1-Anilino-8-Naphthalene Sulfonate Anion-Protein Binding Depends Primarily on Ion Pair Formation

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ABSTRACT The ANS^- (1-anilino-8-naphthalene sulfonate) anion is strongly, dominantly bound to cationic groups of water-soluble proteins and polyamino acids through ion pair formation. This mode of ANS^- binding, broad and pH dependent, is expressed by the quite rigorous stoichiometry of ANS^- bound with respect to the available summed number of H^+ titrated lysine, histidine, and arginine groups. By titration calorimetry, the integral or overall enthalpies of ANS^- binding to four proteins, bovine serum albumin, lysozyme, papain, and protease omega, were arithmetic sums of individual ANS^-–polyamino acid sidechain binding enthalpies (polyhistidine, polyarginine, polylysine), weighted by numbers of such cationic groups of each protein (additivity of binding enthalpies). ANS^- binding energetics to both classes of macromolecules, cationic proteins and synthetic cationic polyamino acids, is reinforced by the organic moiety (anilinonaphthalene) of ANS^-. In a much narrower range of binding, where ANS^- is sometimes assumed to act as a hydrophobic probe, ANS^- may become fluorescent. However, the broad overall range is sharply dependent on electrostatic, ion pair formation, where the organic sulfonate group is the major determinant of binding.

INTRODUCTION

1-Sulfonato-8-(1′)anilinonaphthalene (1-anilino-8-naphthalenesulfonate, ANS^-) anion is a much utilized fluorescent "hydrophobic probe" for examining the nonpolar character of proteins and membranes. Stryer (1965) showed that the fluorescence of ANS^-, bound to the known nonpolar sites of apomyoglobin and apohemoglobin, was equal to the fluorescence of an equivalent amount of ANS^- in a number of hydrophobic organic solvents. Subsequently ANS^- has been used in several related but very diverse ways: as a back-titration fluorescence indicator for lipid molecule displacement from lipid carrier proteins (Kane and Bernlohr, 1996), in the detection of "molten globule" (Semisotnov et al., 1991), nonpolar surface patches of proteins (Cardamone and Puri, 1992), and many more. ANS^- fluorescence detected five hydrophobic sites on, or in, bovine serum albumin (BSA) (Daniel and Weber, 1966). Earlier work was well reviewed (Slavik, 1982).

In practically all such work concerning ANS^-, the sulfonate SO_3^- group was viewed as a solubilizing group for what would otherwise be a nearly water-insoluble anilinonaphthalene moiety. Assuming that the $-SO_3^-$ anion is merely a convenience, the sulfonate anion presumptively had minor bearing on, perhaps nothing to do with the thermodynamics or stoichiometry (numbers of binding sites) of ANS^--protein molecule interaction. It also was generally assumed that if ANS^- became brilliantly fluorescent upon binding, host protein binding sites were nonpolar and hydrophobic during association. Here we report ANS^-'s binding equilibria, stoichiometry, and heats of binding to BSA over wide pH ranges, and the heats of ANS^- binding to lysozyme, papain, protease omega, and cationic amino acid polymers at pH 2.

Much of ANS^-'s capacity to bind proteins is assignable to electrostatic and stoichiometric relationships dependent on protein or polyamino acid number of cationic charges, and hence is dependent on pH and amino acid composition. Inasmuch as protein-ANS^- interaction is rather sharply dependent on electrostatic (Coulombic) forces, the sulfonate group of ANS^- appears considerably more than a mere "handle" or solubilizing agent for the anilinonaphthalene organic moiety. This alternative has been carefully addressed by Kirk et al. (1996). It requires study because there are two different apparent ranges over which ANS^- binds to proteins such as BSA. One of these is a broad range involving as many as 100 binding sites at pHs below 5, where most of the bound ANS^- is not fluorescent. This large range of sites brackets or spans the much narrower set of five sites on BSA (Daniel and Weber, 1966), within which ANS^- is brilliantly fluorescent. These two very different ranges of ANS^- binding to the same protein molecule increasingly indicate that more than one set of forces (more than simply hydrophobic forces) are involved in ANS^- binding. Origins of ANS^- interaction in the broad binding range are investigated here by determining the stoichiometry of ANS^- with respect to the number of cation charges (denoted z) on macromolecules, and ANS^-'s thermochemistry of binding via titration calorimetry. The ANS^- anion is a "probe" of sorts for proteins to which it has become bound. However, its probe or environment indicator properties are not simple, strict gauges of macromolecule nonpolar character exclusive of all other properties (such as protein charge), before ANS^- becomes bound. When, after ANS^- becomes bound

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and emits brilliant fluorescence, a few such ANS^-'s possibly reflect local hydrophobicity.

MATERIALS AND METHODS

ANS^- fluorescence intensities with bovine serum albumin

ANS^- was purchased from Aldrich (ammonium salt, purity 97%). BSA was purchased from Sigma.

Fluorescence titration experiments were performed on a Perkin-Elmer 650-10S fluorescence spectrophotometer. The concentration of BSA was 1 or 3 μM. ANS^- was used at y values equal to 3.3 and 33 (ratio of added molar concentrations of ANS^- to BSA). Therefore concentrations of ANS^- were 3.3 and 33 μM for 1 μM BSA, and 10 and 100 μM for 3 μM BSA. In a 1-cm cell the fluorescence inner-filter effect becomes significant at ~30 μM ANS^-. Therefore correction factors were used for the fluorescence of ANS^- when its total concentration was larger than 10 μM. The samples were 30 mM in NaCl and 30 mM in the respective buffers. The buffers used were HCl for pH < 2, sodium formate for pH 2.5–3.7, sodium acetate for pH 4.0–5.2, sodium phosphate for pH 6.2–7.4, and sodium hydroxide for pH > 11. All work was performed at 25°C.

Determination of ν, the number of ANS^- apparently bound per protein molecule (via fluorescence titration), was obtained by using the expression $\nu_{ANS-} = (F_m/F_b)y$ (Daniel and Weber, 1966), in which F_m = fluorescence intensity in given BSA concentrations, 1 and 3 μM, F_b = intensity in excess BSA concentrations, 10 and 30 μM; y = molar ratio of ANS^- addition/BSA.

Dialysis equilibrium of ANS^- anion binding to BSA

Dialysis vessels of ~3 ml volume on each side of the cellulose membrane were used. Various concentrations of BSA were added on one side. In Fig. 2 we report data obtained using 0.24 mM BSA. Both sides included 40 mM NaCl. High concentrations of NaCl were avoided because Cl^- anions may compete with ANS^- for the binding sites on the BSA molecule. Equilibrium dialysis was performed overnight, and the concentration of ANS^- was analyzed by spectrophotometry ($\epsilon_{360} = 5000$). No buffer was used, but the pH was adjusted to the desired value by adding HCl or NaOH after the addition of ANS^-. BSA concentration was determined by using values $E_{280}1\% = 6.67$, MW = 65,500.

Integral heats of ANS^- binding to BSA dependent on pH

Isothermal titration calorimetry was performed with a Microcal (Northampton, MA) Omega calorimeter. The cell volume was 1.447 ml. Two milliliters of BSA solution (usually 0.03 mM) in buffer (30 mM NaCl, 30 mM buffer, same as for fluorescence titrations) was added to the cell. ANS^- (concentration 30 mM, in the same buffers) was injected in 44 portions of 6.25 μl. Heats of binding and binding constants were calculated with Origin software. The heat of ANS^- dilution into buffer alone was negligible. The calorimeter was calibrated with Tris-HCl ($\Delta H = -11.3$ kcal/mol acid neutralization).

ANS^- binding to cationic papain, protease Ω, and lysozyme

Lysozyme and papain were purchased from Sigma and used without further purification. Protease omega was isolated from crude papain (obtained from Calbiochem, catalog no. 5125) and chromatographically purified on a Biorex 70 cation exchange column (Dubois et al., 1988; Sylverstein and Kezdy, 1975). Buffers used for binding studies were the same as those described for fluorescence titration. The concentration of lysozyme, papain, and protease omega in the cell of the titration calorimeter was 0.2 mM. The injection size was the same as for BSA.

ANS^- binding to derivatized amino acids and alkane amines

Derivatized amino acids (L-arginine methyl ester, *N*-α-acetyl-L-arginine, *N*-α-acetyl-L-lysine methyl ester, *N*-acetyl-L-histidine, *N*-α-acetyl-glycyl-L-Lysine methyl ester, *N*-α-p-tosyl-L-arginine methyl ester, and *N*-ε-*p*-tosyl-L-lysine ethyl ester) were purchased from Sigma. Their concentration in the titration calorimeter cell was 4 mM in 25 mM NaCl at pH 2.5. The concentration of ANS^- in the injection syringe was 30 mM in 25 mM NaCl at pH 2.5. The injection volume was 12.5 μl.

Calorimetry of ANS^- binding with cationic polymeric amino acids

Polylysine, polyarginine, and polyhistidine were purchased from Sigma. The degree of polymerization for polylysine was 36 or 80 (± 20%) (results were not significantly different when polymers of these two different average degrees of polymerization were used) (polyarginine, 66; polyhistidine, 81). The concentration of polycationic amino acids, in monomer, was 3 mM. The pH was adjusted with HCl to 2.0, in 20 mM NaCl. The concentration of ANS^- in the syringe was 30 mM, in 20 mM NaCl, pH 2.0.

RESULTS

ANS^- fluorescence intensities with bovine serum albumin

Using the notation y = moles ANS^- added/mole protein, ν = moles ANS^- apparently bound/mole protein, fluorescence intensities of ANS^- in the presence of BSA dependent on pH are plotted in Fig. 1 for $y = 3.3$ and $y = 33$. These relative concentrations were chosen to fall somewhat under and over the number of fluorescent sites for BSA-

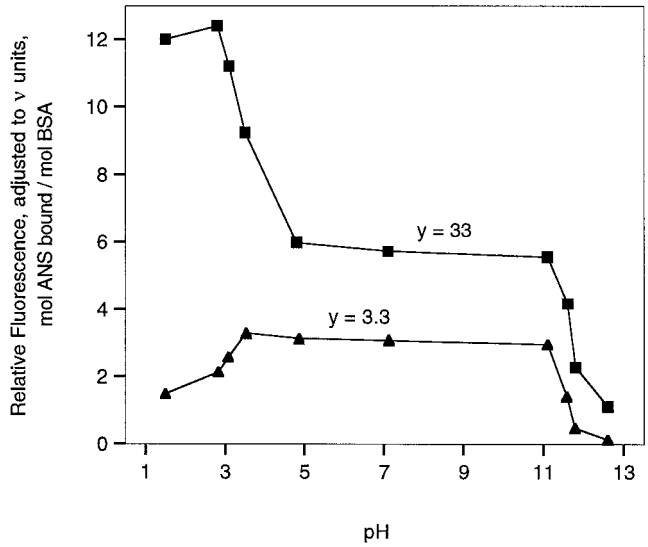

FIGURE 1 Relative fluorescence intensities of ANS^- in the presence of 3 μM BSA as a function of pH, adjusted to ν-mol bound ANS^- per mol BSA (see Materials and Methods). y, mol ANS^- added per mol BSA. ▲, $y = 3.3$; ■, $y = 33$.

ANS^- interaction according to the Daniel-Weber (1966) reports of five fluorescing ANS^- sites in the pH range of 5–7. The level of $y = 3.3$ ANS^-/BSA (Fig. 1) demonstrates that all measurable ANS^- is bound. The level $y = 33$ ANS^- added/BSA returns 5.5 ± 0.5 sites where bound ANS^- brightly fluoresces, $\nu_{ANS^-} \cong 6$, in the pH range of 4–11. Hence these results are consistent with the Daniel-Weber estimate of $n = 5$ such sites for ANS^- in the presence of enough ANS^- to nearly saturate the fluorescence-active sites. The BSA molecule in pH 4–10 is maximally compact and moderately hydrated (Tanford et al., 1955a). Hence BSA within the pH range of 4–10 is not expected to engage in major or global conformational changes that may reflect on ANS^- fluorescence production or the number of available sites, corresponding to the flat plateaus in Fig. 1 in the same pH range. However, outside the pH range of 4–10, the BSA molecule engages in well-characterized conformational expansion (Tanford et al., 1955a). The large increase in protein net positive charge Z_H+ in pH below 4 increases ν_{ANS^-} to ~12 ANS^- bound. In the alkaline region above pH 11 there is a sharp decrease in ANS^- fluorescence.

Dialysis equilibrium of ANS^- anion binding to BSA

Fig. 2 plots numbers of ANS^- anions bound to BSA, ν, versus y moles ANS^- added/mole BSA at three pHs that confer three very different net charges Z_H+ on the protein molecule. When the maximum number of ANS^- capable of binding to BSA under each condition is estimated by the point of apparent saturation, ~100 ANS^- can bind to the protein in acid pH ranges when the protein acquires close to 100 net cationic charges ($Z_H+ = z = 100$). Short of these extreme values, strong binding of ANS^- anions is evinced where the slope of such a plot is close to 1.0, i.e., where $\nu \cong y$ for ANS^-. This behavior holds up to ~90 such sites, but with a lower slope in isoionic pH, where the protein molecule retains most individual cationic H^+ titrated groups, although its overall charge is zero. In strongly alkaline pH, very little ANS^- is bound (up to seven sites at pH 11.6). These results are consistent with primarily electrostatic binding.

Integral heats of ANS^- binding to BSA dependent on pH

The ANS^- anion binds to BSA, producing rather large exothermic heats, dependent on initial pH before mixing and on the mole ratio of ANS^- to BSA (y_{ANS-}), plotted in Fig. 3. Exothermic heat magnitudes are increased with lowered pH. Therefore ANS^- binding depends on electrostatic factors, governed, in turn, by the relation of pH to the protein's pI, to overall protein titration charge Z_H+, and to the number of positive charges on the protein molecule, z. In the lower pH regions where the protein molecule amino acid side chains are cationic, and the carboxyl groups are neutralized to their COOH form, integral heats of binding ANS^- yield several kcal/mole of bound ANS^-. Table 1 summarizes these integral or overall heats for ANS^-, filling the apparent maximum number of available sites for each pH. In alkaline pH well above BSA's pI, the remaining cationic sites persist in binding a few ANS^- anions, even though the net overall protein charge Z_H+ is quite negative.

Altogether, Fig. 3 indicates ~100 heat-producing sites for ANS^- binding, found from the abscissal y value, where all of the plots converge, generating very little additional heat when y exceeds 100. In the very alkaline pH 12.6, only ~6 ANS^- anions bind, producing relatively small heats. The apparent stoichiometry for the maximum number of sites for ANS^- binding in Fig. 3 thus agrees with Fig. 2,

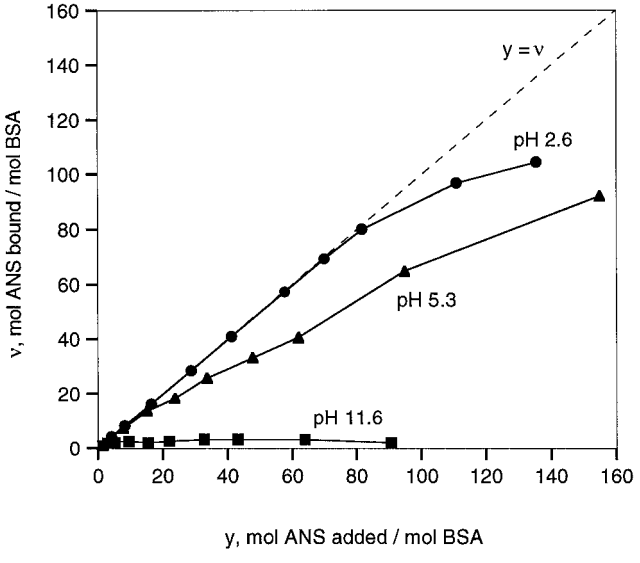

FIGURE 2 Dialysis equilibrium of ANS^- binding to BSA at various pHs. ●, pH 2.6; ▲, pH 5.3; ■, pH 11.6.

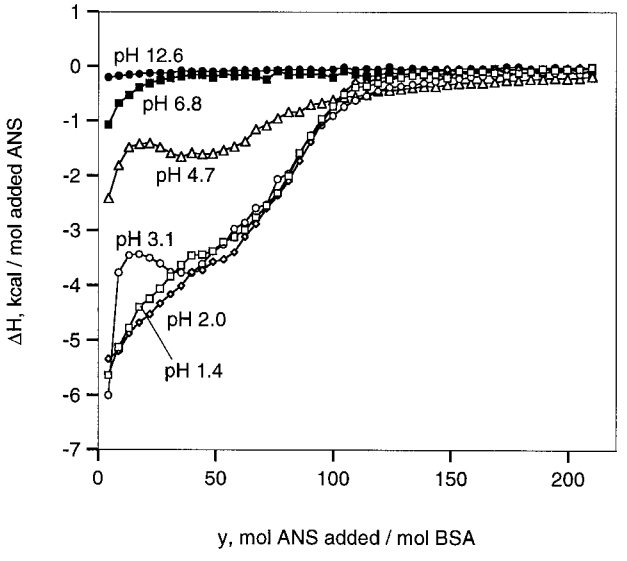

FIGURE 3 Titration calorimetry of BSA with ANS^- at various pHs. Maximum exothermic heat is produced at acidic pH. □, pH 1.4; ◇, pH 2.0; ○, pH 3.1; △, pH 4.7; ■, pH 6.8; ●, pH 12.6.

TABLE 1 Overall integral heats of binding ANS^- to bovine serum albumin, pH dependence, *n* (binding stoichiometry), moles ANS^-/mole BSA, *z* (number of positive charges on BSA molecule), and Z_{H^+} (overall charge of BSA), at 25°C

pH	ΔH (kcal/mol BSA)	*n*	*z*	Z_{H^+}
1.4	−331	70	100	+100
2.0	−341	73	100	+95
3.1	−322	77	100	+60
4.7	−174	87	95	+10
6.8	−40	~15	70	−10
12.6	−14	~6	10	−90

which also indicated approximately the same number of total sites from the direct binding data in acid and neutral pH.

ANS^- binding to cationic papain, protease omega, and lysozyme

ANS^- interaction with these three proteins via titration calorimetry in acid pH regions was evaluated in three respects: numbers of positively charged amino acid side chains in acidic pH 2, presumed binding sites for electrostatic ANS^- binding (first column of Table 2); number of ANS^- bound, denoted *n* (second column) from deconvolution of the calorimetric data; and integral heats of saturation of all four proteins with ANS^- per mole protein (third column).

The numbers of total sites, apparent from the first two kinds of estimates for each protein, agree within a few percent of one another for all four proteins. The integral heats of ANS^- binding for the four proteins are roughly in proportion to their molecular weights, which perhaps is to be expected if they bear more cationic amino acids than the smaller proteins. The apparent total number of sites, *n*, in Table 2 indicates that a large number of ANS^- can bind to all of these proteins if ANS^- concentrations are adequate (millimolar ranges), even if many of such bound anions do not fluoresce. The later, presumably weaker sites (on average) develop appreciable exothermic heats for ANS^- binding, 3–6 kcal/mol of bound ANS^-, on rough average.

TABLE 2 Stoichiometry of ANS^- binding to proteins (*n*), number of cationic amino side-chain sites at pH 2, and calorimetric enthalpies of ANS^- saturation for four proteins

Protein	No. cationic amino acids per protein (composition)	*n* (mole ANS^- bound/mol protein) (measured)	ΔH (kcal/mol protein)
BSA	100	79	−341
BSA (0.1 mM)	100	94	−386
Lysozyme	19	17	−77
Papain	25	21	−97
Protease omega	38	36	−129

ANS^- binding to derivatized amino acids and alkane amines

L-Arginine methyl ester, *N*-α-acetyl-L-arginine, *N*-α-acetyl-L-lysine methyl ester, *N*-acetyl-L-histidine, and *N*-α-acetyl glycyl-L-lysine methyl ester were used because derivatization of these amino acids on the α nitrogen negates the possibility of ion pair formation between the α-ammonium group and sulfonate of ANS^-. Virtually no heat was produced upon mixing of ANS^- with these derivatized amino acids, because negligible association occurs. However, the presence of a large tosyl group on arginine and lysine increased the association and enabled the production of large exothermic enthalpies upon mixing with excess ANS^-: *N*-α-*p*-tosyl-L-arginine methyl ester (guanidinium group positively charged) and *N*-ε-*p*-tosyl L-lysine ethyl ester (α-amino group positively charged) produced (−)11 and (−)8 kcal heat/mol, respectively, with an apparent stoichiometry of association of one amino cation per ANS^-.

Interaction of ANS^- with C_8, C_{10}, and C_{12} alkane ammonium cations in excess ANS^-, in which the available RNH_3^+ cations were saturated with ANS^- anions, produced exothermic enthalpies. Table 3 lists the observed heats, association constants, and apparent stoichiometry (*n* value) for these compounds in acidic pH (2.5) and neutral pH (7.1), where the amines are cations, but carboxylate groups are anionic. Above the eight-carbon series, increased association constants ranging from 1000 to 30,000 M^{-1} and increased binding enthalpies ranging from −3 to −9 kcal/mol of alkane ammonium were produced as a result of increasing alkane amine chain lengths. Mixing aliphatic carboxylate anions with ANS^- produced negligible association and zero enthalpy. If hydrophobic interaction were the only reason for binding, one would not expect to see an appreciable difference between ANS^- binding to alkane carboxylate and alkane ammonium with the same chain length.

The foregoing pertains to ANS^- association with alkane ammonium cations in water. On the addition of *tert*-butanol to both components before mixing, enabling isocratic mixing when components were brought together, the ANS^- association constant with decylammonium cation decreases slightly over the range 0–9% *t*-butanol. However, the enthalpies (exothermic ΔH production) increase rather dramatically, as shown in Table 3. The apparent stoichiometries (*n* values) remain essentially constant, remaining close to unity throughout (average $n \cong 0.98$).

Calorimetry of ANS^- binding with cationic polymeric amino acids

Three homopolymers, polylysine, polyhistidine, and polyarginine, all at pH 2, where they are cationic, were calorimetrically titrated with ANS^-. The ANS^- injections were carried to a twofold excess ($y \cong 2$) of added ANS^- per amino acid positive charge to fully saturate all binding sites. The heats of ANS^- interaction with polyamino acids are

TABLE 3 ANS^-, alkane ammonium binding stoichiometry (n), association constants (K_b), and enthalpies of binding (ΔH) for aqueous and aqueous t-butanol cosolvent mixtures

Alkane amine, alkane carboxylate		pH	n	K_b (M^{-1})	ΔH (kcal/mol bound ANS^-)
n-Octylamine	$C_8H_{17}NH_3^+$	2.5	~1	~1000	−3
n-Decylamine	$C_{10}H_{21}NH_3^+$	2.5	0.97	5500	−5.1
n-Dodecylamine	$C_{12}H_{25}NH_3^+$	2.5	0.94	30000	−8.5
n-Decylamine	$C_{10}H_{21}NH_3^+$	7.1	0.95	5000	−5.6
n-Decanoate	$C_9H_{19}COO^-$	7.1	0	~0	0
n-Decylamine, $C_{10}H_{21}NH_3^+$, in aqueous *tert*-butanol (vol/vol %)					
0		2.5	0.97	5500	−5.1
2.4		2.5	1.07	4200	−6.1
4.7		2.5	0.98	3400	−7.4
7.0		2.5	0.99	3600	−8.7
9.0		2.5	0.95	3300	−10.4

shown in Fig. 4. The polyamino acids remain in true solution in the range of y from ~0.1 to 0.8, generating the bottom plateaus plotted in the figure. When y is larger than 0.8, the ANS^- polyamino acid complex precipitates. However, it is the heats for the polymer-ANS^- interaction in true solution on which the additivity comparisons (below) are based. Table 4 lists fitting parameters, stoichiometries (n value), association constants K_b, and calorimetric heats for the polymers in ANS^- association, obtained from the data in these same figures. Table 5 lists thermodynamic parameters, enthalpies of binding (ΔH), free energies of binding (ΔG; calculated from association constants K_b using $\Delta G = -RT \ln K_b$), and entropies of binding ($T\Delta S$; calculated via $\Delta G = \Delta H - T\Delta S$).

In the early stage after the addition of ANS^- to the homopolymers, particularly polylysine, where $y < 0.3$ moles of ANS^- added/mole lysine cation side chain, heat generation does not fully develop until some amount of ANS^- is added, approaching the threshold $y \cong 0.3$.

All three cationic amino acid polymers bound one ANS^- per positively charged amino acid (1:1 stoichiometry). Overall shapes of polyhistidine and polyarginine calorimetric titration curves in Fig. 4 are similar. Polylysine bound ANS^- with the smallest association constant and smallest exothermic enthalpy. Polyarginine bound ANS^- with the largest association constant and exothermic enthalpy. Polyhistidine fell in the middle.

ANS^- polyamino acid interactions, in general, like a majority of anion-binding reactions with positively charged macromolecules, where electrostatic forces are primary, are enthalpy driven (exothermic), compensating for positive entropies (in all cases listed in Table 5). The contrast in K_b association constant magnitudes (~2.5×10^5 for poly-Arg versus 2.0×10^4 for poly-Lys) in ANS^- association apparently is assignable to enhanced exothermic enthalpies and smaller positive entropies to the advantage of the poly-Arg versus poly-Lys polymer.

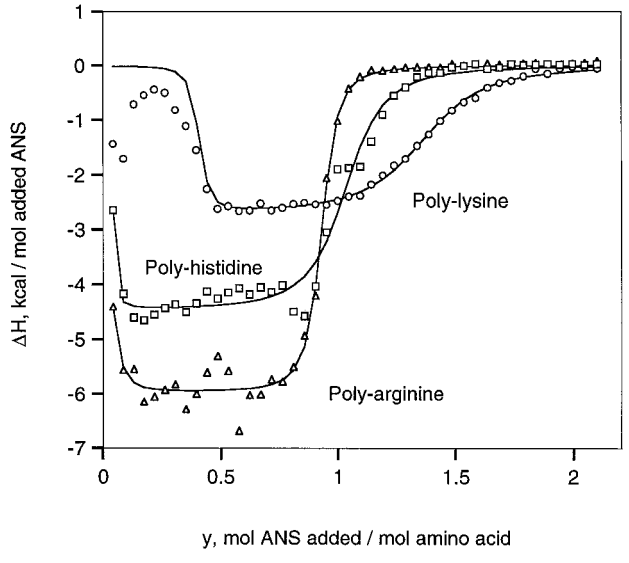

FIGURE 4 Calorimetry of ANS^- association with polylysine (○), polyhistidine (□), and polyarginine (△) at pH 2.0.

Additivity of enthalpies of ANS^- binding to cationic groups of four proteins

Individual molar enthalpies of ANS^- interaction with histidine, lysine, and arginine cations in the homopolymers polyhistidine, etc. in Table 5 provide a basis for calculating the summed heats for ANS^- binding to equivalent numbers

TABLE 4 Stoichiometries of binding (n), association constants (K_b), and enthalpies of ANS^- association (ΔH), to polyamino acids at pH 2.0 and 25°C

Polyamino acid	n (± 0.1)	K_b (± 10%) (M^{-1})	ΔH (± 10%) (kcal/mol)
poly-Lys	0.97	19,000	−2.7
poly-His	0.95	150,000	−4.5
poly-Arg	0.88	260,000	−6.0

See curves in Fig. 4.

TABLE 5 Thermodynamic quantities: ANS^- binding to cationic polyamino acids, at pH 2.0, 25°C

Polyamino acid	ΔH (kcal/mole a.a.)	K_b (M^{-1})	ΔG (kcal/mole a.a.)	$T\Delta S$ (kcal/mole a.a.)	ΔS (cal/(mol*K))
poly-Lys	-2.75 ± 0.3	$(2.0 \pm 1) \times 10^4$	-5.9 ± 0.5	3.2 ± 0.8	10.7 ± 2.7
poly-His	-4.5 ± 0.5	$(2.0 \pm 1) \times 10^5$	-7.3 ± 0.5	2.8 ± 1.0	9.4 ± 3.4
poly-Arg	-6.0 ± 0.5	$(2.5 \pm 1) \times 10^5$	-7.4 ± 0.3	1.4 ± 0.8	4.7 ± 2.7

a.a., amino acid. Averages of two independent experiments.

of cationic sites on protein molecules. For example, the BSA molecule bears 17 histidine side chains, 58 lysines, 24 arginines, and one N-terminal amine in the amino acid sequence of Carter and Ho (1994). These compositional parameters are very close to the cationic side-chain composition obtained from the precise H^+ titration of BSA (Tanford et al., 1955b). Hence the calculated overall or integral heat for saturating one mole of BSA with ANS^- in acid pH 2.0, from ANS^- site enthalpies from Table 5, are: $4.5 \times 17 + 2.75 \times 58 + 6.0 \times 24 = 380.0$ kcal (exothermic).

The measured calorimetric enthalpy of BSA saturation with ANS^- is $(-)341.3$ kcal/mol BSA at pH 2.0. Thus, taking the molar individual site enthalpies of ANS^- binding to the homopolymers as a basis, and weighting them according to the amino acid composition of BSA, the calculated overall heats agree fairly well (within 10% at pH 2) with the directly observed overall heat. This would not be expected if ANS^- were weakly bound, or if it had to seriously compete with many strongly, previously bound counterions such as Cl^- for it to bind.

The same "additivity" for calculating integral heats for ANS^- saturation of four acid proteins, factoring in their known amino acid compositions—their numbers of cationic sites (protonated histidine, lysine, and arginine), enables us to construct Table 6. The rightmost column of Table 6 lists the measured values for ANS^- saturating enthalpies for the proteins, $\Delta H_{ANS^-,Sat.}$, for comparison with $\Delta H_{ANS^-,Calc.}$ (calculated) values. The two sets of enthalpies for the four proteins agree within 10% (measured versus calculated integral heats; lysozyme: ±13%). The comparison for each protein is predicated on ANS^- interaction with charged sites in 1:1 stoichiometry. The enthalpies from these interactions are largely determined by the electrostatic component of ANS^--protein interaction: not the absolute magnitudes of such enthalpies, but in numbers of ANS^- anions and of protein side chains acting as sites—the apparent stoichiometry.

DISCUSSION

The primary determinant of ANS^- binding to proteins and polyamino acids, in the overall broad range of ANS^- binding, is electrostatic, Coulombic, in origin. Expression of this behavior is clearly seen in the stoichiometry: one ANS^- sulfonate group is attracted to one macromolecule cationic group, even in water, averaging from ~ -3 to -6 kcal/mole ANS^- bound through the ranges of all sites of variable affinity, stronger and weaker. The organic anilinonaphthalene moiety of ANS^- perhaps determines part of the magnitude of these enthalpies—hence the other contributions to the thermodynamics of such binding. However, the stoichiometry over the broad range of ANS^- binding, from sparse binding up to saturation, is determined by the sulfonate group of ANS^- in conjunction with the number of cationic (histidine, lysine, arginine side chains) groups donated by the protein molecule. This behavior is similar, nearly congruent, to that of other kinds of organic sulfonate and sulfate ligands able to vigorously bind to protein molecules, such as detergent sulfates and azoaromatic dye sulfonates of many kinds (Matulis et al., 1996; Conroy and Lovrien, 1992). The dominantly electrostatic determinant, the origin, of ANS^- stoichiometry is indicated by the sharp pH dependency of the ANS^- binding process. The overall consequences of organic sulfonate interaction, such as coprecipitation, conformation change, and spectroscopic behavior, are sharply dependent on what the organic moiety is— they are "ligand structure" dependent. But the stoichiometry of binding is surprisingly independent of the precise structure of the organic moiety.

ANS^- as a putative probe for protein structure in water, via fluorescence intensity measurements, as usually conceived (Slavik, 1982), is actually dependent on three factors deserving more attention:

1. The range of ANS^- binding over which ANS^- produces its brilliant fluorescence, generally is far more narrow

TABLE 6 Additives of ANS^- binding site enthalpies based on protein side-chain composition

Protein	Lysine content	Histidine content	Arginine content	Summed $\Delta H_{ANS^-,Calc.}$ (kcal/mol protein)	Measured $\Delta H_{ANS^-,Sat}$ (kcal/mol protein)
BSA (0.1 mM)*	58	17	24	−380	−386
BSA	58	17	24	−380	−341
Lysozyme	6	1	11	−87	−77
Papain	10	2	12	−109	−98
Protease omega	22	4	11	−139	−125

*Measured enthalpy of ANS binding when BSA concentration was larger (0.1 mM).

than the overall range of ANS^- binding set by electrostatic forces primed by ANS^-'s sulfonate group; for example, five ANS^- anions bind to BSA with strong fluorescence production, but straddling that narrow range is BSA's ability to bind up to 100 ANS^- anions, over a considerable pH range.

2. ANS^- fluorescence production is sharply dependent on the absence of water molecule quenching of fluorescence, rather than completely dependent on the polar versus nonpolar nature of ANS^-'s immediate surroundings, its microenvironment.

3. Macromolecules to which ANS^- may bind need not possess large hydrophobic structure patches or globules before ANS^- binding starts. If hydrophobicity is a necessity for brilliant fluorescence (or for the absence of water quenching), it is the status of ANS^- bound in a complex with the protein (or other macromolecule) that counts; that is, the erstwhile hydrophobicity of a protein, evinced by ANS^- fluorescence, is likely a creation of the product (protein with bound ANS^-, the complex), not of the reactant (hydrated protein before ANS^- binds). Oddly, in many reports on other classes of probes and interpretation of their data, the distinction between protein molecule structural status as a reactant versus protein molecule structure-function status as a bound complex with ligands often is not made. That is, protein molecules in solution are seen as static. Protein molecules as reactants before ligands such as ANS^- become bound, versus the products of ligand or probe binding when major conformation changes are made visible, can be represented as

Reactants		**Product**
Ligands	+ Protein (Initial conformation, state of hydration net charge Z_1)	→ Ligand●Protein (Conformation in the bound complex, state of hydration net charge Z_2)

Thus Fig. 1, Fig. 2, the convergence point in Fig. 3, and the enthalpies approaching $y = 1$ in Fig. 4, where ANS^- calorimetrically titrated the available cation-binding sites of each polyamino acid, are clear manifestations of ANS^- acting as a "probe" of protein and polyamino acid molecules as products from such reactants. The primary cue starting the binding reaction is the stoichiometry, the number of sites offered by electrostatic forces, whether or not the reactant is "hydrophobic." After the binding process starts, hydration changes, and hydrophobic interaction may play some role for some of the ligands that become part of the product. In BSA, approximately five such sites are produced and are brilliantly fluorescent, in agreement with the data of Daniel and Weber (1966). The large majority of papers have not connected ANS^--protein associative phenomena with the number of positive charges on protein molecules, because of the belief that ANS^- binding or failure to bind is primarily dependent on protein hydrophobic content. Lysozyme has some hydrophobic content on its surface, and binds up to 17 ANS^- anions in acidic pH. None of them fluoresce.

Recent crystallographic studies of ANS^- complex with adipocyte lipid-binding protein showed that the sulfonate group of ANS^- is bound to the guanidinium group of arginine hidden deep in the lipid-binding cavity (Ory and Banaszak, personal communication). Other crystallographic studies of low molecular weight guanidinium cations with various sulfonates also suggest that the ion pair is formed between these groups (Ojala et al., 1996; Russell et al., 1994).

In the ANS^--protein interaction product, the mutual hydrophobic (apolar) character of the protein and the anilinonaphthalene group may engender a lowered dielectric constant near the ion pair of the sulfonate-protein sidechain cation. A local dielectric constant lowering is expected to add exothermicity in the heats of binding, as observed. Exothermic enthalpy enhancement by alkane longer chain amines, by tertiary butanol when used as a cosolvent, and by the tosyl group substitution on arginine can likewise be explained.

ANS^- binding depends primarily on macromolecule cationic charge, i.e., attraction for the organic sulfonate group of ANS^-. After binding, ANS^- fluorescence depends on a tangle of properties, including how the ground state or the excited state interfaces with its microenvironment. Whether the anilinonaphthalene moiety is coplanar or appreciably distorted, and whether water molecules in contact are immobilized, or remain mobile and can quench, are structural factors to which ANS^- fluorescence production is quite sensitive (Kirk et al., 1996). These factors are either poorly or incompletely summarized by hydrophobicity. One is led to believe that the anilinonaphthalene moiety of ANS^- is relatively nonpolar, "hydrophobic," or hydrophobic site-seeking. However, the overall binding process and its consequences, including fluorescence production or lack thereof, are dependent on much more than simple nonpolar character, whether of ANS^- or of macromolecules, whatever their conformation is before binding starts.

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