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1-Anilino-8-Naphthalene Sulfonate as a Protein Conformational Tightening Agent

Abstract: 1-Anilino-8-naphthalene sulfonate (ANS) anion is conventionally considered to bind to preexisting hydrophobic (nonpolar) surfaces of proteins, primarily through its nonpolar anilino-naphthalene group. Such binding is followed by an increase in ANS fluorescence intensity, similar to that occurring when ANS is dissolved in organic solvents. It is generally assumed that neither the negative sulfonate charge on the ANS, nor charges on the protein, participate significantly in ANS-protein interaction. However, titration calorimetry has demonstrated that most ANS binding to a number of proteins occurs through electrostatic forces, in which ion pairs are formed between ANS sulfonate groups and cationic groups on the proteins (D. Matulis and R. E. Lovrien, *Biophys. J.*, 1998, Vol. 74, pp. 1–8). Here we show by viscometry and diffusion coefficient measurements that bovine serum albumin and γ -globulin, starting from their acid-expanded, most hydrated conformations, undergo extensive molecular compaction upon ANS binding. As the cationic protein binds negatively charged ANS anion it also takes up positively charged protons from water to compensate the effect of the negative charge, and leaves the free hydroxide anions in solution thus shifting pH upward (the Scatchard-Black effect). These results indicate that ANS is not always a definitive reporter of protein molecular conformation that existed before ANS binding. Instead, ANS reports on a conformationally tightened state produced by the interplay of ionic and hydrophobic characters of both protein and ligand. © 1999 John Wiley & Sons, Inc. *Biopoly* 49: 451–458, 1999

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INTRODUCTION

The ANS anion (1-anilino-8-naphthalene sulfonate) is a much utilized “hydrophobic probe” for proteins, membranes, etc.^{1,2} ANS in solvents such as isopropa-

nol is brilliantly fluorescent, but practically nonfluorescent in water. This behavior has often been extrapolated to mean that the anilinonaphthalene group, when it fluoresces upon addition to a protein, binds in a nonpolar (hydrophobic) site that exists in the protein

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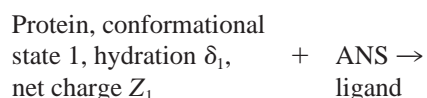
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before binding starts. In fact, ANS binding to proteins depends on protein cationic charge and solution pH, and occurs largely through the ANS sulfonate group.³ The dependence of ANS binding on electrostatic interaction between the sulfonate group and protein cationic groups indicates that ANS binding does not require preexisting hydrophobic sites on or in protein molecules to start the binding reaction. Moreover, ANS fluorescence is quenched by water,⁴ so fluorescence is profoundly affected if a change in protein hydration or ANS accessibility to water occurs during binding.

The binding process is determined by the mutual reactivity of the reaction partners, ANS, and protein. A potential contributor to mutual reactivity is a conformational change in the targeted protein molecule. This was recently presented in the following way³:



Reactants

Protein–ANS complex,
conformational state 2,
hydration δ_2 , net charge
in the bound complex Z_2

Product

This paper presents the evidence that conformational changes indeed are seen, in bovine serum albumin (BSA) and γ -globulin upon ANS binding. They are large enough to produce appreciable effects on protein hydrodynamic properties, its diffusion coefficient, and effective hydrodynamic volume, under conditions in which the protein molecule starts from an expanded conformation (state 1). Such hydrodynamic changes in solution are the most direct evidence of conformational tightening caused by ANS.

Additional but less direct evidence for conformational tightening is that ANS is a strong coprecipitating agent (a matrix ligand) when ANS binding approaches its ion pairing limit.⁵ Fink⁶ also noted that ANS precipitates proteins, consistent with the ability of many kinds of anions to conformationally tighten proteins on the way to eventually coprecipitating them. The reactions induced by ANS and other matrix ligands also protect protein molecules against severe denaturing conditions such as thermal and acid pH shock.

Our experimental methods measure ANS–protein interaction at equilibrium. Engelhard and Evans,⁷ investigating ANS–BSA interaction by kinetic measurements, arrived at a conclusion similar to ours: the protein does not behave as a static particle when ANS binds to it. Rather, ANS can induce rate-limited conformational changes that, when completed, bring the macromolecule to a state that enables a few ANS ligands to fluoresce.

MATERIALS AND METHODS

Proteins and ANS

BSA (Sigma, St. Louis MO) is sensitive to anions that may already be bound to it. Accordingly it was rendered isoionic, salt free, and counterion free by the Dintzis mixed bed exchange method, as recently reviewed.⁸ γ -Globulin, ovalbumin, and catalase were purchased from Sigma and used without further purification. ANS (Aldrich, Milwaukee WI) was used as ammonium salt.

ANS Binding to BSA

ANS binding was measured by equilibrium dialysis as previously described.³ The pH of BSA solution was started at 2.5 and allowed to change upon addition of ANS for correlating the binding data with the Scatchard–Black effect measurements. The previously reported results of equilibrium dialysis at pH 2.5 (kept constant) were used to correlate viscometry and dynamic light scattering data with ANS binding to BSA.

Viscometry

Viscometry was performed at 25°C in a temperature-controlled water bath using Cannon–Fenske capillary viscometers. Reduced specific viscosities, $\eta_{sp} = (\tau - \tau_o)/\tau_o c$, where τ and τ_o are flow times of protein solutions and solvent respectively, c is the weight concentration of protein (g/mL), were measured as a function of relative ANS concentrations $y_{ANS} = \text{moles ANS anion added/mole protein}$. ANS concentrations were low to avoid protein coprecipitation. The intrinsic viscosity $[\eta]$, the limit of η_{sp} extrapolated to zero BSA concentration, was measured for acid expanded BSA, at a number of ANS concentrations. Concentrations of BSA ranged from 5 to 24 mg/mL (most measurements performed at 12 mg/mL). Viscosities of isoionic (pH 5.2), salt-free BSA, and of HCl-expanded BSA (19 mM HCl added, pH 2.5) were measured. In the ANS–acid–BSA interaction, viscosities were measured with three sequences of ANS addition to confirm reversibility: (1) ANS added first to BSA before acid expansion, (2) after acid expansion (HCl added before ANS), and (3) addition of isoionic BSA to an ANS–HCl mixture sufficient to arrive at the desired pH and y_{ANS} value.

Viscosities of γ -globulin, catalase, and ovalbumin solutions were measured similarly to viscosities of BSA solutions, although none but γ -globulin and BSA showed significant acid expansion.

Dynamic Light Scattering

Dynamic light scattering measurements were performed with a Lixel 95-3 model argon ion laser, 488 nm wavelength, power output 100–250 mW. Homodyne time autocorrelation functions of the photocurrent⁹ were collected with a Brookhaven Instruments BI-9000AT model correlator at a scattering angle of 90°. The data were analyzed with the CONTIN program¹⁰ to obtain the distribution of translational diffusion coefficients D .

BSA Hydrodynamic Size and Shape

The BSA molecule was modeled as three linearly connected spheres.¹¹ Spheres were moved apart from each other to model expansion, and closer together to model ANS-induced contraction. Theoretical viscosity and diffusion coefficient values were calculated with the HYDRO program of Garcia de la Torre et al.,¹² varying the distances between the three spheres until calculated values matched the measured ones most closely.

Scatchard–Black Effect: pH Shifts of BSA Solutions Induced by ANS

The Scatchard–Black effect¹³ is the production of OH⁻ ions, an alkaline shift in pH, induced by organic anion binding to protein molecules. The concentration of BSA was 16 mg/mL (0.24 mM) in 40 mM NaCl, 22 mM added HCl; initial pH was 2.6 before ANS addition. Upward shifts of pH, resulting from ANS binding, were followed as a function of y_{ANS} , the number of ANS anions added per BSA molecule. The difference between the total charges of the BSA molecule (Z_{H^+}) from the titration data¹⁴ at the start and end of each ANS addition gives the number of released hydroxyl anions ($\nu_{\text{released OH}^-}$) from water molecules per BSA molecule.

Differential Scanning Calorimetry

A Microcal MC-2 calorimeter was used to scan heat capacities of ANS–BSA complexes as a function of temperature. BSA concentration was 2 mg/mL; solvent 0.15M NaCl, 10 mM sodium phosphate buffer, pH 6.8. The reference cell contained no protein and no ANS, but simply buffer and NaCl. The temperature of the maximum heat capacity was assigned as the melting temperature T_m of the protein–ANS complex.

RESULTS

Viscometry: Acid Expansion of BSA, Contraction by ANS

Near neutral pH 5–9, BSA normally exhibits intrinsic viscosity $[\eta] = 3.7 \text{ cm}^3/\text{g}$. It fits a model of three

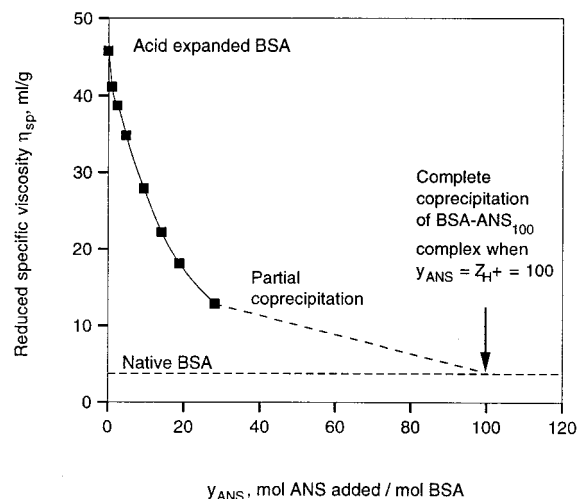


FIGURE 1 Reduced specific viscosities of acid-expanded BSA tightened by ANS dependent on y_{ANS} , relative added ANS concentration.

domains in a linear array, which separate along their linked linear axis as pH is reduced.^{11,15} A further decrease of pH in a second stage of expansion below 3.6¹⁶ starting from $[\eta] = 4.5 \text{ cm}^3/\text{g}$ produces large intrinsic viscosities reaching 20–30 cm^3/g . These $[\eta]$ values may vary considerably with added salt concentration because of chloride binding with effective neutralization of BSA protein cationic charge.¹⁷ Alternatively, $[\eta]$ may be decreased by the normal sensitivity of expanded charged protein molecules to solvent and salt penetration, involving protein intramolecular electrostatic forces.¹⁶ Unfolded, expanded BSA with intact disulfide bonds in 6M guanidine hydrochloride has $[\eta] = 23 \text{ cm}^3/\text{g}$. When all of BSA's disulfide bonds are broken and reduced, giving yet further expansion and solvent penetration, $[\eta] = 52 \text{ cm}^3/\text{g}$.¹⁸

Figure 1 shows the dependence of reduced specific viscosity η_{sp} on the relative amount of added ANS. Reduced specific viscosity values are up to two times larger than corresponding values of intrinsic viscosity. For example, $[\eta] = 27 \text{ cm}^3/\text{g}$ (at $y_{\text{ANS}} = 0$ and $[\text{BSA}]$ approaching 0 mg/mL, while $\eta_{\text{sp}} = 46 \text{ cm}^3/\text{g}$ (at $y_{\text{ANS}} = 0$ and $[\text{BSA}] = 12 \text{ mg/mL}$). When ANS is added, with y_{ANS} ranging from 0 to 30 starting from the acid-expanded form, η_{sp} decreases to values not far from those of the compact, neutral protein. The pH was maintained constant at 2.5. If relative concentrations of ANS are much higher than 30, the protein starts to aggregate and coprecipitate with ANS. The ANS anion is strongly bound in the acid pH region, such that at our added levels of ANS, $\nu_{\text{ANS}} \cong y_{\text{ANS}}$, where $\nu_{\text{ANS}} = \text{moles ANS bound/mole BSA}$. In the acid pH region, levels of bound ANS and added ANS

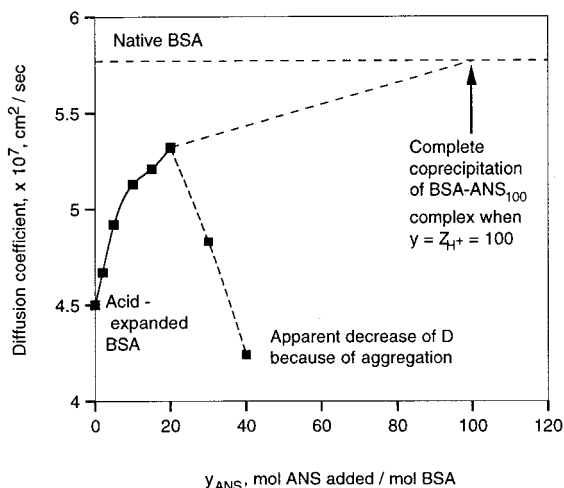


FIGURE 2 Increase in diffusion coefficients of acid-expanded BSA caused by ANS.

are quite close to one another. ANS binding in the acid region is nearly stoichiometric with respect to the number of positive charges on the protein z .³ The total number of positive charges per BSA molecule is exactly equal to 100 (from amino acid sequence). When y_{ANS} is near 100 all BSA coprecipitates, but some aggregation is observed at y_{ANS} as low as 40, thereby obstructing viscosity measurements.

Throughout this strong ANS binding region while all the components remain in solution, and $y_{\text{ANS}} \leq z$, dramatic decreases occur in reduced specific viscosities from the grossly expanded conformation. The conformational tightening, indicated by the viscosity decrease, starts at as low as $y_{\text{ANS}} \cong 3-5$. As y_{ANS} is increased, while the protein remains in solution, the reduced viscosities in Figure 1 fall below $10 \text{ cm}^3/\text{g}$, not far from the value for a compact native-like BSA. Because of the Scatchard-Black effect, ANS addition to BSA shifts the pH upward. Accordingly, additional HCl was added to maintain the pH at 2.5.

Diffusion Coefficients of BSA Expanded by Acid and Contracted by ANS

The diffusion coefficients (D) of compact BSA at neutral pH and acid-expanded BSA at pH 2.5, in 20 mM NaCl, are 5.8×10^{-7} and $4.5 \times 10^{-7} \text{ cm}^2/\text{s}$, respectively. These are in fair agreement with the values reported by Gaigalas et al.¹⁹ and Raj and Flygare²⁰ at comparable salt concentrations and pH values. Figure 2 plots D at pH 2.5 as ANS is added up to $y_{\text{ANS}} \cong 30$, after which aggregation begins. The pH was maintained constant at 2.5 by adding HCl. At even higher relative concentrations of ANS, $y_{\text{ANS}} \cong$

100, BSA and ANS completely coprecipitate. The increase in D with y_{ANS} , indicating a decrease in hydrodynamic volume, is consistent with ANS-induced tightening of BSA from an expanded to a more compact, less hydrated state.

Expansion of γ -Globulin Molecule by Acid and Contraction by ANS

The γ -globulin protein molecule in neutral pH has a reduced specific viscosity close to that of BSA, $\sim 4 \text{ mL/g}$. On addition of HCl, the reduced specific viscosity of γ -globulin solution increases to 13 mL/g at pH 2.0. This is less than the tenfold increase in η_{sp} of BSA in going from compact to maximally expanded form, but it is a large increase nonetheless. γ -Globulin molecules have a smaller mole fraction of cationic charges relative to BSA. Figure 3 shows the dependence of reduced specific viscosity η_{sp} of acid-expanded γ -globulin solution (constant pH 2.0) on the relative amount of added ANS, y_{ANS} . As ANS anions are added to initially expanded γ -globulin in the range where ANS binds by sulfonate anion pairing to protein cationic groups, η_{sp} steadily decreases up to the point of overt aggregation near $y_{\text{ANS}} \sim 40$. The most contracted ANS- γ -globulin complexes achieve η_{sp} values quite close to the native compact form.

Viscosity of ovalbumin solution did not increase appreciably upon addition of acid, indicating that its expansion at pH as low as 2.0 is insignificant. Catalase expanded slightly, but even a small addition of ANS caused it to aggregate. Hence, investigation of ANS-induced conformational tightening was not feasible with these proteins.

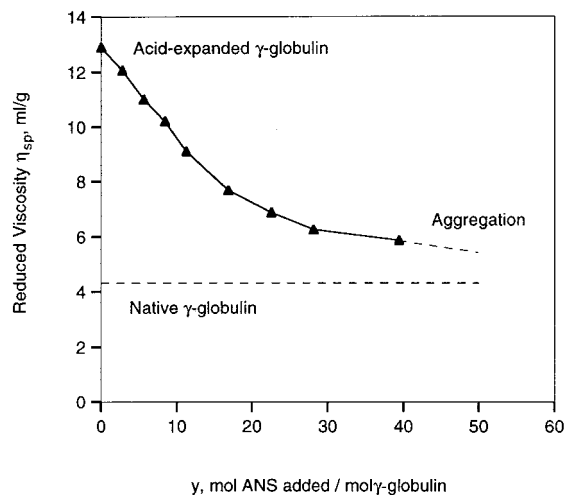


FIGURE 3 Reduced specific viscosities of acid-expanded γ -globulin in response to relative ANS concentration, y_{ANS} .

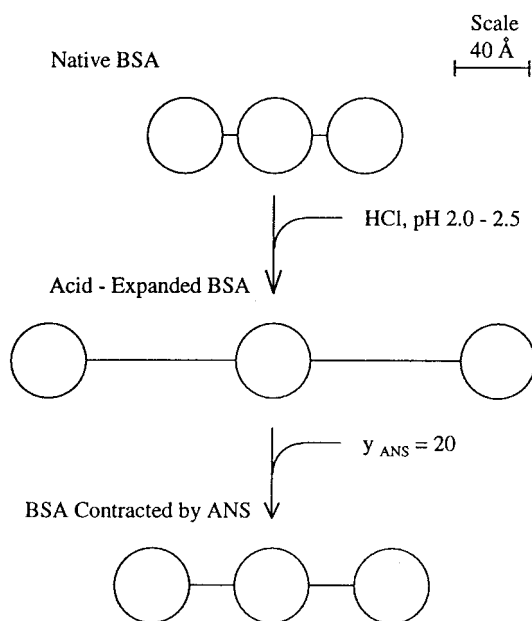


FIGURE 4 Schematic illustration of BSA expansion by acid and contraction by ANS in acid.

Conformational Modeling of Hydrodynamic Changes of BSA

To calculate D and $[\eta]$ with the HYDRO program,¹² BSA was modeled as three spheres, which for simplicity were chosen to be linearly arranged and of equal radius. Similar models, reviewed by Peters,²¹ have been used recently in connection with other techniques for estimating BSA size and shape. The best fits of measured and calculated viscosities and diffusion coefficients were obtained with a sphere radius of 20 Å. In native BSA the center-center distance of adjacent spheres is 50 Å. In acid-expanded BSA the center-to-center spacing becomes 125 Å, while in BSA, contracted by ANS ($y_{\text{ANS}} = 20$) at pH 2.5, the spheres approach to 68 Å spacing. These models are drawn to scale in Figure 4, with values given in Table I. HYDRO generally does not calculate $[\eta]$ as accurately as D , but both hydrodynamic quan-

ties agree that BSA at pH 2.5 exists in an extended or expanded state with large hydrodynamic volume. Addition of ANS, forming ion pairs with even part of the available cationic sites, immediately produces major contraction.

Scatchard-Black Effect: pH Shifts of BSA Solutions Induced by ANS

Strongly binding anions, particularly organic anions that hardly can become protonated themselves (such as organic sulfonate anions¹³), produce considerable upward pH shifts when these anions bind to the protein molecule. The probable mechanisms are discussed below. The observed pH shifts, ΔpH , are several tenths of a pH unit in magnitude and more when y_{ANS} ranges above about 30 ligands/protein molecule starting from acid pH ranges. Using the detailed titration data for BSA by Tanford et al.¹⁴ and the measured ΔpH shifts generated by ANS binding to BSA, the numbers of OH^- released per protein molecule as a function of added ANS, y_{ANS} , are plotted in Figure 5. They are compared with the number of ANS bound per BSA molecule, ν_{ANS} . Initially, when modest amounts of ANS are added, $y_{\text{ANS}} \leq 40$, the pH shifts and the released amounts of OH^- are relatively small. However, through this lower range of ANS addition, the protein molecule's major hydrodynamic changes, conformation tightening and average radii decreases take place. As y_{ANS} is increased much above 50 to a region where nearly one ANS anion is provided per BSA molecule's original positive charge, $Z_{\text{H}^+} \sim 100$, large upward pH shifts were observed. In this region of y_{ANS} , 60–100, ν_{OH^-} approaches one OH^- generated per added ANS anion. The slope of OH^- released vs y_{ANS} steepens, approaching unity in the range where y_{ANS} is about 70–100. Further addition of ANS anions, beyond $y_{\text{ANS}} = 100$, where the protein molecule is neutralized, brings the complex to the verge of coprecipitation. Such coprecipitated complexes form in constant composition, 100 ANS anions bound per BSA molecule of maximal total charge

Table I Comparison of Measured (Expt) and Calculated (Calc) Diffusion Coefficients (D) and Viscosities of BSA($[\eta]$) at $y_{\text{ANS}} = 20$

Form of BSA	Center-to-Center Distance, Å	$D, 10^{-7} \text{ cm}^2/\text{s}$		$[\eta], \text{ mL/g}$	
		Expt	Calc	Expt	Calc
Native	50	5.8	5.9	3.7	5.9
Acid-expanded	125	4.5	4.5	27	22
ANS-contracted	68	5.3	5.3	10	8.6

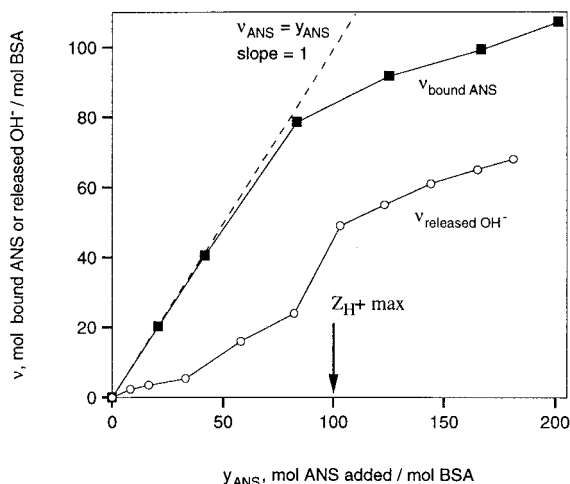


FIGURE 5 ANS binding to BSA (ν_{ANS} , mol ANS bound/mol BSA) determined by dialysis equilibrium (■; no additional acid was added, the pH was not maintained constant at 2.5); the number of released hydroxyl anions from water per BSA molecule (○) as function of relative ANS concentration (y_{ANS}) in the Scatchard–Black Δ pH effect.

+100. Equilibrium dialysis shows that the slope of ν_{ANS} bound vs y_{ANS} added is nearly unity through the region of ANS addition up to a point where the BSA molecule must be nearly saturated (Figure 5).

ANS Stabilization of BSA in Differential Scanning Calorimetry

Melting temperatures T_m of native-like, nonexpanded BSA as a function of ANS addition up to $y_{ANS} = 30$ at pH 6.8, are plotted in Figure 6. Addition of ANS raises T_m sharply at relatively low ratios, $y_{ANS} = 5$ –7, beyond which a more gradual increase in T_m occurs. Increases of the T_m of the protein–ligand complex relative to the T_m of the protein without ligands is evidence that protein is stabilized by bound ligands. ANS binds more strongly to the native state of BSA than to the temperature-unfolded state. Similar behavior of ribonuclease stabilization by bound ligand was described by Brandts and Lin.²²

DISCUSSION

ANS binding to two proteins, BSA and γ -globulin, under conditions where the protein molecule conformationally expands by acid is not a simple reaction in which ANS associates with a preformed, optimally shaped binding site. A conformational tightening of the protein is triggered by ANS: a shrinkage of hy-

drodynamic volume large enough to be readily measured as shown in Figures 1–3.

Binding of ANS to proteins comes from both its nonpolar anilinonaphthalene and the negatively charged sulfonate groups. ANS binds stronger to BSA than inorganic anions. Enhanced binding is at least partially ascribable to the large anilinonaphthalene organic moiety of ANS. It was recently shown that, for the 100 potential sites on BSA capable of binding ANS, the dominant factor that drives binding is ion pair formation.³ Only about 5 of the 100 ANS binding sites, 5% of the total, produce ANS fluorescence, in agreement with the results of Daniel and Weber.²³ It is important to realize that ANS fluorescence may not require immersion of the anilinonaphthalene group in hydrophobic environment inside the protein. It requires only removal of the ANS organic moiety from water, which is an effective quencher of ANS fluorescence.⁴ The intensity, quantum yield, and lifetime of ANS fluorescence depend on the geometry of the phenyl ring twist about the bond connecting it to the naphthalene moiety, as also discussed by Kirk et al.⁴

We conclude that there are at least two modes of ANS interaction with proteins. These are schematically illustrated in Figure 7. In the first mode, ANS has its anilinonaphthalene group located so that it is mostly or completely occluded from water quenching and therefore fluorescent. There are five such ANS anions bound to BSA. In the second mode, ANS has its anilinonaphthalene group located such way that it is mostly or completely exposed to water quenching and therefore nonfluorescent. There are up to about 95 such ANS anions bound to BSA.

Similar to the number of ANS anions bound to bovine serum albumin in the first mode, there are

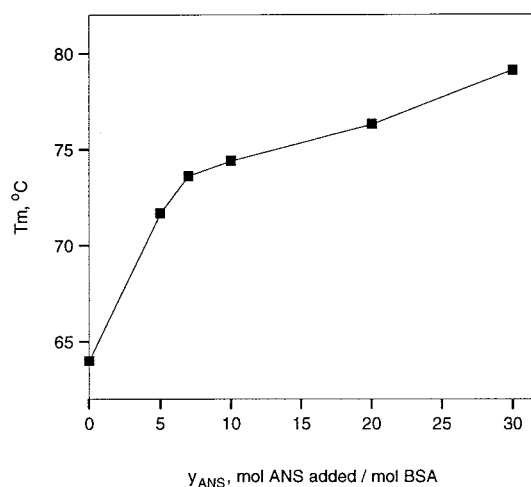


FIGURE 6 Increase in the temperature of BSA denaturation (T_m) upon addition of ANS.

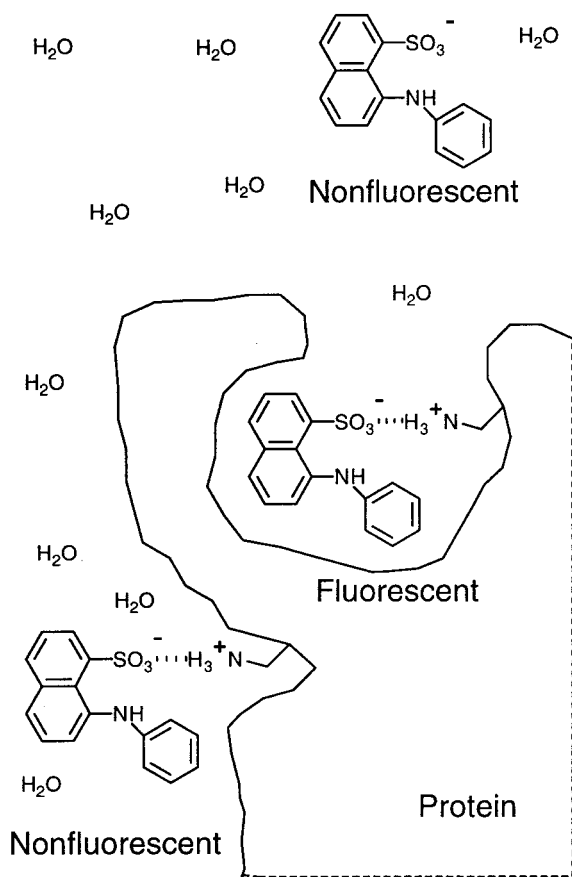


FIGURE 7 Two modes of ANS anion binding to proteins. In the first mode, the anilinonaphthalene group of bound ANS anion is occluded from quenching by water. These ANS are fluorescent. There are 5 such binding sites on BSA molecule. In the second mode, the anilinonaphthalene group of bound ANS is accessible to water quenching. There are 95 such binding sites on BSA molecule, and these ANS anions are nonfluorescent. Conformational tightening results from ANS anions bound in both modes. All 100 ANS anions are bound by ion pairs between the sulfonate groups and the positively charged amino acid side chains.

approximately 2–5 sites for several kinds of nonionic nonpolar molecules. For example, 1–4 butane molecules bind to BSA depending on pH.^{24,25} Westphal²⁶ reviewed over thirty papers on binding of testosterone, progesterone, androstanes, and other steroids to bovine and human serum albumin. All results centered around 2–5 nonionic steroids bound/protein molecule. Boman and Lovrien (unpublished observations) measured 1–5 uncharged polynuclear aromatic hydrocarbons, phenanthrene, naphthalene, and biphenyl, bound to serum albumin. Only in some of these studies were conformational changes accompanying neutral hydrocarbon or steroid binding looked for. However, when they were monitored (e.g., butane gas

vs nitrogen gas binding to BSA²⁵), viscometry and Scatchard–Black pH shifts indicated an ability of bound neutral molecules to generate appreciable conformational responses in the direction of tightening. Wishnia²⁷ assigned some of the thermodynamics and specificity for binding neutral hydrocarbons to induced fitting reactions²⁸: mutual accommodation of neutral hydrocarbon and target protein nonpolar groups to one another, similar to the action of large organic anions. That is, conformation changes are part of the binding process.

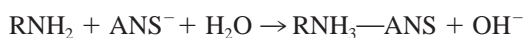
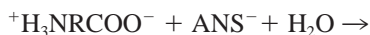
It appears from these earlier results that ion pair formation may not be necessary for the ANS anions bound in the first mode. However, the strong anionic properties of organic sulfates and sulfonates greatly increase their ability to bind and change protein conformation in response to the way in which pH controls protein titration charge. The enthalpies of ANS binding in the first mode are very similar to the enthalpies in the second mode of binding.³ Therefore it appears that ANS anions, which are bound in the first mode (fluorescing), are also bound by ion pairs. ANS binding first occurs here because ion pairs are stronger in cavities with lower dielectric constants.²⁹

Numbers of fluorescent ANS anions bound to BSA in the first mode increase from 5 to about 12 when BSA is expanded by acidic pH change from 4 to 2.³ Expansion of BSA molecule opens more ANS binding sites where the anilinonaphthalene group is occluded from water quenching. Binding of these 12 ANS anions is accompanied by the conformational change. Therefore binding occurs through the mechanism of induced fit rather than through the mechanism of conformationally static lock and key.

ANS anions, which are bound in the second mode, act as strong matrix-forming protein-coprecipitating agents. Their behavior is similar to that of many other organic sulfonate and sulfate anions. Matrix ligands are characterized by a set of properties including strong binding, induced conformational change, and coprecipitation.^{5,6} Coprecipitation in this manner requires association between ligand nonpolar tails, to draw protein–ANS complexes together. Matrix ligands also displace water from protein molecules, which helps drive the conformational tightening from the highly expanded form. Matrix ligands upon binding to proteins produce large Scatchard–Black effects, increase protein melting temperatures in solution, generate large exothermic binding enthalpies in solution, coprecipitate proteins, and exhibit pH-dependent behavior that correlates with protein titration charge.

The Scatchard–Black effect, an increase in pH accompanying strong organic anion binding, is a convenient experimental tool for monitoring the binding

process. Such pH shifts occur from ion pair formation between ANS and positively charged protein amino acid side chains, or by protonation of a carboxylate group, depending on the pH. A water molecule is hydrolyzed in the process.



The protein molecule binds protons in order to neutralize the effect of negatively charged ANS anions. However, the number of bound protons is only about half of bound ANS anions. Therefore overall charge of the protein molecule becomes less positively charged. Weaker electrostatic repulsion between positive charges enables protein molecules to shrink, and conformationally tighten.

These considerations, based on pH changes, differential scanning calorimetry, and hydrodynamic measurements, support related conclusions reached by Engelhard and Evans⁷ based on spectroscopic, kinetic studies of ANS-protein interaction—namely, that ANS may have marked effects on protein conformation, and does not bind to a merely static protein molecule.

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