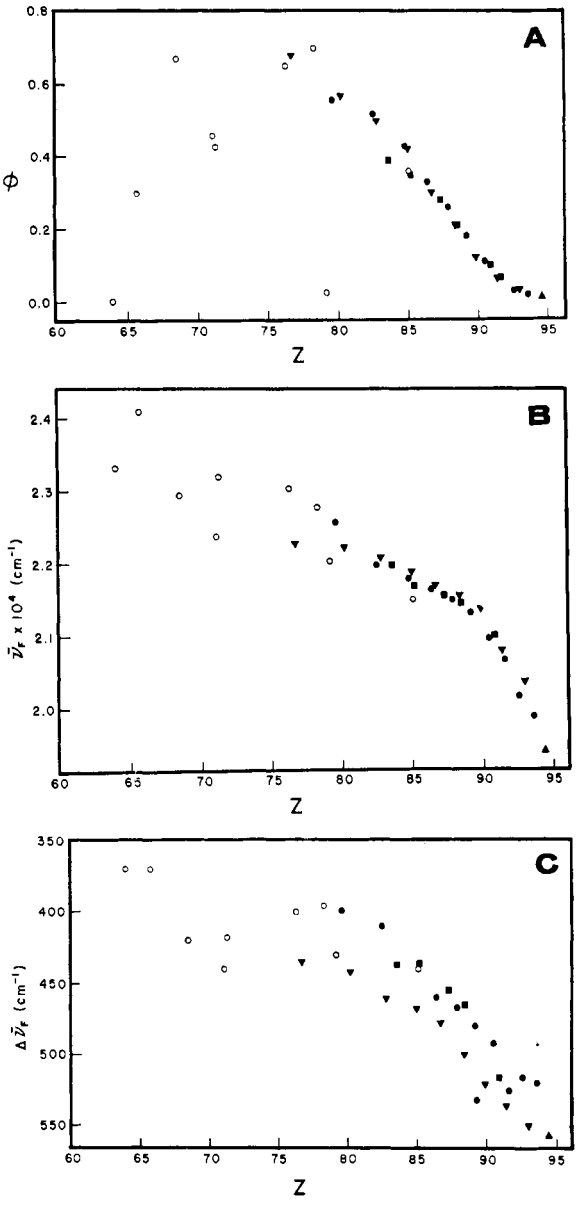

FIGURE 2: Plots of quantum yield, transition energy, and bandwidth of fluorescence *vs.* the empirical solvent polarity scale, Z (Kosower, 1958). The solid points designate organic solvent–water mixtures, as follows: ethanol (●), dioxane (▼), and methanol (■). Water is indicated by ▲. Other solvents are denoted by O. The data are taken from Table I.

values for the other three probes; (2) the average Z values estimated from the three remaining dyes lie between 81 and 87 for almost all proteins studied; (3) there is reasonably good agreement among the values for 1,5-ANS, 1,7-ANS, and 2,6-TNS for any one protein, so that it can be said, for example, that the polarity of the chymotrypsinogen site(s) exceeds that of the chymotrypsin site(s), and that all dehydrogenases studied have binding sites of similar polarity ($Z \simeq 85$). It should be noted that no large changes in emission wavelengths of bound dyes were observed as increasing aliquots of protein were added.

Studies on the Mechanism of the Polarity Effect. Stryer (1966) suggested that excited-state proton trans-

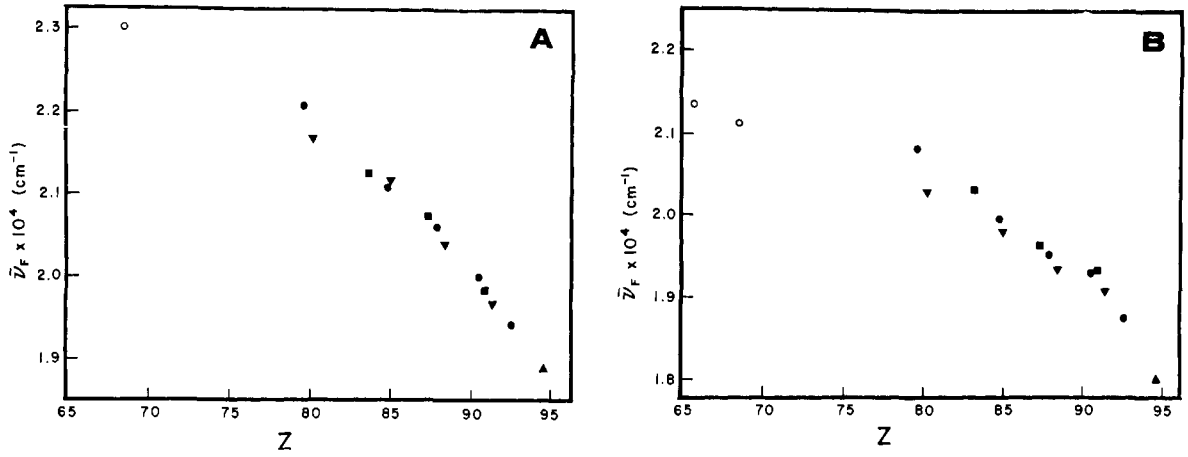

FIGURE 3: Plots of the transition energy of fluorescence as a function of the empirical solvent polarity scale, Z (Kosower, 1958) for 1,5-ANS (A) and for 1,8-ANS (B). The data are taken from Table II, and the solvents are denoted as in Figure 2.

TABLE II: Fluorescence of 1,5-ANS and 1,8-ANS in Various Solvents.

	1,5-ANS			1,8-ANS		
Solvent (%)	Quantum Yield (ϕ)	$\bar{\nu}_F$ ($\times 10^4$ cm^{-1})	$\Delta\bar{\nu}_F$ ($\times 10^2$ cm^{-1})	Quantum Yield (ϕ)	$\bar{\nu}_F$ ($\times 10^4$ cm^{-1})	$\Delta\bar{\nu}_F$ ($\times 10^2$ cm^{-1})
Water	0.0099	1.889	5.48	0.0032	1.802	5.2
Ethanol (20)	0.027	1.942	5.06	0.0072	1.876	5.3
Ethanol (40)	0.083	2.000	5.20	0.020	1.931	4.5
Ethanol (60)	0.20	2.061	5.11	0.050	1.953	4.6
Ethanol (80)	0.38	2.109	4.62	0.11	1.996	4.98
Ethanol	0.69	2.209	4.41	0.40	2.083	4.74
Dioxane (20)	0.050	1.968	5.08	0.012	1.908	4.7
Dioxane (40)	0.15	2.040	5.04	0.038	1.934	4.9
Dioxane (60)	0.33	2.118	4.98	0.099	1.980	4.7
Dioxane (80)	0.54	2.169	4.65	0.23	2.028	4.9
Dioxane	0.74	2.292	4.22	0.57	2.118	4.48
Methanol (50)	0.084	1.984	5.08	0.029	1.934	4.9
Methanol (80)	0.27	2.074	4.91	0.077	1.964	5.10
Methanol	0.63	2.127	4.62	0.17	2.032	4.96
Acetone				0.39	2.137	4.57
Dimethylformamide	0.70	2.302	4.28	0.39	2.114	4.64
Ethylene glycol	0.38	2.066	4.70	0.12	1.968	4.88

fer (Weller, 1961) might be the mechanism responsible for quenching of ANS fluorescence in polar solvents. In order to obtain further evidence in regard to the mechanism of quenching, we have measured the fluorescence yield, emission maximum, and band width of 1,7-ANS as a function of pH. They were found to be invariant over the pH range 1.5–12.0. Below pH 1.5 the fluorescence decreases as the 350-mμ absorbance decreases, reflecting ground-state protonation. Above pH 12 the quantum yield first decreases without changes in band width or emission maximum and in 10 N NaOH a new highly fluorescent species with an emission maximum at 463 mμ appears. These findings indicate that the naphthylanilide ion forms only at very high pH, if at all, and is of little importance in the general solvent effect.

Fluorescence studies of 1,7-ANS and several related dyes in H_2O and in D_2O are shown in Table IV. All the ANS dyes studied show a higher yield in D_2O than in H_2O. However, 2,6-MANS which has no ionizable proton shows almost the same enhancement as 2,6-ANS which does have an ionizable proton. 1,5-DNS and 1,5-DNS-glycine, a compound with fluorescence properties similar to the *N*-arylaminonaphthalenesulfonates (Chen, 1967), also show enhanced fluorescence. The enhancement with 1,5-DNS has also been observed by Förster and Rokos (1967). These findings support

TABLE III: Estimation of the Polarity of Protein Binding Sites from the Emission Maxima of Bound *N*-Arylaminonaphthalenesulfonates.

Protein	Protein Concn (mg/ml)	1,8-ANS		1,7-ANS		1,5-ANS		2,6-TNS	
		$\bar{\nu}_F$ ($\times 10^4$ cm^{-1})	Z_{est}	$\bar{\nu}_F$ ($\times 10^4$ cm^{-1})	Z_{est}	ν_F ($\times 10^4$ cm^{-1})	Z_{est}	$\bar{\nu}_F$ ($\times 10^4$ cm^{-1})	Z_{est}
Alcohol dehydrogenase (horse liver)	2.0	2.036	80–83	2.145	89	2.123	84.5	2.198	85
Alcohol dehydrogenase (yeast)	0.8	2.070	76–80.5	2.169	86.5	2.114	85	2.273	82.5
Glutamate dehydrogenase	1.20	2.044	79–82	2.193	84	2.118	84.5	2.232	84
Lactate dehydrogenase	1.5	2.074	76–80.5	2.179	85.5	2.105	86	2.208	85
Chymotrypsinogen	0.9	2.087	74–79	2.198	84	2.159	82	2.174	86
Chymotrypsin	1.1	2.040	79–82.5	2.100	91	2.066	87.5	2.155	86.5
Trypsin	5	2.053	77–82	2.193	84	2.092	86.5	2.222	84
Phosphofructokinase	1.5	2.053	77–82	2.193	84	2.114	85	2.257	83
Hexokinase	0.19			2.193	84			2.262	83
Luciferase	0.1	2.070	76–80.5	2.242	74–81			2.370	64–77
Myokinase	0.5	2.087	74–79	2.227	77–81.5	2.150	82.5	2.288	81.5
Ribonuclease	2.2	2.105	70–77	2.252	73–80			2.247	83.5
Deoxyribonuclease	0.76	2.061	76–81	2.193	84	2.137	83.5	2.273	82.5
Adenosine deaminase	0.2	2.083	75–79.5	2.227	81			2.252	84
Enolase	0.75	2.070	76–80.5	2.212	82.5	2.174	79–81.5	2.268	83
Adolase	2.00	2.061	76–81	2.212	82.5	2.123	84.5	2.268	83
Phosphoglucose isomerase	0.40			2.208	82.5			2.242	84
Lysozyme	1.00			2.155	88			2.217	84.5
Plasma albumin (bovine)	3.00	2.074	76–80.5	2.208	82.5	2.118	84.5	2.273	82.5
Alkaline phosphatase	0.1	2.087	74–79					2.283	82.5

TABLE IV: Ratio of Quantum Yield in D_2O to That in H_2O for N-Substituted Aminonaphthalenesulfonates.

Compound	$\phi D_2O/\phi H_2O$[a]
1,7-ANS	2.8
1,5-ANS	3.6
1,8-ANS	2.4
2,6-ANS	2.4
1,5-DNS-glycine	2.4
1,5-DNS	1.6
2,6-MANS	2.6

[a] Since in all cases there was no difference in the shape of the emission bands in the two solvents, this ratio was calculated by comparing peak heights, not areas, of the fluorescence spectra.

the idea that excited-state proton transfer is not involved in quenching of ANS fluorescence in polar solvents.

Fluorescence of Related Compounds. A comparison of solvent effects on quantum yields and emission transition energies for several dyes related to the three ANS isomers is indicated in Table V. Besides the ANS isomers (Tables I and II), only 2,6-ANS, 2,6-TNS, 2,6-MANS, and 1,8-AmNS show the phenomenon of very low yield in water and very high fluorescence in ethanol. The other compounds have high yield in water and in several cases actually have a higher yield in 60–80% ethanol than in pure ethanol. The strong polarity dependence of the 2,6-MANS quantum yield underscores again that loss of an amine proton during the excited state cannot explain the polarity effects on ANS fluorescence. The results for 1,8-AmNS may reflect interaction between the amino and sulfonate groups. Certain regularities are apparent in the values for the transition energies: (1) for all four ANS isomers the difference in transition energy between ethanol and water ($\bar{\nu}_F$(ethanol) − $\bar{\nu}_F$(water)) exceeds that for the corresponding aminonaphthalenesulfonates; (2) that most of this difference is attributable to a greater red shift of the ANS spectrum; and (3) that derivatives of 1- and 2-naphthylamine have similar properties.

Discussion

The polarity at the active sites of enzymes must be important both in binding of substrates and cofactors and in the actual catalytic action. While many reagents are available to evaluate the importance of specific chemical groups at the active site region, the development of probes for polarity has so far received little attention. One useful approach has been to attach a dye covalently to a functionally important site on a protein. The spectroscopic properties of the dye then "report" information about polarity (Horton and Koshland, 1967). Since the fluorescence of *N*-arylaminonaphthalenesulfonate dyes is very sensitive to polarity and since these dyes also adsorb specifically to active sites of some proteins, it was of interest to examine their fluorescence in more detail. The initial work of Stryer (1965) and McClure and Edelman (1966) has been extended to enable us to estimate the polarity of binding sites more closely and to compare results obtained with four different probes.

If spectroscopic characteristics of a bound dye are to be quantitatively related to polarity they must first be shown to approximate a reasonable function of some polarity scale. We understand polarity to encompass all the general molecular interactions which the solvent and solute can undergo, including dispersion forces, dipole–dipole interactions, and dipole–induced dipole interactions. Ionization and complex formation which lead to a change in the nature of the solute are excluded (Reichardt, 1965). This definition has the strength that it includes all the general molecular interactions even those which are usually not reflected by macroscopic scales of solvent polarity such as dielectric constant or refractive index. The value of establishing a quantitative relationship between the fluorescence of ANS dyes and Kosower's *Z* scale is that it permits an unambiguous estimate of the polarity of an unknown environment. To illustrate this, if we measure the emission transition energy of 1,7-ANS bound to a protein to be 2.17×10^4 cm^{-1}, we can estimate from Figure 2B the *Z* value of the environment around the dye to be 86 ± 1. However, if we were to consult a plot of transition energy of 1,7-ANS *vs.* dielectric constant, the estimate of the dielectric constant of the binding site would be 27 ± 8, even when nonaqueous solvents are ignored. The value of quantitatively relating ANS fluorescence to the empirical *Z* scale is that these measurements can be compared with many other solvent effects on spectroscopic parameters and on organic reaction mechanisms. This is just the information desired to relate the solvent polarity at the active sites to the rate and mechanisms of enzyme-catalyzed reactions.

Our data indicate, then, that in certain solvents at least, the fluorescence properties of ANS dyes can be used as measures of the *Z* value of the solvent. Considering Figure 2A, one could imagine a line drawn through those points which represent aqueous mixtures of ethanol, methanol, and dioxane. Such a line may be regarded as an "upper limit" on the quantum yield (McClure and Edelman, 1966). At any given value of *Z*, no value of ϕ falls above this line, but there are several examples of solvents in which the ϕ is well below this line. We believe that the line through the points for aqueous mixtures defines the "general" polarity effect of the solvent, on the quantum yield of 1,7-ANS, and that deviations are ascribable to "specific" solvent effects. The same sort of reasoning can be applied to the plots of $\bar{\nu}_F$ and $\Delta\bar{\nu}_F$ against *Z*. The solvents which show the greatest deviations from the general relationship are dipolar aprotic solvents. Similar deviations have been observed by Parker (1962) who has rationalized such behavior in terms of solvent structure.

The existence of functional groups on proteins which might alter the fluorescence of 1,7-ANS makes it

TABLE V: Fluorescence of Isomeric Aminonaphthalenesulfonates and Derivatives.

Compound	Emission Max ($\bar{\nu}_F$) ($\times 10^4$ cm^{-1})					
	Water	20% Ethanol	40% Ethanol	60% Ethanol	80% Ethanol	Ethanol
1,4-AmNS	2.37	2.38	2.40	2.41	2.42	2.45
1,5-AmNS	1.92	1.94	1.96	2.00	2.04	2.13
1,6-AmNS	2.12	2.15	2.17	2.19	2.22	2.27
1,7-AmNS	2.06	2.08	2.12	2.15	2.18	2.23
1,8-AmNS	1.92	1.93	1.94	1.96	2.00	2.06
2,6-AmNS	2.39	2.40	2.42	2.43	2.44	2.46
2,6-ANS	2.07	2.16	2.23	2.29	2.32	2.41
2,6-TNS	2.00	2.06	2.11	2.16	2.20	2.34
2,6-MANS	1.84	1.93	2.03	2.13	2.19	2.32
	Absolute Quantum Yield (ϕ)					
1,4-AmNS	0.80	0.79	0.86	0.80	0.87	0.76
1,5-AmNS	0.18	0.38	0.37	0.52	0.52	0.73
1,6-AmNS	0.29	0.40	0.48	0.52	0.87	0.56
1,7-AmNS	0.55	0.60	0.74	0.73	0.86	0.66
1,8-AmNS	0.026	0.051	0.097	0.15	0.25	0.51
2,6-AmNS	0.47	0.48	0.48	0.47	0.46	0.42
2,6-ANS	0.011	0.040	0.19	0.32	0.35	0.55
2,6-TNS	0.002	0.0097	0.030	0.083	0.22	0.55
2,6-MANS	0.0078	0.025	0.086	0.22	0.32	0.48

important to be able to distinguish specific from general effects. The fact that all three fluorescence parameters vary with the Z value of the solvent but may not all be susceptible to the same specific effects, may be useful inasmuch as Z values obtained with one measurement may be compared with the other two. Although the quantum yield shows the most dramatic changes and correlates best with Z, it is also the most susceptible to specific solvent effects. It is also the most difficult to measure with proteins because of the need for an accurate binding constant and the value of the extinction coefficient of the bound dye.

It is of interest that three of four dyes can be used to give consistent estimates of binding site polarity for a variety of proteins (Table III). Certain classes of enzymes, such as kinases and dehydrogenases, appear to have characteristic binding site polarities. In order to make further comparisons it is necessary to establish that the dyes bind to the same site, as has been done for horse liver alcohol dehydrogenase (Brand *et al.*, 1967), and to obtain quantitative equilibrium binding data. We have no explanation for the apparently anomalous values obtained with 1,8-ANS.

The precise mechanism for the effect of solvent polarity on the fluorescence of ANS isomers remains to be elucidated. The data presented here indicate that a common mechanism may be involved in the effect of solvent on the three fluorescence parameters measured. A bathochromic shift of the fluorescence maximum in polar solvents is expected in those cases in which the first singlet excited-state dipole moment is greater than that of the ground state (Lippert, 1966; McClure and Edelman, 1966). The correlation of $\bar{\nu}_F$ with Z indicates that the emission process in 1,7-ANS, like the absorption process in 1-alkylpyridinium iodides, approximates an "ideal extreme process" (Kosower, 1968), in which a very polar state is changed to a neutral one. A plausible explanation for the relationship of quantum yield to the emission maximum shift can be given if it is assumed that the dipole moment of the first excited triplet state is less than that of the first excited singlet. This seems reasonable in light of Murrell's calculations for aniline (Jackson and Porter, 1961). The difference in energy between the first excited singlet and triplet states is a key factor in determining the rate of intersystem crossing (Kasha, 1960). Since the difference in energy between the excited singlet and triplet states would be expected to decrease as solvent polarity is increased, the rate of intersystem crossing, and hence of fluorescence quenching, would increase in polar solvents. A reasonable explanation for the relation between band width and emission maximum has already been given by McClure and Edelman (1966).

A quite different mechanism to explain the very low yield of ANS in water has been suggested by Stryer (1966). This explanation, involving excited-state proton transfer, does not seem reasonable in the case of the dyes studied in this report since their fluorescence was pH independent over a wide range and since related chromophores without an ionizable proton show a D_2O fluorescence enhancement and a similar fluorescence dependency upon polarity.

The lowered energy of the first excited state in ANS relative to AmNS (Table V) can be ascribed to the

increased possibilities for electron delocalization upon the addition of another aromatic ring which stabilize the excited state resulting from an intramolecular charge-transfer transition. We have already provided an explanation of the polarity effect on ANS fluorescence which requires the lowering of the energy of the first excited singlet so that intersystem crossing is possible in polar solvents. The data for 1,8-AmNS in Table V suggest that the presence of a negatively charged group very close to the nitrogen with its net positive charge may also decrease the energy of the lowest lying (charge-transfer) excited state, and thus facilitate quenching in polar solvents *via* intersystem crossing. The fact, noted by McClure and Edelman (1966) for TNS, and shown by us for 1,7-ANS, that many solvents in which ϕ is low have unpaired electrons, does not appear to be related to the general polarity effect, for no wavelength shifts accompany quenching in those cases.

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