Binding of 8-Anilinonaphthalene Sulfonate to Dimeric and Tetrameric Concanavalin A: Energetics and Its Implications on Saccharide Binding Studied by Isothermal Titration Calorimetry and Spectroscopy

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Received: November 2, 2005; In Final Form: December 13, 2005

The binding of 8-anilinonaphthalene sulfonate to concanavalin A has been investigated. Isothermal titration calorimetry (ITC) and circular dichroism studies have been performed under different experimental conditions to understand the binding quantitatively and evaluate contributions of different forces responsible for it. Isothermal titration calorimetric results of concanavalin A with ANS at pH 5.2 and 2.5, where it exists as a dimer, indicated binding heterogeneity and two classes of noninteracting sites. Enhancement of the binding constants from native to pH 2.5 suggests that the ANS binding is strongly influenced by the protein charge and the favorable alteration in the structure of concanavalin A as suggested by near-UV CD results. No binding was observed with the tetrameric form of concanavalin A, indicating shielding of sites due to dimerization of canonical dimers. The results have also demonstrated existence of a hydrophobic binding site that is distinct from the saccharide binding site.

1. Introduction

8-Anilinonaphthalene-1-sulfonate (ANS), a charged hydrophobic fluorescent molecule, has been extensively used as an extrinsic probe for monitoring the protein conformation. The extensive use of this fluorophore is due to the dramatic enhancement of its fluorescence[1] and the shift of the emission maximum to shorter wavelengths when surrounded by nonpolar amino acid residues of proteins. 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, [2] in the detection of molten globule intermediates,[3] as nonpolar surface patches of proteins, and many more. ANS has also been used recently to investigate the structural and physicochemical characteristics of the aggregates of transthyretin (TTR) to provide insights into the mechanism of protein misfolding and misassembly.[4]

Currently, ANS is being used as a standard probe for investigating the population of compact partially folded intermediate states of proteins.[5−11] We recently demonstrated the use of ITC in characterizing the partially folded state of the protein.[12] Hydrophobicity is no longer considered to be the only determinant of the fluorescence enhancement or the sole driving force of ANS binding to proteins. The importance of electrostatic interactions between the sulfonate group of ANS and positive charges on the polypeptide has been clearly demonstrated.[13]

Interest in understanding the different protein conformations led us to investigate ANS−concanavalin A interactions with particular attention to its dependence upon the solution pH. A good deal is known about the static structure of concanavalin A. Concanavalin A is a lectin isolated from jack beans that exists as a tetramer at physiological pH and as a dimer at pH values less than 6. Each monomer (M_r = 26 500) possesses one saccharide binding site as well as a transition-metal ion site S1 that typically binds Mn^{2+} and a site S2 that binds Ca^{2+}.[14]

The three-dimensional structure of the lectin at 1.75 Å resolution has been determined by X-ray diffraction analysis[15] and further refined at 1.2 Å.[16]

Isothermal titration calorimetry (ITC) and circular dichroism experiments have been performed under different experimental conditions as a function of temperature and pH to understand the binding quantitatively and evaluate contributions of different forces responsible for it. Isothermal titration calorimetric results of binding of ANS with concanavalin A at pH values where the protein exists as a dimer indicated binding heterogeneity with two classes of noninteracting sites. To understand the nature of the binding site and the implication of ANS binding on the affinity of the protein for saccharide, we also tried to rationalize the data in terms of estimates of comparative binding of ANS to the native and maltose-bound lectin. These results suggest the possible use of ANS−con A complexes as markers for analysis of the dynamics and flexibility of cell surface receptors and the behavior of surface glycoproteins.

2. Materials and Methods

Concanavalin A, sodium acetate, sodium chloride, glycine, 2,2,2-trifluoroethanol, sodium chloride, and ANS of best available purity grade were purchased from Sigma-Aldrich Chemical Co. Manganese chloride and calcium chloride were extra pure analytical reagent grade and obtained from Merck limited. The mass fraction purity of TFE checked by gas chromatography was 0.99. A Sartorius BP 211D digital balance of readability ±0.01 mg was used for the mass measurements. The water used for preparing the solutions was double distilled and then deionized using a Cole-Parmer research mixed-bed ion-exchange column. The protein was dialyzed extensively against 20×10^{-3} mol dm^{-3} glycine−HCl at pH 2.5, sodium acetate at pH 5.2, HEPES at pH 7.2 containing 1×10^{-3} mol dm^3 calcium chloride, and 0.1 mol dm^3 manganese chloride with at least four changes of the buffer. The reported pH is that of the dialysate measured on a Standard Control Dynamics pH meter at room

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10.1021/jp0563179 CCC: $33.50
Published on Web 03/09/2006

temperature. The concentration of concanavalin A was determined spectrophotometrically on a Shimadzu double beam spectrometer UV 265 at 280 nm using $A^{1\%,1cm} = 12.4$ for concanavalin A at pH 2.5 and 5.2 and $A^{1\%,1cm} = 13.7$ at pH 7.2 and expressed in terms of monomer.[17]

2.1. Isothermal Titration Calorimetry. ITC measurements were carried out on a VP− ITC titration calorimeter (Micro Cal, Northampton, MA). Before loading the solutions were thoroughly degassed. The reference cell was filled with the respective degassed buffer. The protein was kept in the sample cell, and aqueous ANS was filled in the syringe of volume 250 μL. The ANS solution was added sequentially in 10 μL aliquots (for a total of 25 injection, 20 s duration each) at 4 min intervals. Sequential titrations were performed to ensure full occupancy of the binding sites by loading and titrating with the same ligand without removing the samples from the cell until the titration signal was essentially constant. The titrations were linked together for data analysis using ConCat32 software distributed from MicroCal, Inc. The heats of dilution were determined in parallel experiments by injecting (i) 7.5×10^{-3} mol dm^{-3} solution in buffer, (ii) buffer solution in to 0.15×10^{-3} mol dm^{-3} con A, and (iii) buffer into buffer solution. The respective heats of dilution were subtracted from the corresponding con A−ANS experiments prior to curve fitting. Origin 5.0 software was used to fit the thermodynamic parameters to the heat profiles. The binding of ANS to concanavalin A was analyzed using two classes of independent binding sites

$$\text{con A} + n_1\text{ANS} = [\text{conA}\cdot n_1\text{ANS}] \quad (1)$$

$$\text{con A}\cdot n_1\text{ANS} + n_2\text{ANS} = [n_2\text{ANS}\cdot\text{conA}\cdot n_1\text{ANS}] \quad (2)$$

where n_1 and n_2 represent the number of moles of ANS molecules bound to the first and second set of sites, respectively.

This model defines the equilibrium association constants as

$$K_1 = \theta_1/(1 - \theta_1)L \quad (3)$$

$$K_2 = \theta_2/(1 - \theta_2)L \quad (4)$$

θ is the fractional ligand occupancy

$$L_t = L + M_1(n_1\theta_1 + n_2\theta_2) \quad (5)$$

where L_t is the total ligand concentration, K is the apparent association constant

$$L_t = L + n_1M_tLK_1/(1 + LM_t) + n_2M_tLK_2/(1 + LK_2) \quad (6)$$

The Q after any injection i is then expressed as

$$Q_i = M_tV_0(n_1\theta_1\Delta H_1 + n_2\theta_2\Delta H_2) \quad (7)$$

Binding models were fit to the data by a nonlinear least-squares Marquardt algorithm until constant χ^2 values were achieved.

To test the possibility of overlapping sites we performed competition experiments in which the protein is titrated with maltose to near saturation, followed by titration with ANS. To evaluate role of hydrophobic interactions in lectin−sugar binding we first titrated protein with ANS to near saturation followed by titration with maltose. In parallel experiments the respective heats of dilution were also determined.

2.2. Circular Dichroism Experiments. The CD experiments were performed on a Jasco-810 CD spectropolarimeter. The protein concentration and path length of the cell used were 10 μM and 0.1 cm for far-UV CD and 30 μM and 1 cm for near-UV CD measurements. The spectropolarimeter was purged with N_2 prior to the experiment. Each CD plot was an average of three accumulated plots. The plots were baseline corrected. The molar ellipticity was calculated from the observed ellipticity θ as $100\theta/cl$ where c is the concentration of the protein solution in molarity and l is the path length of the cell in centimeters.

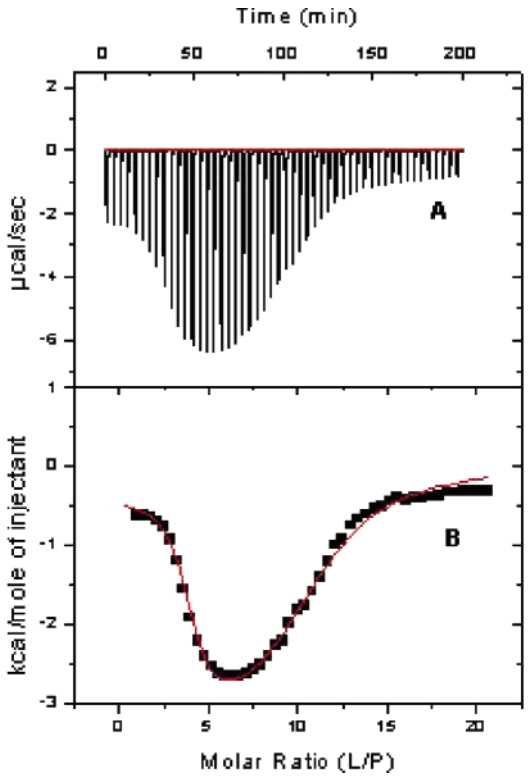

Figure 1. Titration of 7.5×10^{-3} mol dm^{-3} ANS with 0.15×10^{-3} mol dm^{-3} concanavalin A at pH 5.2 and 298.15 K, showing the calorimetric response as successive injections of ligand are added to the reaction cell. (B) Isotherm of the calorimetric titration shown in A. In B the solid curve represents the best nonlinear least-squares fit to a two-site model.

2.3. Fluorescence Experiments. The fluorescence experiments were done on a Perkin-Elmer LS-55 spectrofluorimeter. The protein concentration in all the experiments was kept at 12.8×10^{-6} mol dm^{-3}. The excitation and emission slit widths were fixed at 5 nm. The ANS concentration was varied from 0.1 to 2.25×10^{-3} mol dm^{-3} using an extinction coefficient of ANS as $E_{350}^{1\%} = 5000$ M^{-1} cm^{-1}.

3. Results

3.1. Calorimetric Titrations of ANS with Concanavalin A at pH 5.2. Representative calorimetric titration of ANS with concanavalin A at pH 5.2 is shown in Figure 1. Each peak in the binding isotherm (Figure 1A) represents a single injection of ANS solution. The negative deviations of the heat signal from the baseline on addition of ANS indicate that heat was evolved. As the titration progresses, the area under the peaks progressively becomes smaller due to an increased occupancy of binding sites on concanavalin A with ANS molecules. In Figure 1B the plot of the amount of heat generated per injection as a function of the molar ratio of ligand to protein is shown. A model of two independent sites fits adequately to the calorimetric data. The enthalpy change associated with each injection of ANS was plotted against the ANS/concanavalin A molar ratio, and $\Delta H°$, $\Delta S°$, and K_a were obtained. The smooth solid line shown in Figure 1B is the best fit to the experimental data according

TABLE 1: Binding Affinities *K*, Enthalpies ΔH, Entropies ΔS, and Stoichiometry of Binding *n* Accompanying Binding of ANS to Concanavalin A at Temperature *T* and pH 5.2[a]

T (K)	K_1 (M^{-1})	n_1	ΔH_1 (cal mol^{-1})	ΔS_1 (cal K^{-1} mol^{-1})	K_2 (M^{-1})	n_2	ΔH_2 (cal mol^{-1})	ΔS_2 (cal K^{-1} mol^{-1})
283.15	$(2.9 \pm 0.6) \times 10^5$	11.3 ± 0.1	-48.6 ± 6.8	24.9 ± 0.2	$(4.7 \pm 0.8) \times 10^3$	11.2 ± 0.4	-1055 ± 57	13.1 ± 0.9
288.15	$(2.5 \pm 0.7) \times 10^5$	11.3 ± 0.2	-71.5 ± 12.3	24.4 ± 0.1	$(4.4 \pm 0.8) \times 10^3$	11.0 ± 0.7	-1628 ± 57	11.0 ± 0.5
293.15	$(2.2 \pm 0.5) \times 10^5$	13.1 ± 0.2	23.3 ± 16.6	24.5 ± 0.4	$(3.5 \pm 0.5) \times 10^3$	12.6 ± 0.7	-2110.79 ± 164	9.0 ± 0.1
298.15	$(1.7 \pm 0.4) \times 10^5$	16.6 ± 0.3	30.9 ± 17.2	24.0 ± 0.4	$(4.1 \pm 0.7) \times 10^3$	16.0 ± 0.8	-1954 ± 153	10.0 ± 0.3
303.15	$(6.5 \pm 0.1) \times 10^4$	17.3 ± 0.5	130.1 ± 23.0	22.5 ± 0.2	$(4.8 \pm 0.8) \times 10^3$	20.2 ± 1.1	-1557 ± 131	11.7 ± 0.2
308.15	$(2.4 \pm 1.3) \times 10^4$	20.6 ± 0.6	355.2 ± 177.0	21.3 ± 0.6	$(5.1 \pm 0.6) \times 10^3$	22.4 ± 0.9	-2298 ± 698	9.4 ± 0.5

[a] 1 cal = 4.184 J.

to two independent binding site models. The model with a single binding site or three independent binding sites did not fit the experimental data. As can be observed from Table 1, binding was more favorable at lower temperature at the first binding site whereas it remained unchanged for the second binding site. Binding constant K_a for the first site is higher than that at the second binding site. The values of enthalpy change for the first binding site at temperatures of 283.15 and 288.15 K were found to be slightly negative, whereas at the other temperatures they were positive.

Binding of ANS at first site seems to be driven primarily by an entropic contribution. To assess the molecular nature of the changes that occur upon ANS binding to protein, the values of heat capacity (ΔC_p) were calculated from the slope of a linear plot of measured ΔH versus temperature for the first binding site. The value of ΔC_p thus calculated is $\Delta C_P = 15.01 \pm 3.6$ cal mol^{-1} K^{-1} for the first site.

The number of ANS molecules binding to protein (stoichiometry of binding) increases with increasing temperature. Whereas binding constant K_a for the second class of binding site remains unaffected with the increase in temperature, binding appears to be more enthalpically driven at this site.

3.2. Calorimetric Titrations of ANS with Concanavalin A at pH 2.5. The representative calorimetric titrations of ANS with concanavalin A at pH 2.5 are shown in Figure 2.

As can be seen from the Table 2, binding at this pH value was more favorable at lower temperature. A positive change in heat capacity ($\Delta C_P = 7.44 \pm 1.73$ cal K^{-1} mol^{-1}) was calculated from the slope of a plot of ΔH against *T*. The binding at the second site at 308.15 K appears to be entropically opposed.

A negative change in heat capacity ($\Delta C_P = -60 \pm 12.4$ cal mol^{-1} K^{-1}) was calculated from the slope for the second class of site. The number of ANS molecules bound to protein (stoichiometry of binding) remained constant with increasing temperature.

3.3. Calorimetric Titrations of ANS with Concanavalin A at pH 7.2. Figure 3 shows the isothermal titration calorimetric profile for the titration of ANS with concanavalin A at pH 7.2. The negative deviation from the stable baseline upon addition of ANS was very small toward the negative side, indicating that the process is slightly exothermic.

The enthalpy change associated with each 10 μL injection of ANS plotted against the ANS to concanavalin A molar ratio is shown in Figure 3B. No variation in the heat evolved with the increase in the molar ratio was observed.

3.4. Protein Conformation at Different pH Values. The near-UV CD spectra of native concanavalin A are shown in Figure 4. The aromatic CD spectra of concanavalin A at pH 5.2 and 7.2 showed two positive bands with maxima at 250 and 320 nm, the phenyl alanine bands at 262−268 nm, and tryptophan and tyrosine bands at 282−291 nm. The spectrum at pH 7.2 shows less positive ellipticity than that at pH 5.2, whereas at pH 2.5 the positive bands are not prominent. The difference in the near-UV CD spectra reflects local environment differences in the vicinity of tyrosine and tryptophan residues. The far-UV CD spectra of these samples showed very minor variations.

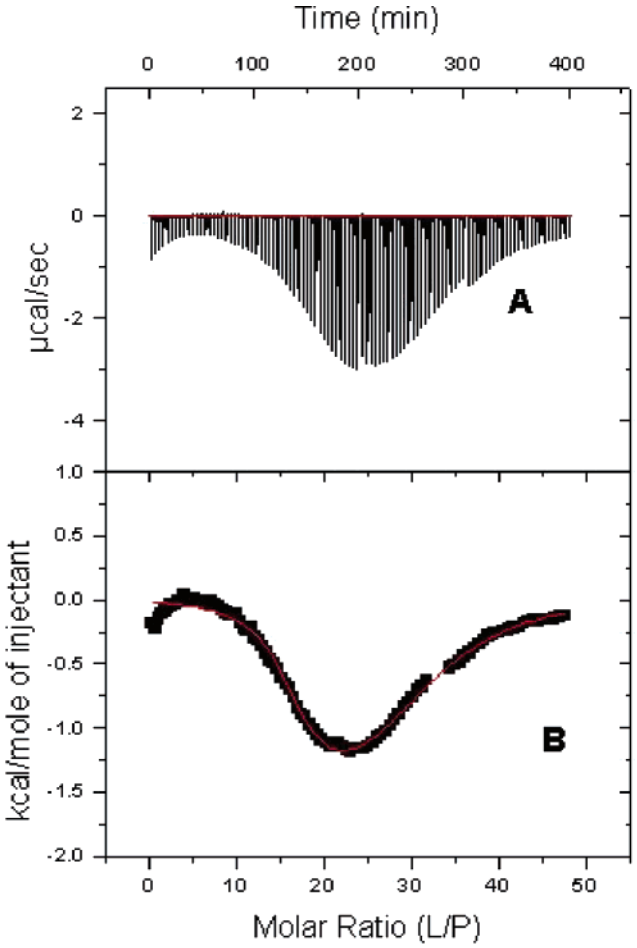

Figure 2. Titration of 7.5×10^{-3} mol dm^{-3} ANS with 0.15×10^{-3} mol dm^{-3} concanavalin A at pH 2.5 and 298.15 K, showing the calorimetric response as successive injections of ligand are added to the reaction cell. (B) Binding isotherm of the calorimetric titration shown in A. In B the solid curve represents the best nonlinear least-squares fit to a two-site model.

3.5. Calorimetric Titrations of ANS with Maltose-Bound Concanavalin A at pH 5.2. Figure 5 compares the binding isotherms of ANS with 0.15×10^{-3} mol dm^{-3} concanavalin A in the absence and presence of maltose. The exothermic heat pulses caused by the binding of ANS were reduced in the presence of maltose. Binding of ANS to concanavalin A in the presence of maltose seems to produce a conformational change at the second binding site (Table 3), leading to a reduction in the stoichiometry and entropy of binding.

3.6. Calorimetric Titrations of Maltose with ANS-Bound Concanavalin A at pH 5.2. Figure 6 shows the binding

TABLE 2: Binding Affinities *K*, Enthalpies Δ*H*, Entropies Δ*S*, and Stoichiometry of Binding *n* Accompanying the Binding of ANS to Concanavalin A at Temperature *T* and pH 2.5[a]

T (K)	K_1 (M^{-1})	n_1	ΔH_1 (cal mol^{-1})	ΔS_1 (cal K^{-1} mol^{-1})	K_2 (M^{-1})	n_2	ΔH_2 (cal mol−^{1})	ΔS_2 (cal K^{-1} mol^{-1})
283.15	$(2.1 \pm 1.1) \times 10^5$	2.5 ± 0.1	-370.6 ± 98.8	27.7 ± 1.1	$(3.7 \pm 0.8) \times 10^4$	6.5 ± 0.3	-2520 ± 99.7	2.0 ± 0.7
288.15	$(6.4 \pm 2.0) \times 10^5$	3.5 ± 0.1	-398.8 ± 83.6	25.2 ± 0.5	$(1.2 \pm 0.1) \times 10^4$	7.9 ± 0.2	-2700 ± 90.6	9.4 ± 0.4
293.15	$(5.7 \pm 1.6) \times 10^5$	3.4 ± 0.2	-379.0 ± 55.4	25.0 ± 0.2	$(1.2 \pm 0.2) \times 10^4$	5.1 ± 0.2	-3327 ± 172	7.3 ± 0.2
298.15	$(5.1 \pm 1.5) \times 10^5$	3.8 ± 0.1	-382.3 ± 80.7	24.8 ± 0.3	$(9.3 \pm 1.1) \times 10^3$	7.1 ± 0.2	-3620 ± 149	6.0 ± 0.5
303.15	$(5.0 \pm 1.1) \times 10^5$	4.4 ± 0.2	-304.8 ± 54.8	25.1 ± 0.5	$(9.1 \pm 0.9) \times 10^3$	8.1 ± 0.3	-3690 ± 137	5.9 ± 0.2
308.15	$(5.8 \pm 2.7) \times 10^4$	6.4 ± 1.5	-250.6 ± 109.0	21.0 ± 0.2	$(3.3 \pm 0.8) \times 10^3$	7.6 ± 1.4	-8909 ± 101	-12.8 ± 0.4

[a] 1 cal = 4.184 J.

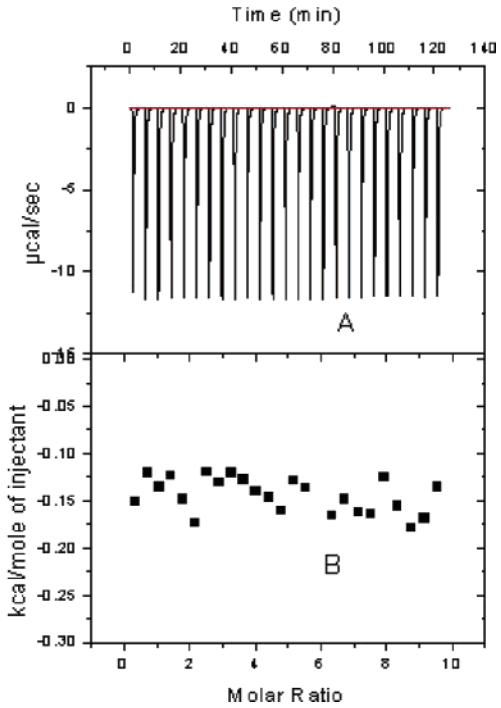

Figure 3. Titration of 7.5×10^{-3} mol cm^{-3} ANS with 0.15×10^{-3} mol dm^{-3} concanavalin A at pH 7.2 and 298.15 K, showing the calorimetric response as successive injections of ligand are added to the reaction cell. (B) Isotherm of the calorimetric titration shown in A.

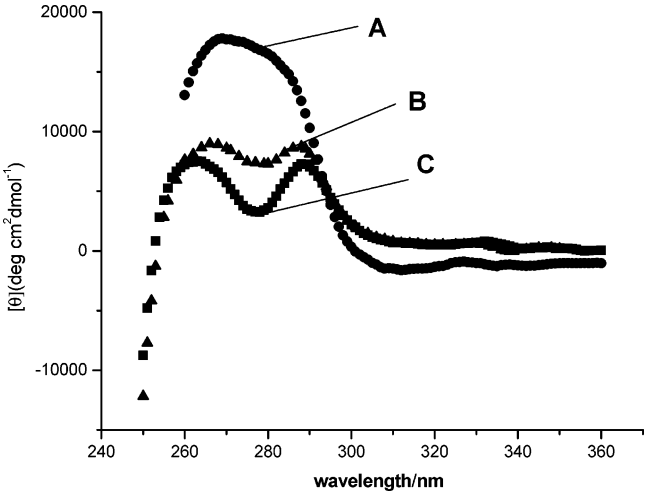

Figure 4. Near-UV CD spectra of 20×10^{-6} mol dm^{-3} concanavalin A pH (A) 2.5, (B) 5.2, and 7.2 (C).

isotherm of maltose with concanavalin A in the absence and presence of ANS. As seen from Table 4, there is no significant change in the binding affinity of maltose to concanavalin A in the presence of ANS. However, in the presence of maltose, heat

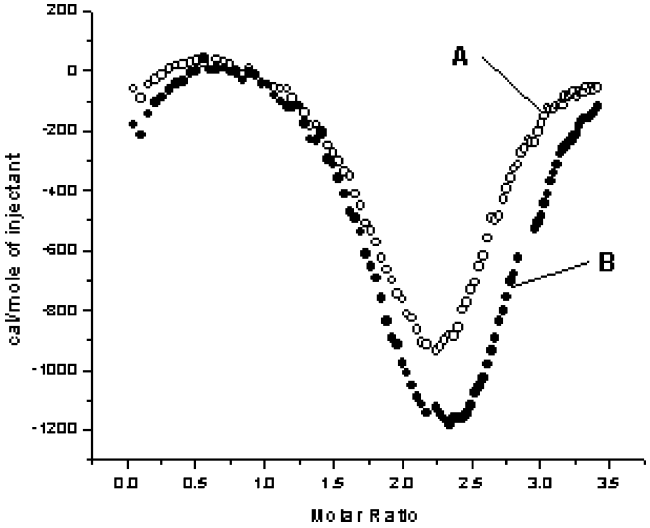

Figure 5. Binding isotherm for the titration of 7.5×10^{-3} mol dm^{-3} ANS with 0.15×10^{-3} mol dm^{-3} concanavalin A in the presence (A) and absence (B) of 8.5×10^{-3} mol dm^{-3} maltose at 298.15 K.

pulses are less exothermic and favorable entropy is offset by less favorable enthalpy.

4. Discussion

8-Anilinonaphthalene-1-sulfonate is a much utilized fluorescent "hydrophobic probe" for examining the nonpolar character of proteins and membranes. It is commonly used in studies of protein denaturation as an indicator of allowed penetration to the protein core. It is also known to bind amyloid fibrils.[18]

Con A is particularly useful for the study of the interactions of fluorescent probes with proteins because both its amino acid sequence and its three-dimensional structure at atomic resolution are known.[14]

In this work we studied the interaction of ANS with concanavalin A under different experimental conditions. Isothermal titration calorimetric results have demonstrated that ANS binds to concanavalin A at pH 2.5 and 5.2, where it exists as a dimer and displays heterogeneity with two classes of noninteracting binding sites as shown by eqs 1 and 2. The results also indicate that ANS does not bind to tetrameric concanavalin A.

4.1. Binding of ANS with Concanavalin A at pH 5.2. At all the studied temperatures at pH 5.2 for the first binding site Δ*S*° values contributed favorably to the free energy of binding, indicating a strong hydrophobic contribution to the binding process. A small positive value of heat capacity change accompanying ANS binding at the first site in the protein indicates that a slight conformational change takes place upon binding, leading to exposure of hydrophobic residues. Possible reasons for a change in heat capacity upon binding are (1) conformational changes in protein or ligand, (2) changes in ionization,[19−21] (3) changes in the water network in the binding

TABLE 3: Binding Affinities *K*, Enthalpies ΔH, Entropies ΔS, and Stoichiometry of Binding *n* Accompanying the Binding of ANS to Concanavalin A in the Absence and Presence of 8.5×10^{-3} mol dm^{-3} Maltose[b] at Temperature *T* and pH 5.2[a]

T (K)	K_1 (M^{-1})	n_1	ΔH_1 (cal mol^{-1})	ΔS_1 (cal K mol^{-1})	K_2 (M^{-1})	n_2	ΔH_2 (cal mol^{-1})	ΔS_2 (cal K^{-1} mol^{-1})
298.15	$(1.7 \pm 0.4) \times 10^5$	16.6 ± 0.3	30.9 ± 8.2	24.0 ± 0.4	$(4.1 \pm 0.7) \times 10^3$	16.0 ± 0.7	-1954 ± 115	10.0 ± 0.7
298.15[b]	$(4.3 \pm 0.20) \times 10^4$	18.5 ± 0.5	2.8 ± 0.6	21.2 ± 0.6	$(2.1 \pm 0.6) \times 10^3$	6.5 ± 0.5	-3954 ± 80	1.9 ± 0.2

[a] 1 cal = 4.184 J. [b] In the presence of maltose.

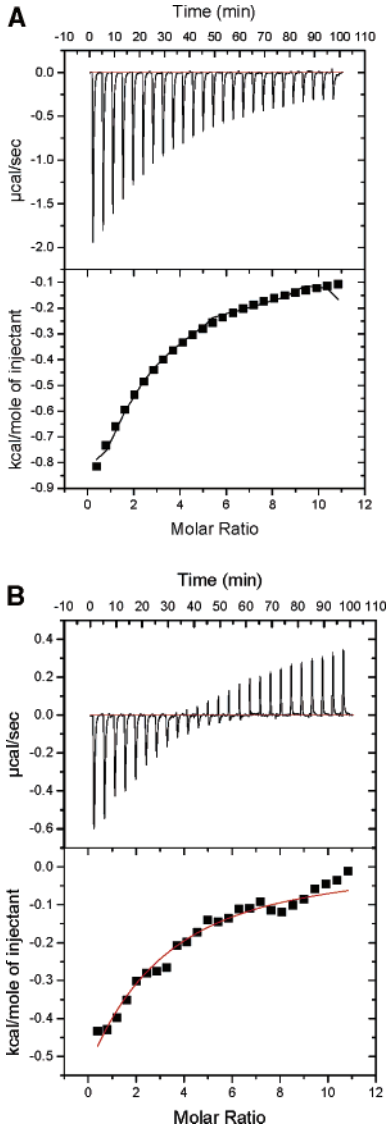

Figure 6. Binding isotherm for titration of 8.5×10^{-3} mol dm^{-3} maltose with 0.15×10^{-3} mol dm^{-3} concanavalin A in the absence (A) and presence (B) of ANS at 298.15 K.

TABLE 4: Binding Affinities *K*, Enthalpies ΔH, Entropies ΔS, and Stoichiometry of Binding *n* for Binding of 8.5×10^{-3} mol dm^{-3} Maltose to Concanavalin A in the Absence and Presence of ANS[b] at Temperature *T* and pH 5.2[a]

T (K)	K_1 (M^{-1})	n_1	ΔH_1 (cal mol^{-1})	ΔS_1 (cal K^{-1} mol^{-1})
298.15	$(1.34 \pm 0.2) \times 10^3$	1.0 ± 0.1	-3824 ± 162	1.5 ± 0.3
298.15[b]	$(1.22 \pm 0.1) \times 10^3$	1.0 ± 0.1	-3250 ± 180	3.2 ± 0.8

[a] 1 cal = 4.184 J. [b] In the presence of ANS.

site, and (4) release of water from the hydrophobic surface upon binding. Since the number of ANS molecules bound to protein (stoichiometry of binding) increases with the increase in temperature, it indicates a considerable exposure of the hydrophobic surface of the protein with a rise in temperature. However, the increase in temperature does not have a significant effect on the binding of ANS with the protein at the second site where binding appears to be more enthalpically driven. On the basis of thermodynamic parameters, some insights into the factors that contribute to the binding of ANS to the concanavalin A can be obtained. In principle, a variety of noncovalent forces such as hydrophobic interactions, electrostatic interactions, van der Waals interactions, and hydrogen-bonding interactions are responsible for the binding.[22]

The negative values of ΔH and positive (though small) values of ΔS are consistent with the characteristics of a combination of van der Waals, hydrophobic, and electrostatic interactions in the binding process. The temperature dependence of the binding affinity was used to calculate the van't Hoff enthalpy, which did not match the calorimetric enthalpy. The ratio of van't Hoff to calorimetric enthalpy, which is greater than one changes with temperature, indicates deviation from two-state binding. Possibilities for this deviation also suggest conformational change in the protein as a function of temperature affecting the binding site, leading to nonadherence to van't Hoff dictates.

4.2. Binding of ANS with Concanavalin A at pH 2.5. Binding at the first site at pH 2.5 is more entropically driven than the enthalpic contribution. Since binding at the second site, especially at 308.15 K, appears to be entropically opposed, it could be ascribed possibly to hydrogen-bond formation, a decrease in the number of isoenergetic conformations, and a decrease in soft internal vibrational modes.

The main contributors to a negative value of ΔC_p for the second site could be due to an increase in hydrophobic interaction, the burial of nonpolar surface area from water, and the presence of water molecules buried in the interface and/or local folding due to binding.[23−26]

4.3. Probable Sites for Binding of ANS to Concanavalin A. On the basis of crystallographic data it has been postulated that con A protomer has a site where the hydrophobic molecules can bind.[17] The hydrophobic cavity is surrounded by the amino acid chains of tyr 54, leu 81, val 89, val 91, phe 111, ser 113, val 179, ile 181, phe 191, phe 212, and ile 214. This cavity may be assumed to be one of the probable sites where ANS binds. Since the value of n_1 is significantly large, the possibility of other nonspecific sites cannot be excluded.

4.4.Circular Dichroism Spectroscopy. Near-UV CD spectra suggest favorable alteration in the structure of concanavalin A at pH 2.5. In particular, the protein at pH 2.5 appears less structured than at the other values of pH, while at pH 7.2 the spectrum is very similar to that of the native concanavalin A at pH 5.2. Enhancement of the binding constants when going from pH 5.2 to 2.5 suggests that ANS binding is strongly influenced by the protein charge and by the favorable distortion in the structure of concanavalin A as suggested by near-UV CD spectra.

4.5. Role of Dimer and Electrostatic Effects. Significant binding at pH values where concanavalin A exists as a dimer suggests that dimerization of concanavalin A does not affect the access of ANS to the binding sites. The ITC data at pH 2.5 shows higher binding affinity than at pH 5.2. Electrostatic

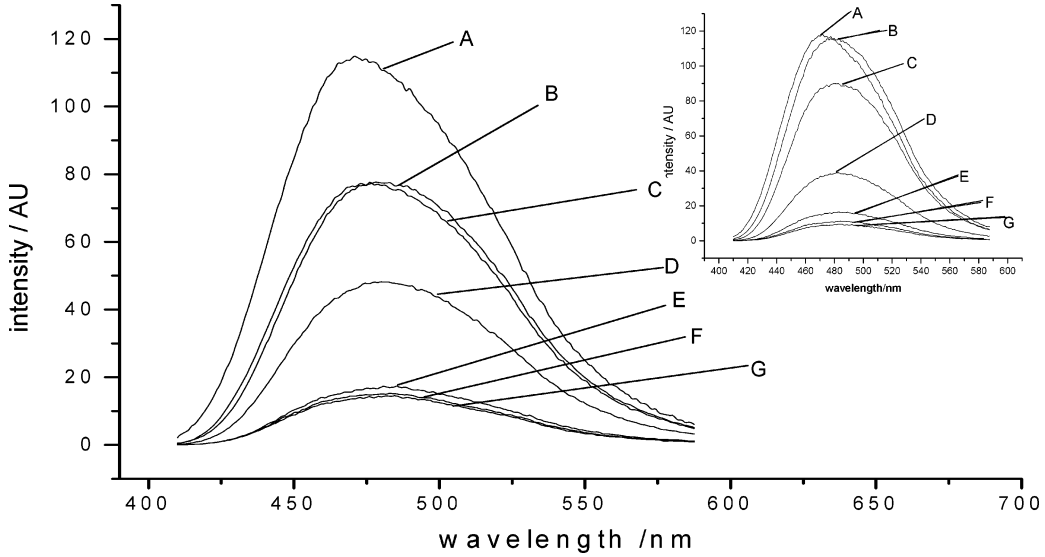

Figure 7. Emission spectra of 12.8×10^{-6} mol dm^{-3} concanavalin A at pH 2.5 (inset pH 5.2) in the presence of increasing molar ratios of ANS: 0.25 (A), 0.5 (B), 0.75 (C), 1.0 (D), 1.5 (E), 2.0 (F), and 2.25 (G).

interactions are expected to play a major role since ANS carries one unit of negative charge and concanavalin A carries positive charge at pH 2.5, which is below the isoelectric point; therefore, it is reasonable to assume that the effective free energy for the association process stems from two contributions, electrostatic interactions and nonelectrostatic interactions that include all other interactions.

4.6. Effect of Maltose on ANS Binding. Since the binding affinity of ANS for concanavalin A in the presence of maltose decreases for both classes of sites, this could be attributed to stabilization of concanavalin A upon maltose binding, leading to a more compact state of the protein, thereby leading to burial of hydrophobic residues and hence reduction in binding affinity.

Maltose binding specificity with concanavalin A is not altered in the presence of ANS since the binding constant remained unchanged. This is based upon observations that when ANS is titrated with maltose-bound protein its affinity is slightly reduced; however, when maltose is titrated with ANS-bound protein, its binding affinity is not altered. This indicates that the ANS and saccharide-binding site are independent of each other.

4.7. Binding of ANS with Concanavalin A at pH 7.2. ITC results at pH 7.2 indicate an absence of appreciable binding. The concanavalin A tetramer is formed when canonical dimers associate with the central parts of their back sheets in a perpendicular manner, and the absence of appreciable binding in the tetramer indicates that quaternary association between two canonical dimers leads to shielding of sites where ANS binds.

The absence of appreciable ANS binding to the tetrameric form of concanavalin A has also been demonstrated by fluorescence results. Fluorescence titrations of ANS with concanavalin A at pH 2.5, 5.2, and 7.2 are shown in Figures 7 and 8.

ANS has been widely used as a putative probe for examining the protein structure via fluorescence intensity measurements.[1] It has been extensively used as a hydrophobic probe to study biological membranes and nonpolar sites on protein. Recently, fluorescence has been employed to monitor the binding of ANS to proteins in ice and has been proposed as a useful monitor of ice-induced strains on the native fold.[27] Comparison of the emission spectra at pH 2.5, 5.2, and 7.2 indicates substantial enhancement of fluorescence intensity accompanying ANS binding to dimeric concanavalin A at pH 2.5 and 5.2 compared

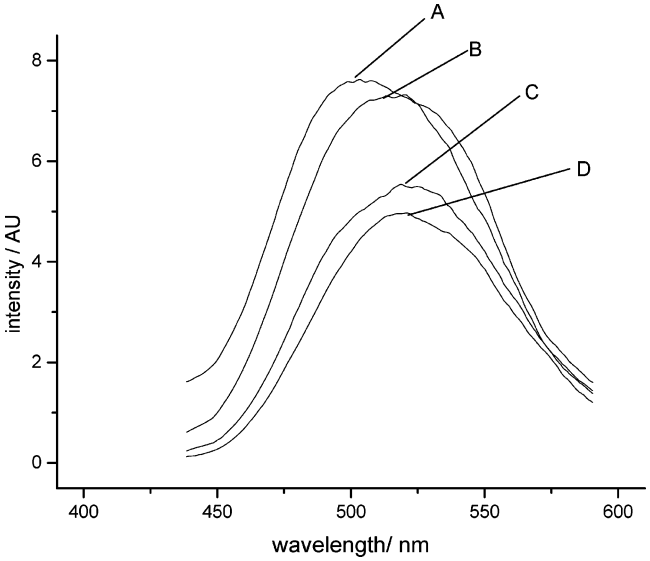

Figure 8. Emission spectra of 12.8×10^{-6} mol dm^{-3} concanavalin A at pH 7.2 in the presence of ANS: 0.25 (A), 0.5 (B), 0.75 (C), and 1.0 (D).

to that at pH 7.2, indicating an absence of appreciable binding to the tetrameric form of concanavalin A. Fluorescence and ITC results correlate well with each other, indicating the absence of ANS binding to the tetrameric concanavalin A.

4.8. Possible Biological Implications of ANS Binding with Concanavalin A. The study of hydrophobic binding properties of lectins may be relevant to the biological role of these proteins in plants as plants produce a variety of hydrophobic secondary metabolites which could potentially bind to these sites. In addition to a saccharide binding site a second binding site on the con A protomer is present that is capable of interacting with hydrophobic ligands. This raises the possibility that con A may interact with the cell membrane via a hydrophobic portion of the molecular surface that alone would not bind; this secondary interaction may provide the surface alterations necessary for some of the lectin's biological effects. Demonstration of a hydrophobic binding site that is distinct from the carbohydrate binding activity has a number of implications for interpretation of experiments based on the use of con A as an adventitious mitogen for lymphocytes.

5. Conclusions

Isothermal titration calorimetric measurements have demonstrated that ANS binding to concanavalin A displays heterogeneity with two classes of noninteracting binding sites. Enhancement of the binding constants and fluorescence intensity from native to pH 2.5 suggests that ANS binding is influenced by the protein charge and by the favorable alteration in the structure of concanavalin A as suggested also by near-UV CD spectra. ANS does not bind to concanavalin A at pH 7.2, where it exists as a tetramer, which indicates that quaternary association between two canonical dimers leads to shielding of sites where ANS binds. ANS binding experiments in the presence of maltose leads to a decrease in binding affinity. The results have demonstrated a hydrophobic binding site that is distinct from the saccharide binding site. The above results suggest that it may be possible to use ANS−con A complexes to measure the properties of these molecules bound to cell surface receptors and, by implication, the properties of the receptors themselves. These experiments may provide insight into the differing biological properties of con A.

Acknowledgment. This work was supported by funding from the Department of Science and Technology, New Delhi, India

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