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# An Electrospray Ionization Mass Spectrometry Investigation of 1-Anilino-8-Naphthalene-Sulfonate (ANS) Binding to Proteins

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The binding of 1-anilino-8-naphthalene-sulfonic acid (ANS) to various globular proteins at acidic pH has been investigated by electrospray ionization mass spectrometry (ESI-MS). Maximal ANS binding is observed in the pH range 3–5. As many as seven species of dye-bound complexes are detected for myoglobin. Similar studies were carried out with cytochrome *c*, carbonic anhydrase, triosephosphate isomerase, lysozyme,  $\alpha$ -lactalbumin, and bovine pancreatic trypsin inhibitor (BPTI). Strong ANS binding was observed wherever molten globule states were postulated in solution. ANS binding is not observed for lysozyme and BPTI, which have tightly folded structures in the native form.  $\alpha$ -Lactalbumin, which is structurally related to lysozyme but forms a molten globule at acidic pH, exhibited ANS binding. Reduction of disulfide bonds in these proteins leads to the detection of ANS binding even at neutral pH. Binding was suppressed at very low pH (<2.5), presumably due to neutralization of the charge on the sulfonate moiety. The distribution of the relative intensities of the protein bound ANS species varies with the charge state, suggesting heterogeneity of gas phase conformations. The binding strength of these complexes was qualitatively estimated by dissociating them using enhanced nozzle skimmer potentials. The skimmer voltages also affected the lower and higher charge states of these complexes in a different manner.

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Electrospray ionization mass spectrometry (ESI-MS) provides a powerful means to study noncovalent interactions in the gas phase [1–3]. The mild ionization procedure permits protein molecules to retain “conformational memory” of their solution state structures in the gas phase [4–9]. Different folded states in solution produce different charge states in the ESI-MS spectrum. ESI-MS has been used extensively to establish binding stoichiometries for many protein–ligand and protein–protein complexes. We have recently demonstrated in a preliminary report [10], that ESI-MS can be used to detect noncovalent complexes of the widely used fluorescent dye 1-anilino-8-naphthalene-sulfonate (ANS) [11, 12] to proteins in the gas phase. An important issue that has been addressed in several studies is the correlation between protein conformation in solution and the nature of the structural states detected in the gas phase [6, 13–19]. Although hydrophobic interactions are generally major contributors to the stabilization of folded structures and protein–ligand complexes in aqueous solutions, it is clear that they will become less important in the gas phase. On the contrary, electrostatic effects and hydrogen bonds, which appear less dominant in water because of

solvent competition, are likely to assume importance in the gas phase.

ANS is widely used as a hydrophobic probe to study solution state folding behavior. Completely folded and unfolded states of proteins often do not bind ANS with appreciable affinity. Binding is maximal under conditions where they exist in partially unfolded “molten globule” states, which are known to provide exposed hydrophobic sites for ANS binding [12]. Although ANS binding to partially unfolded states has been widely investigated by optical spectroscopic methods, there is comparatively little information regarding the stoichiometry of binding. Recent studies suggest that ANS binds nonspecifically to hydrophobic sites and the interaction is mediated by formation of ion pairs [20, 21].

In this study, we analyze the interactions of ANS with diverse proteins using ESI-MS to detect gas phase complexes when solutions of protein and dye are electrosprayed under a variety of conditions.

## Experimental Procedures

### Chemicals

Myoglobin (horse heart), cytochrome *c* (bovine), lysozyme (hen egg white),  $\alpha$ -lactalbumin (bovine), carbonic anhydrase (bovine), and BPTI (bovine) were

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obtained from Sigma (St. Louis, MO) and were used without further purification. *Plasmodium falciparum* triosephosphate isomerase was expressed in *E. coli* and purified as described before [22]. The ANS ammonium salt (Sigma) was recrystallized from ethanol before use.

### *Unfolding and ANS Binding*

pH dependent unfolding was carried out using 25 mM ammonium acetate buffer and the pH adjusted using glacial acetic acid and ammonia solution. Each protein (15  $\mu$ M) was incubated with ANS (100  $\mu$ M) at the desired pH for 30 min at 25 °C prior to mass spectral analysis.

### *Reduction of Disulfide Containing Proteins*

Lysozyme, BPTI, and  $\alpha$ -lactalbumin (50  $\mu$ M) were reduced by adding 100 mM DTT and heating at 45 °C for 1 h. Excess DTT was removed by passing the solution through a G10 column. Protein samples were stored in an inert atmosphere and checked by ESI-MS to confirm complete reduction.

### *Mass Spectrometry*

ESI-MS was carried out on a Hewlett Packard (model HP-1100) electrospray mass spectrometer. Ammonium acetate buffer adjusted to the required pH was used as the mobile phase at a flow rate of 10  $\mu$ L/min. Approximately 150 pmol of sample was injected per run. Electrospray was carried out using a capillary with an internal diameter of 0.1 mm. The tip was held at 5000 V in positive ion mode. Nebulization was assisted by N<sub>2</sub> gas (99.8%) at a flow rate of 10 L/min. The spray chamber was held at 200 °C. The ion optics zone was optimized for maximal ion transmission. The instrument has two variable skimmers. Skimmer 2 was held constant at 10 V, and skimmer 1 (declustering potential) was adjusted for optimal detection. Maximal binding was detected when skimmer 1 was held at 30 V ( $\Delta V = 20$  V). Data was acquired across mass range 500–3000  $m/z$  with a quadrupole cycle time of 3 s. The spectrometer was tuned using five calibration standards provided by the manufacturer. Data processing was done using the deconvolution module of the Chemstation software. A minimum of five peaks well above the baseline was used for molecular mass determination.

## **Results and Discussion**

### *Gas Phase Detection of ANS–Protein Complexes*

The binding of ANS to proteins, in principle, should lead to increase in mass by 299 Da for each bound anion of ANS. In the ESI-MS spectrum, protein-dye adducts can be detected as satellite charge states around each protein charge state. The various charge states of a protein may show different affinities for ANS, leading

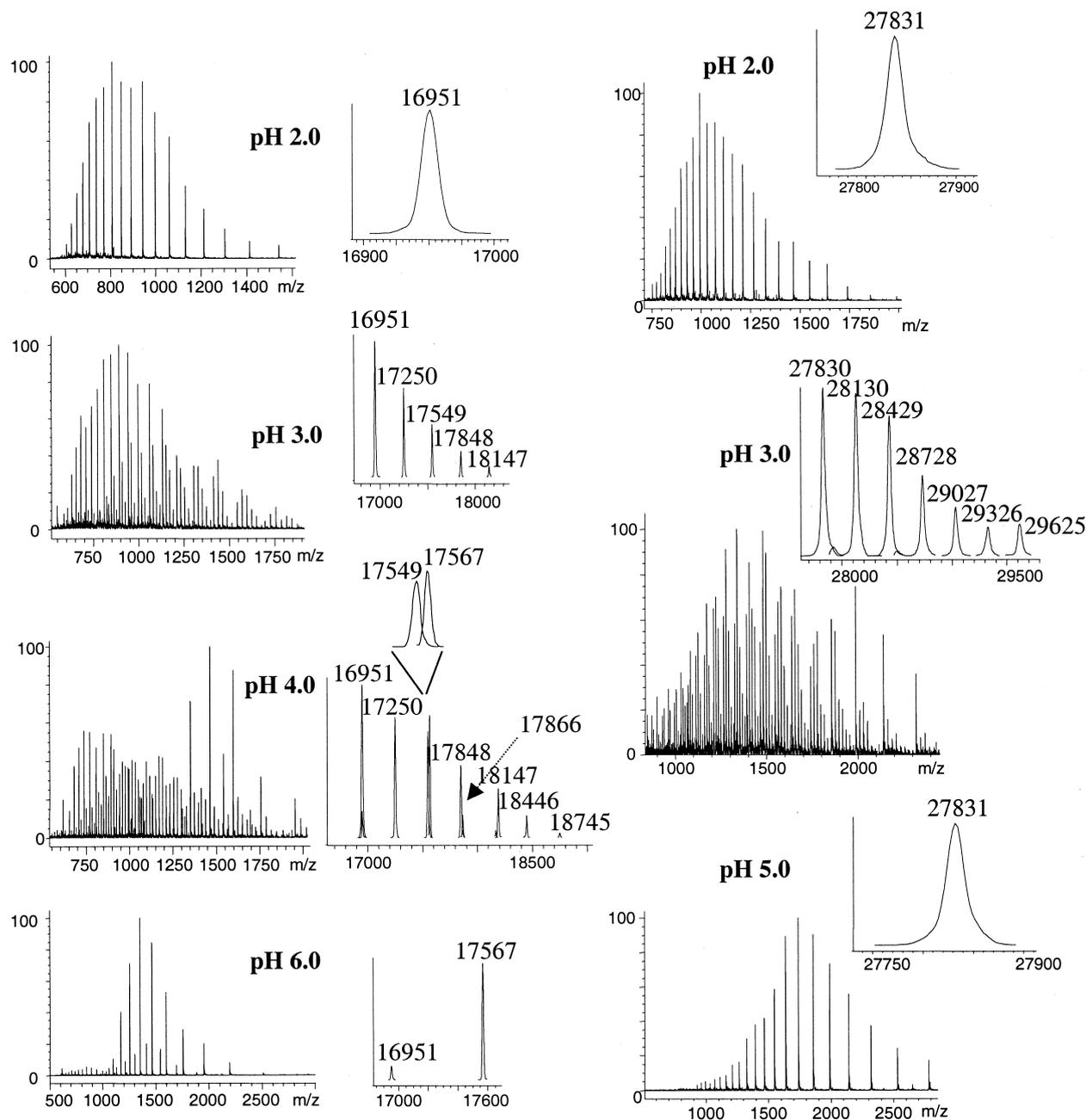
to variation in the intensity of the ANS satellite peaks. The number and the distribution of molecular masses obtained upon processing (deconvoluting) the raw spectrum provides a qualitative measure of bound moieties that survive the desolvation/ionization process.

### *Myoglobin*

Myoglobin is a globular all-helical protein with a molecular mass of 16,951 Da (for the apo form) and binds heme (616 Da) in its native state. Extensive mass spectral studies of myoglobin have focused on refolding behavior [23], H–D exchange [24], gas phase stability of the heme bound complex [25], proton transfer reactivities [26], and sensitivity to proteolysis [27]. Tsui et al., were able to detect an intermediate during myoglobin unfolding using quench flow ESI-MS [28].

Under mild electrospray source conditions and a solution pH of 2.0, myoglobin ions are highly charged showing a charge state distribution from +8 to +30 centered at +19 (Figure 1). Heme binding is not observed. At low pH, the prosthetic group is known to dissociate due to titration of the heme binding histidine residues. At pH 3.0, four distinct sets of charge states appear, which deconvolute to masses of 16,951 Da, 17,250 Da (apomyoglobin + 1ANS), 17,548 Da (apomyoglobin + 2ANS), 17,849 Da (apomyoglobin + 3ANS), and 18,146 Da (myoglobin + 4ANS). At pH 4.0, there is a very low population of free apomyoglobin and most of it is bound to one or more ANS molecules. Some of the protein now binds to heme and yields a molecular mass of 17,567 Da (holomyoglobin). It is interesting to note that a new species having a mass of 17,867 Da corresponding to holomyoglobin bound to one ANS was detected. This species has not been detected previously because the heme bound to myoglobin interferes in optical spectroscopic studies of ANS binding. At pH 6.0, only holomyoglobin is observed with a very small proportion of apomyoglobin still visible. The spectrum at pH 6.0 has fewer charge states, suggesting a considerable compaction of the molecule upon heme binding (Figure 1). At pH 2.0 the ESI-MS spectra do not reveal any bound ANS. This is likely to be due to titration of the sulfonic acid group of ANS, resulting in a loss of electrostatic contributions to binding. This feature is observed in all the proteins studied.

These data suggest, that between pH 4.0 and 3.0, dissociation of heme is almost complete and multiple ANS molecules bind to apomyoglobin. The observation is consistent with the finding that removal of heme leads to the formation of a “molten globule like” floppy structure of apomyoglobin as reported by others using 2-D NMR [29]. It is noteworthy, that the single ANS bound species is much more intense than apomyoglobin peak itself under these electrospray conditions. Between pH 6.0 and 4.0, the heme bound form of myoglobin undergoes acid expansion resulting in exposure of a new hydrophobic binding site for ANS. The



## Myoglobin

## Triosephosphate Isomerase

**Figure 1.** ESI mass spectrum of myoglobin (left) and triosephosphate isomerase (pTIM) (right) at different pH values after incubation with excess ANS. The masses derived by deconvolution are shown to the right of the spectra. For spectra showing multiple species, the mass values obtained by deconvolution are indicated. In the case of myoglobin at pH 4.0, the species A\* and B\* correspond to holomyoglobin and holomyoglobin + 1ANS, respectively.

observation of a species at pH 4.0, which corresponds to holomyoglobin with a single bound ANS molecule, suggests a secondary binding site distinct from the classical ANS binding site at the heme pocket. This also points to the existence of a minor pathway for myoglobin unfolding, where a surface for dye binding is exposed at a place other than the heme-binding pocket,

creating an additional binding site for ANS. The mass spectral measurements avoid interference from the heme, which is an unavoidable feature of optical spectroscopic measurements.

In fact, H-D exchange experiments on myoglobin provided strong proof of the floppy nature of the protein, which undergoes rapid exchange with the

solvent [24]. Time resolved ESI-MS of the acid unfolding of holomyoglobin demonstrates that myoglobin undergoes acid expansion following titration of the histidine residues bound to the heme [30].

### *Triosephosphate Isomerase*

Triosephosphate isomerase (PftIM) used in this study was cloned from *Plasmodium falciparum* and expressed in *E. coli*. PftIM is a homodimeric  $\beta 8/\alpha 8$  barrel protein, whose folding pathway has been studied in various denaturants [31, 32]. PftIM has been demonstrated to form a molten globule state at pH 3.0. Fluorescence studies indicate that the pH 3.0 form of PftIM shows strong ANS binding. PftIM upon pH unfolding exhibits a shift in charge state distribution from higher  $m/z$  values to lower  $m/z$  values. ANS binding studies carried out with pftIM (Figure 1 right panel) shows that the protein indeed binds a large amount of ANS at this pH where it is known to exist as a molten globule. The protein binds up to six ANS molecules. Below pH 2.5, the protein binds no ANS. ANS binding is also not detectable at pH 5.0. At pH 2.0, the protein does not bind ANS and the charge state distribution shifts to very low  $m/z$  values suggesting exposure of all the residues that are likely to be protonated at this pH. Indeed, solution studies indicate that PftIM does not bind ANS at this pH. PftIM does not yield an ESI-MS spectrum at pH 7.0, presumably because it forms a strong dimer, whose charge states lie outside the quadrupole mass range (3000 Da) of the instrument. It is interesting to note that even at pH 3.0, some charge states are observed at the high end of the quadrupole (2700–3000  $m/z$ ), which do not show any satellite peaks arising from bound ANS molecules. Close inspection of the charge states at pH 3.0 clearly shows a bimodal charge state distribution for the monomeric protein. There are two maxima; one at 1849  $m/z$  and the other at 1341  $m/z$ . Chowdhury et al. [5] attributed such a bimodal distribution in cytochrome *c* to be due to distinct protein structural states, which are populated in solution, which then produce distinct charge state distribution in the electrospray mass spectrum.

### *Carbonic Anhydrase*

Carbonic anhydrase is a 29 kDa monomeric zinc containing protein. Figure 2 left panel shows the mass spectra of carbonic anhydrase incubated with 100  $\mu$ M ANS and recorded at pH values of 2.5, 6.0, and 3.0. At pH 2.5, the protein appears to be unfolded and yields a mass of 29,020 Da, which corresponds to the mass of the protein without the zinc. Indeed, the zinc is held by four histidines (His3, His94, His96, His119), which are protonated at low pH leading to the release of zinc. At pH 6.0, the protein exhibits a charge state distribution that is shifted towards the higher  $m/z$  values and yields a mass of 29,083 Da, indicating that the protein is in its zinc bound form and is compacted sufficiently. At pH

5.0, the protein undergoes mild acid expansion, although much of the protein still remains in the holo form (not shown). The holo form did not bind any ANS at pH 6.0 and 5.0. At pH 4.0, some charge states corresponding to an ANS bound form were detected. At pH 3.5, the protein binds up to four ANS molecules per protein molecule; the single ANS bound form being the most predominant.

It is interesting to note that the onset of ANS binding is correlated to the release of zinc from the protein. Below pH 3.0, very little ANS binding was detected. Similar behavior is observed in studies of carbonic anhydrase binding to inhibitors when monitored by ES-MS [33].

### *Cytochrome c*

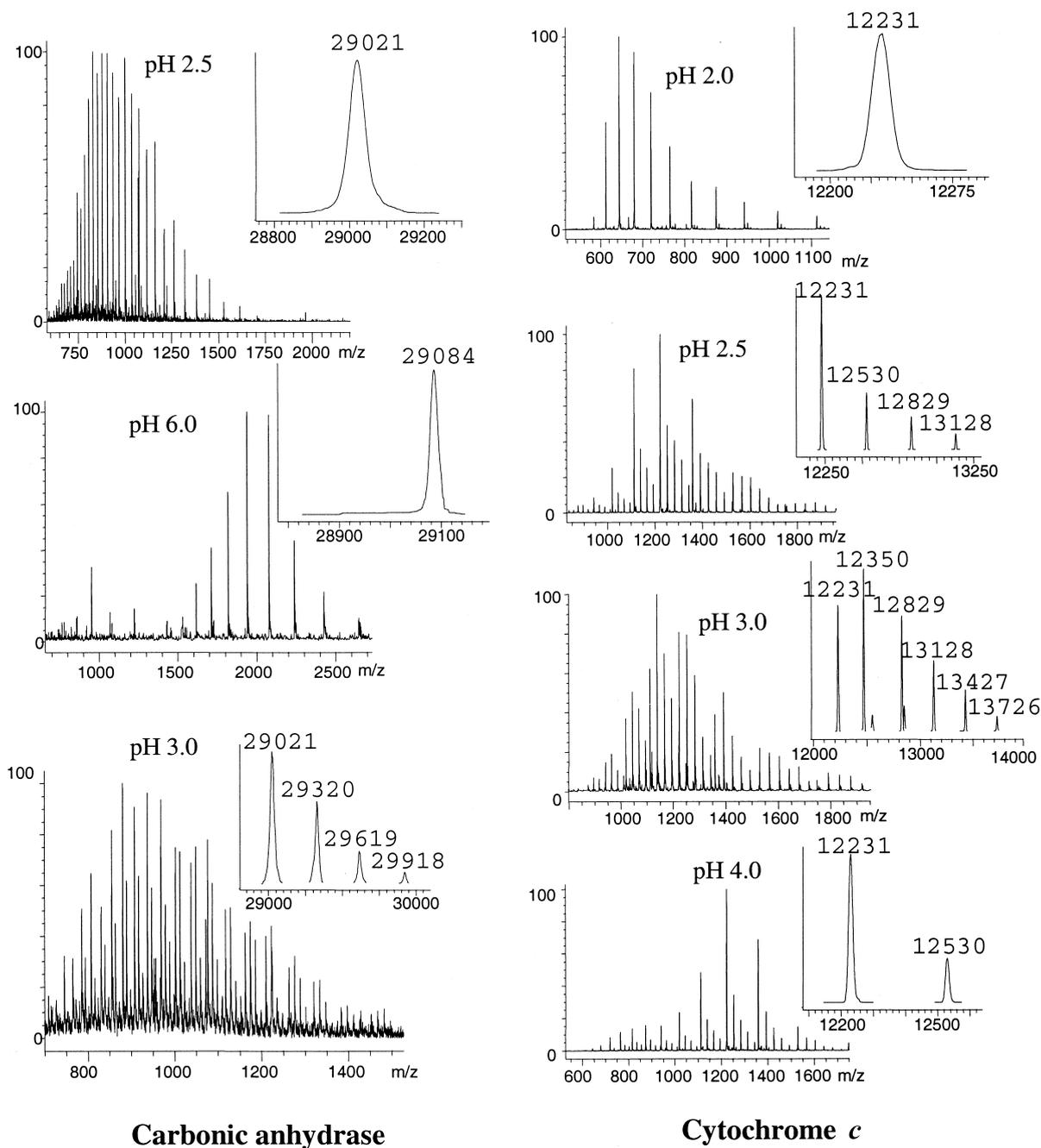
Cytochrome *c* is probably one of the best characterized proteins both in solution and in the gas phase [14–17, 34–37] because of its small size and relative ease of ionization. Different conformations are known to coexist in the absence of solvent [17].

Binding of ANS to bovine cytochrome *c* monitored by ESI-MS, when incubated at different pH is shown in the right panel of Figure 2. At pH 2.0, no ANS binding is observed and the spectrum deconvolutes to the molecular mass of free cytochrome *c* (12,231 Da). At pH 2.5, four sets of charge states are observed, one of which is the free protein. The other charge states correspond to one, two, and three ANS bound forms, respectively. At pH 3.0, at least five different ANS bound species are detectable, the species with two molecules of bound ANS being the most abundant. At pH 4.0, only one ANS bound species is observed and its abundance in solution is drastically reduced when compared with that at pH 3.0. At pH 5.0, most of the protein exists in free form with only a very small fraction still bound to ANS. At pH 6.0, the +8 charge state is the most intense peak. The folded form of cytochrome *c* is known to give predominantly charged states +7 to +9 in solution, whereas for unfolded states the charge states range from +12 to +21 [5, 9].

These results suggest, the presence of a “molten globule like” conformation with exposed hydrophobic patches for cytochrome *c* at pH 3.0 as evidenced by its enhanced affinity for ANS. Notably, molten globule like states for cytochrome *c* have been implicated only in mixed aqueous-organic solvents [46], or under membrane mimetic conditions [47]. The use of other spectroscopic probes suggests a cooperative unfolding transition with decrease in pH, in aqueous medium containing a low concentration of methanol (3%). It is conceivable that the low pH “unfolded states” of cytochrome *c* at pH 3.0 may still possess sufficient structure to permit dye binding.

### *Lysozyme*

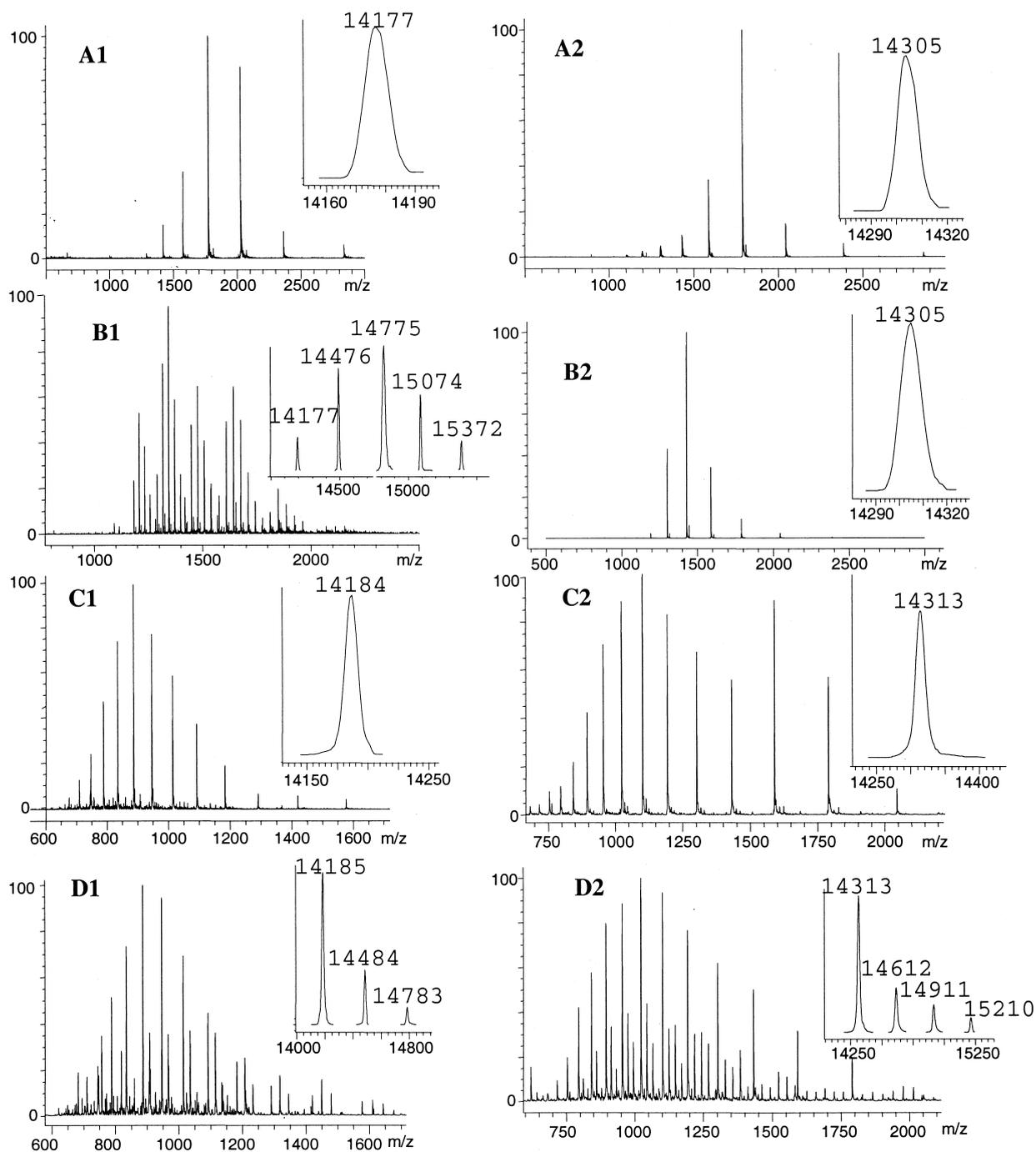
The folding of lysozyme has been extensively studied. It is a small structurally robust monomeric protein (14,305 Da). Much of its stability is due to the presence of the



**Figure 2.** ESI mass spectrum of carbonic anhydrase (left) and cytochrome *c* (right) at different pH values in the presence of excess ANS. The masses obtained upon deconvolution are shown to the right of each spectra. For carbonic anhydrase spectra are shown for pH values 2.5, 3.0, and 6.0, whereas for cytochrome *c*, spectra at pH 2.0, 2.5, 3.0, and 4.0 are shown.

four disulfide crosslinks. Reduction of these disulfides leads to dramatic destabilization of the protein [38]. Acid unfolding of the oxidized and reduced forms in the presence of ANS monitored by ESI-MS spectra clearly revealed major structural differences (Figure 3 right panel). Oxidized lysozyme (the native protein), exhibited very little shift in charge states and did not bind ANS at any pH. The observed charge states (+8 to +13) is much less than the number of putative charge-

able sites on the protein (18 positively charged residues + the free N-terminus). This suggests that even at low pH the protein remains substantially compact and structured. Upon reduction with 200 mM DTT, the spectra becomes distinctly rich in charge states with states bearing up to 21 positive charges appearing at pH 2.0. The reduced form did not bind ANS between pH 2.0 and 5.0. At pH 7.0, it shows a bimodal distribution of charge states, which deconvolute to 14,314.7 Da



**Figure 3.** ANS binding to oxidized and reduced forms of  $\alpha$  lactalbumin (left) and lysozyme (right): (A1) Oxidized  $\alpha$ -lactalbumin at pH 7.0. (B1) Oxidized  $\alpha$ -lactalbumin at pH 3.0. (C1) Reduced  $\alpha$ -lactalbumin at pH 3.0. (D1) Reduced  $\alpha$ -lactalbumin at pH 7.0. (A2) Oxidized lysozyme at pH 7.0. (B2) Oxidized lysozyme at pH 3.0. (C2) Reduced lysozyme at pH 3.0. (D2) Reduced lysozyme at pH 7.0. All spectra were recorded after incubation with ANS.

(weight of the reduced form). The spectrum has two maxima, one at 1591.4  $m/z$  (+9) and the other at 1021.4 (+14), suggesting that the protein after reduction and incubation at pH 7.0, is partially folded and exists in

equilibrium between multiple conformations. Under these conditions, lysozyme bound up to three ANS molecules with the molecular weights 14,614.74 Da (lysozyme + 1ANS), 14,914 Da (lysozyme + 2ANS)

and 15,212 Da (lysozyme + 3ANS) observed upon deconvolution. Many fluorescence studies in solution have shown that reduced lysozyme indeed binds ANS at near neutral pH [39].

### *$\alpha$ -Lactalbumin*

Although structurally similar to lysozyme including the presence of four disulfides,  $\alpha$ -lactalbumin (14,177 Da) has a different pH unfolding profile. It is known to form a molten globule state called the A state at low pH [40]. Other methods of inducing molten globules in  $\alpha$ -lactalbumin include reduction and calcium removal [40]. Unlike lysozyme, four ANS bound complexes were detected with oxidized  $\alpha$ -lactalbumin at pH 3.0 (Figure 3), the most intense being the two ANS bound form. A modest shift in the charge state distribution was observed, suggesting acid expansion of the molecule. Reduction with DTT leads to a marked increase in the number of detectable charge states. Between pH 2.0 and 4.0, no ANS binding is observed with reduced  $\alpha$ -lactalbumin (Figure 3). At pH 5.0, one bound ANS molecule was detected and at pH 7.0, two ANS molecules were bound (Figure 3 D1). Previously reported spectroscopic studies on reduced forms of  $\alpha$ -lactalbumin containing one to three disulfides (3SS, 2SS, and 1SS), indicate that all of them appear to be molten globule like structures, with exposed hydrophobic surfaces, which can bind ANS [41].

### *BPTI*

BPTI is a small monomeric 6 kDa protein, which acts as an inhibitor of bovine trypsin. The protein is stabilized by three disulfide bridges, and the reduction of these disulfide bridges leads to loss of structure. Extensive folding studies have been carried out on BPTI [42]. Mutants of BPTI lacking disulfide bonds form molten globules and show enhancement of ANS fluorescence indicative of dye binding [43, 44]. ESI-MS studies established that BPTI undergoes acid denaturation with a monotonous shift in charge state distribution, without any evidence for any unfolding intermediates. Like lysozyme, oxidized BPTI does not bind ANS across the pH range of 2.0 to 7.0 as monitored by ESI-MS. The reduced form exhibits a marked shift in the charge state distribution towards lower  $m/z$  values. Reduced BPTI bound a single ANS molecule at near neutral pH and none at acidic pH (data not shown) suggesting the presence of a floppy structure at pH 7.0 with not many hydrophobic sites competent for ANS binding.

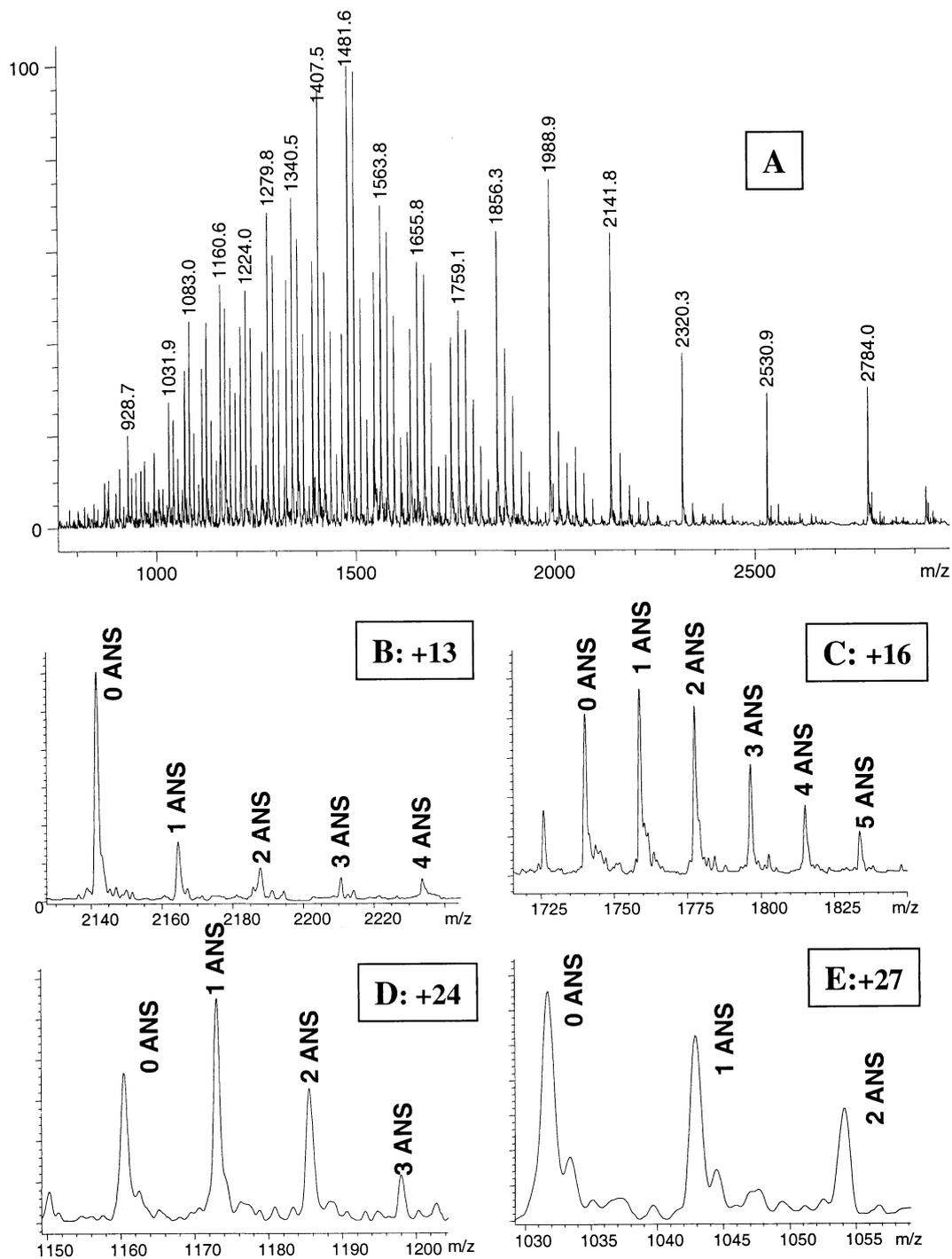
### *Effect of Charge States on ANS Binding*

The results presented above suggest that there is a strong correlation between ANS binding observed in the gas phase and the structural state of the protein in solution. The association of ANS with expanded, molten globule states of proteins in solution, is maintained

under mass spectrometric conditions. The mild ionization conditions facilitate the maintenance of noncovalent interactions that promote dye binding to proteins. In the case of protein molten globule states, it is very difficult to establish stoichiometry of ANS binding in solution using conventional fluorescence methods due to the presence of multiple equilibria and binding site heterogeneity. In proteins like myoglobin and cytochrome *c*, the presence of the heme hampers optical spectroscopic measurements. ESI-MS provides a direct measurement of the number of ANS molecules bound per protein molecule. For example, smaller proteins like myoglobin (6 ANS) and cytochrome *c* (5 ANS) bind a larger number of ANS molecules than a larger protein like carbonic anhydrase (4 ANS). The binding of ANS thus appears to be a property of protein structure rather than the size.

The ready detection of multiple dye-protein complexes in the gas phase suggests that ESI-MS may be used to determine the distribution of various species in solution, assuming that all species ionize with equal efficacy under electrospray conditions. This assumption however may not be generally valid in all cases [1]. Close inspection of the intensities of the satellite peaks around each charge state, suggests that individual charge states have different affinities for ANS. It was observed for cytochrome *c* and myoglobin (data not shown) that the lower charge states have one or two ANS bound species as the major components, whereas in the higher charge states, the free protein dominates. In the case of cytochrome *c*, the +7 charge state has the single ANS bound species as the major component, whereas the +13 charge state has the free protein as the dominant species. Similarly, in the case of myoglobin, the +9 charge state has the single ANS bound species as the most intense component, whereas for the +17 charge state, the free protein peak is most intense.

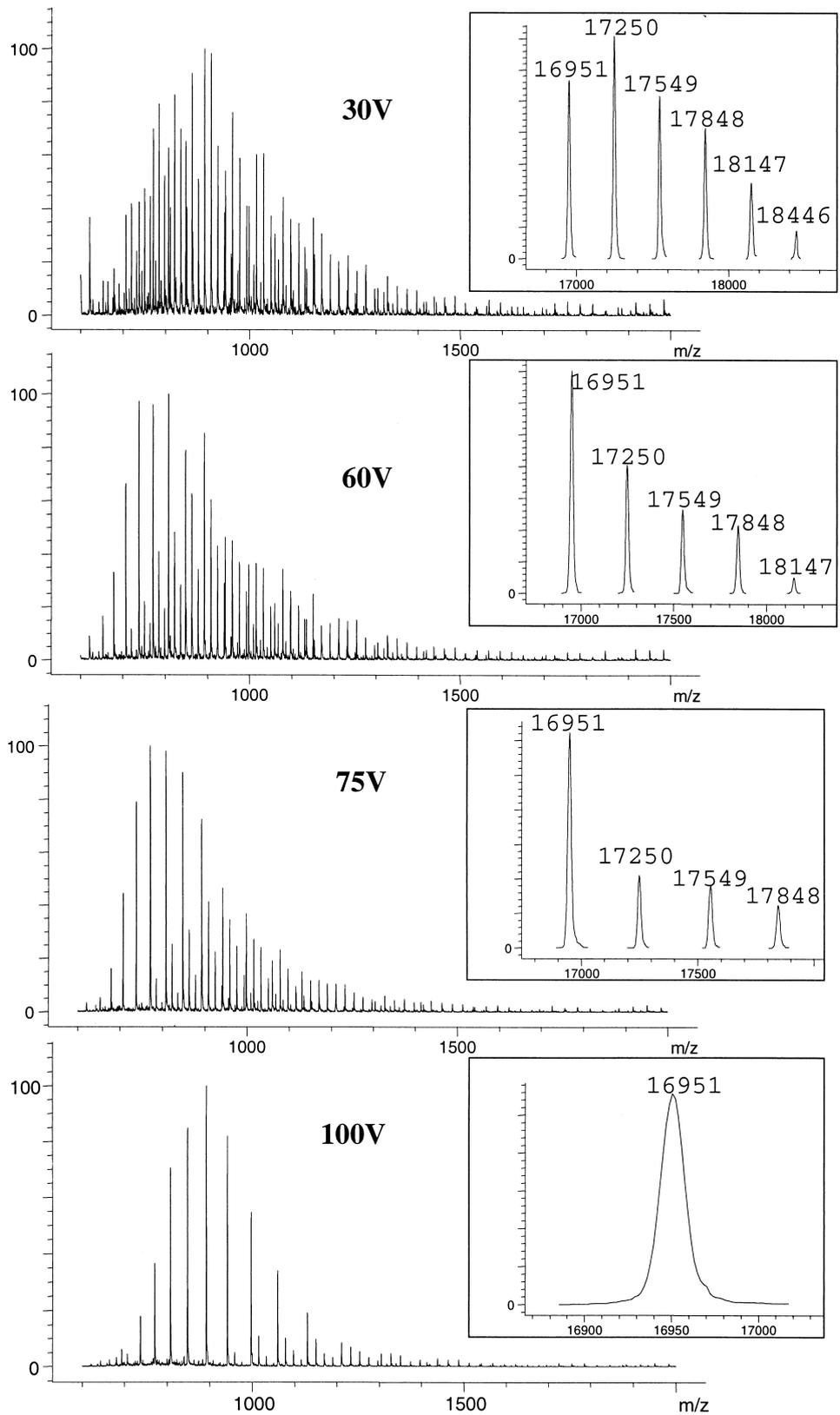
In the case of PftIM, the charge states are complicated by the fact that at pH 3.0, there are probably multiple conformations in equilibrium (Figure 4A). The expanded regions corresponding to four charge states are shown (Figure 4B–E). The charge state +13 has very few low intensity satellite peaks due to bound ANS. The +16 charge state shows several ANS molecules bound to it with the one ANS bound species being the most intense. The +24 charge state shows reduced ANS binding but still has the single ANS bound component as the dominant species. The charge state +27 has the free protein as the most dominant species. The possibility that ANS binding is dependent on the overall resident positive charge of the protein may be discounted since both the lower charge state +13 (Figure 4B) and the higher charge state +27 (Figure 4C) show diminished ANS binding in the case of PftIM, suggesting, that the observed binding may in fact be a property of the structural state of the protein. Indeed, hydrogen-deuterium exchange studies carried on gas phase ions of cytochrome *c* [35], lysozyme, and BPTI [36] support



**Figure 4.** (A) ESI-MS spectrum of pFTIM (15  $\mu$ M) incubated with excess ANS (100  $\mu$ M) recorded at pH 3.0. (B–E) Expanded regions of this spectrum showing charge states +13, +16, +24, and +27, respectively.

the view that the gas phase conformations are different for specific charge states. Furthermore, ion mobility studies by Clemmer et al., on lysozyme ions indicate that lower charge states have different drift times as compared to higher charge states [38]. The higher charge states have been proposed to have an elongated structure as compared to lower charge states; a feature

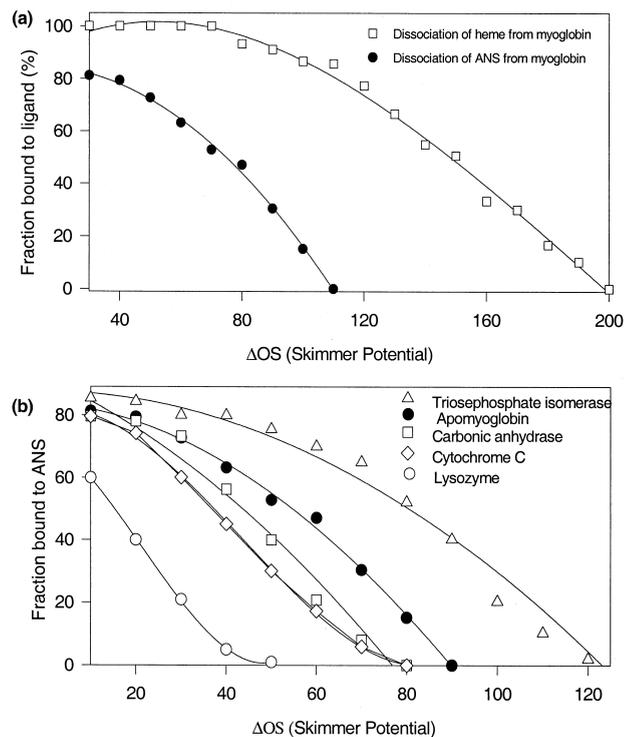
related to the greater internal energies of gas phase protein ions [38]. Ions with greater internal energy have been shown to form poor noncovalent complexes with ligands. Therefore, the effect of increasing the internal energy of the protein molecule on the distribution of ANS can be investigated by increasing the nozzle skimmer potential.



**Figure 5.** ESI mass spectra of ANS adducts from myoglobin (15  $\mu$ M) incubated with ANS (100  $\mu$ M) and recorded at different nozzle skimmer potential ( $\Delta V$ ). Spectra obtained at 30, 60, 75, and 100 V are shown.

## Effect of Nozzle Skimmer Potential

The skimmers are located in the collision zone of the mass spectrometer at the ion sampling interface, which accelerates the ions and makes them collide with the collision gas (nitrogen in this case); this causes an increase in the internal energy of the molecule, which in turn affects conformation of protein ions. For example, in the case of myoglobin, Douglas and co-workers have reported that myoglobin can be unfolded partially in the gas phase by varying nozzle skimmer potentials [45]. Myoglobin and cytochrome *b5* ions expand in size and then lose the heme, giving rise to the apo forms [45]. ANS bound noncovalent complexes were subjected to increasing nozzle skimmer potential to study their relative stabilities. Figure 5 shows the spectra obtained for dissociation of ANS from apomyoglobin as a function of skimmer potential. As expected, it was found that with increasing nozzle skimmer potential the ANS molecules were systematically stripped from the protein. ANS dissociates first from species bearing multiple molecules of ANS. At relatively high skimmer potentials, only single ANS bound species and unbound protein are detectable. Douglas and co-workers [45] have used these potential values to measure gas phase binding energies. For comparison, dissociation of heme from myoglobin was also carried out as a function of nozzle skimmer potential. No attempt was made in the present study to estimate the gas phase binding energy of protein-ANS complexes, because under the current experimental conditions instrumental parameters like drift time, collision cross section area, and gas number density at the sampling orifice could not be determined. Nevertheless a qualitative comparison of heme and ANS dissociation is discussed. Figure 6A shows a plot for dissociation of ANS from apomyoglobin and heme from myoglobin as a function of skimmer potential. Similar plots were obtained for cytochrome *c*, carbonic anhydrase, and lysozyme to study the relative strengths of ANS bound species. The dissociation plots for ANS from these molecules as a function of nozzle skimmer potential is shown in Figure 6B. Although it is difficult to directly compare dissociation profiles obtained for ANS binding to proteins of widely differing masses, some qualitative features may be inferred from the data. ANS is easily dissociated from complexes with the reduced forms of lysozyme at very low nozzle skimmer potential suggesting, a relatively weak association between the dye and the macromolecule. Similar results have been obtained with reduced forms of  $\alpha$ -lactalbumin and BPTI suggesting that these proteins do not have appreciable structure in the absence of disulfide bonds resulting in only weak binding of the dye. It is anticipated that the dye complexes of larger proteins will require higher dissociation voltages precluding a direct comparison with significantly smaller macromolecules. Nevertheless, the data in Figure 6B shows significant differences in dissociation voltages of the proteins triosephosphate isomerase ( $M_r = 27,831$



**Figure 6.** (a) Dissociation curves of ANS and heme from myoglobin as a function of skimmer potential. (b) Dissociation curves showing fraction of ANS bound to different proteins as a function of skimmer potential. The fraction of dye bound to the protein was determined by averaging over the total ion chromatogram and using the intensities of the species obtained at different masses in the deconvoluted spectrum. The fraction bound includes all the multiply bound forms.

Da) and carbonic anhydrase ( $M_r = 29,021$  Da) suggesting that ANS binding is significantly stronger in the former. It is pertinent to note that Hunter et al. [45], observed that although cytochrome *b5* ( $M_r = 15,198$  Da) and myoglobin ( $M_{rapo} = 16,950$  Da) have similar binding affinities for heme binding in solution, the gas phase higher skimmer potentials are necessary for the dissociation of heme from myoglobin than cytochrome *b5*. Inspection of the charge states of the protein-ANS complexes as a function of nozzle skimmer potential suggests that the higher charge states lose the bound ANS before the lower charge states. The higher skimmer potentials probably affect higher charge state structures more than lower charge states causing release of ANS. The pattern dissociation of ANS from carbonic anhydrase was found to be similar to the dissociation of ligand complexes of carbonic anhydrase as reported recently by Gao et al. [46].

## Conclusions

The present study demonstrates the utility of ESI-MS as a rapid and a sensitive tool to detect ANS complexes in the gas phase. For many proteins maximal ANS binding is observed in the pH range 2.5–4.0 where molten

globules have been demonstrated to exist. It is likely that these acid expanded partially unfolded states provide greater surface area for dye binding. The protein-dye complexes do not dissociate under electrospray conditions. The stoichiometries of dye bound complexes can thus be easily established. The robustness of these adducts in the gas phase suggests that electrostatic interactions between the sulfonate moiety of the dye and protein may be important determinants of the stability of these complexes. Although a great body of literature suggests that ANS binds at hydrophobic regions, recent work has emphasized on the electrostatic nature of ANS binding [20]. For a given charge state distribution, binding is observed to be dependent on the net charge on a given ion. No correlation is found between the number of ANS bound complexes and protein size. Carbonic anhydrase (29,021 Da) binds only three, whereas cytochrome *c* (12,230 Da) binds as many as five ANS molecules.

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