1-Anilino-8-naphthalene-sulfonate (ANS) Binding to Proteins Investigated by Electrospray Ionization Mass Spectrometry: Correlation of Gas-Phase Dye Binding to Population of Molten Globule States in Solution

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The binding of 1-anilino-8-naphthalene-sulfonic acid to globular proteins at acidic pH has been investigated by electrospray ionization mass spectrometry (ESIMS). Mass spectra of apomyoglobin recorded in the pH range 2−7 establish that maximal ANS binding is observed at pH 4.0. As many as seven distinct species may be observed in the gas phase which correspond to protein molecules containing one to six molecules of bound ANS. At neutral pH only a single molecule of ANS is bound. In the case of cytochrome c, maximal binding is observed at pH 4.0, with five molecules being bound. Binding is suppressed at neutral pH. In both cases ESIMS demonstrates maximal ANS binding at pH values where the proteins have been reported to exist in molten globule states. ANS binding is not observed for lysozyme, which has a tightly folded structure over the entire pH range. Reduction of disulfide bonds in lysozyme leads to the detection of ANS-bound species at neutral pH. Binding is suppressed at low pH due to complete unfolding of the reduced protein. The results suggest that ESIMS may provide a convenient method of probing the stoichiometry and distribution of dye complexes with molten protein globules.

Introduction

Since the seminal work of Gregorio Weber[1] 1-anilino-8-naphthalene-sulfonate (ANS) has been extensively used as a fluorescent probe of hydrophobic binding sites in diverse biological systems including proteins, membranes, cell organelles and intact cells. The large increase in fluorescence quantum yield and the pronounced blue shift of the emission maximum upon binding to hydrophobic sites have made ANS a versatile probe.[2] More recently, ANS has been shown to bind preferentially to "molten globule" states, which are widely observed equilibrium intermediates in the folding pathway of several proteins.[3] Although enhanced ANS binding has been considered diagnostic of molten globule states of proteins, there is relatively little information on the number and nature of ANS binding sites.[4] We demonstrate in this communication that non-covalent complexes of ANS with proteins can be detected in the gas phase using electrospray ionization mass spectrometry (ESIMS); a "soft" technique that preserves weak non-covalent complexes in the gas phase.[5] We further demonstrate that ANS binding in the gas phase is preferentially observed when the protein in solution exists in molten globule states. Gas-phase dye binding is not observed in the case where folded and unfolded states of the protein are populated in solution. ESIMS may thus provides a convenient way of estimating the stoichiometry of dye binding to expanded molten globule states.

Experimental Section

Solutions of protein (20 μM) were incubated with 300 μM ANS (ammonium salt) at the desired pH (acetate buffer 10 mM) at 25 °C and used directly for ESIMS, carried out on a Hewlett Packard (model HP-1100) mass spectrometer. The mobile phase (acetate buffer) was adjusted to the same pH as the sample. A flow rate of 80 μL/min was used for all the analyses. A volume of 5 μL of protein solution was used for each experiment. Electrospray was carried out using pneumatic assistance with nitrogen gas. The capillary tip was held at 4000 V in positive ion detection mode. The spray chamber used a nitrogen gas flow rate of 10 L/min at 200 °C, since the effluent was aqueous buffer. The skimmer potential was ramped between 50 and 75 V during a cycle time of 3 s. The raw data was analyzed on the deconvolution module of the HP chemstation software using a minimum of seven peaks for unambiguous assignment of molecular weights.

Results and Discussion

Figure 1 shows the electrospray mass spectrum[6] of horse heart apomyoglobin (pI = 7.4) at pH 2.0 in the presence of 15 fold molar excess of ANS. At pH 2.0 no ANS binding is observed, the charge state distribution being characteristic of unfolded apomyoglobin.[7] At pH 2.0, ANS is expected to exist in its protonated form. Binding to proteins is facilitated only at pH values where ANS is anionic. The derived mass 16951.2 Da [right panel] is in agreement with the mass calculated from the apomyoglobin sequence. At pH 3.0, a second species of lower intensity is clearly discernable in the mass spectrum. Deconvolution of these charge states yields two molecular masses of 16951 and 17250 Da, the major component being apomyoglobin. The minor component corresponds to an apomyoglobin species bound to a single ANS molecule [ANS, Mw = 299.8 Da]. At pH 4.0, the mass spectrum becomes distinctly complex with several closely spaced peaks being seen. Deconvolution of the charge states yields as many as six distinct ANS complexes of apomyoglobin, each separated by 299 Da. The mass difference between the various species indicates that ANS

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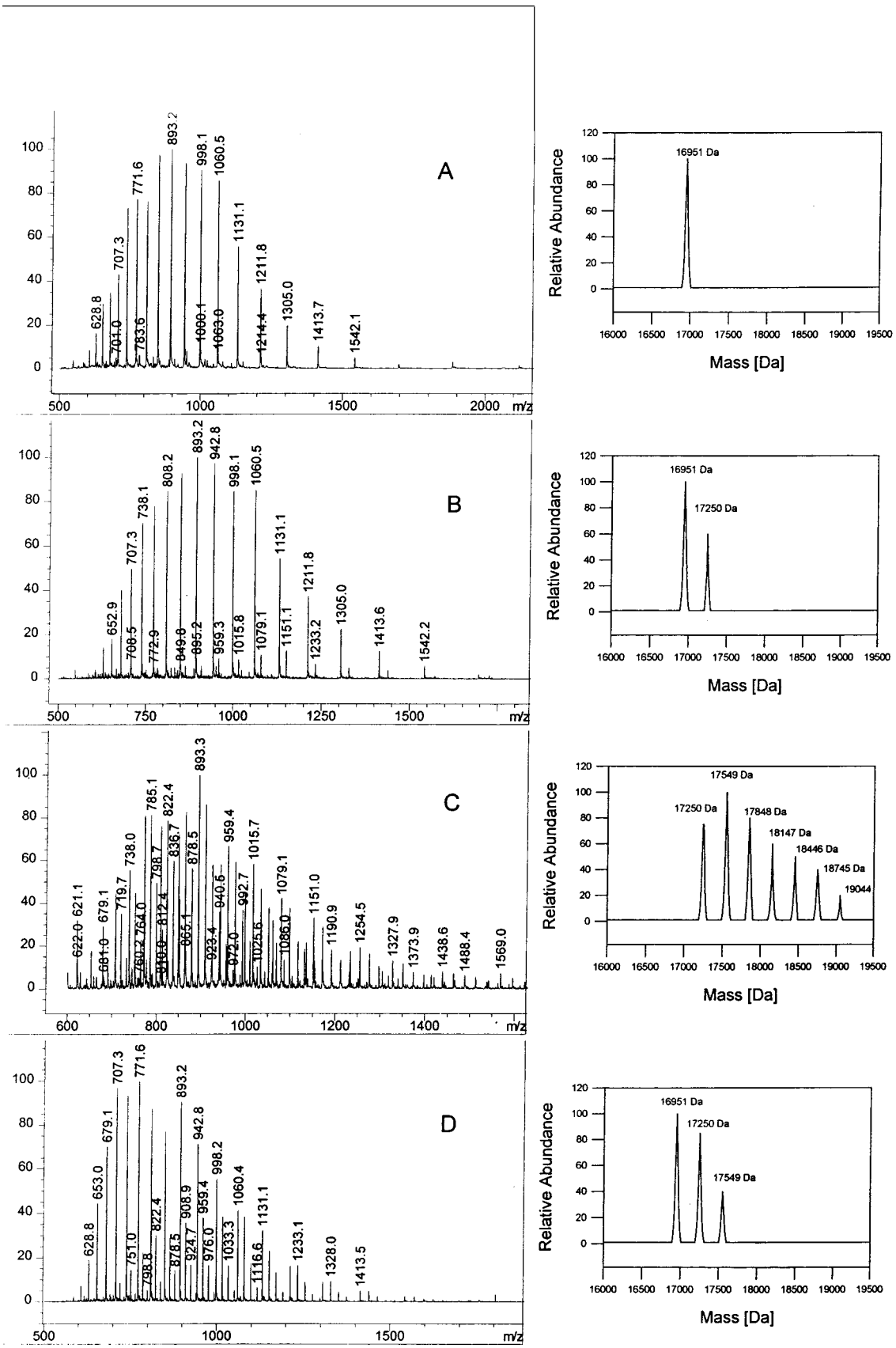

Figure 1. (A) The panel shows the electrospray mass spectrum of apomyoglobin with ANS at pH 2.0. The charge state deconvolutes to yield the expected mass of 16951.2 Da [inset]. (B) ESMS of apomyoglobin with ANS at pH 3.0. The calculated molecular weights are shown in the inset. (C) ESMS of apomyoglobin with ANS at pH 4.0. Masses obtained by deconvolution of the raw spectrum are indicated in the inset. (D) ESMS of apomyoglobin with ANS at pH 6.0. The inset shows the molecular weights obtained after deconvolution. The molecular weights derived from the deconvolution have been normalized in a fashion, where the most abundant species is taken to be 100% and the remaining peaks are expressed relative to the most abundant peak.

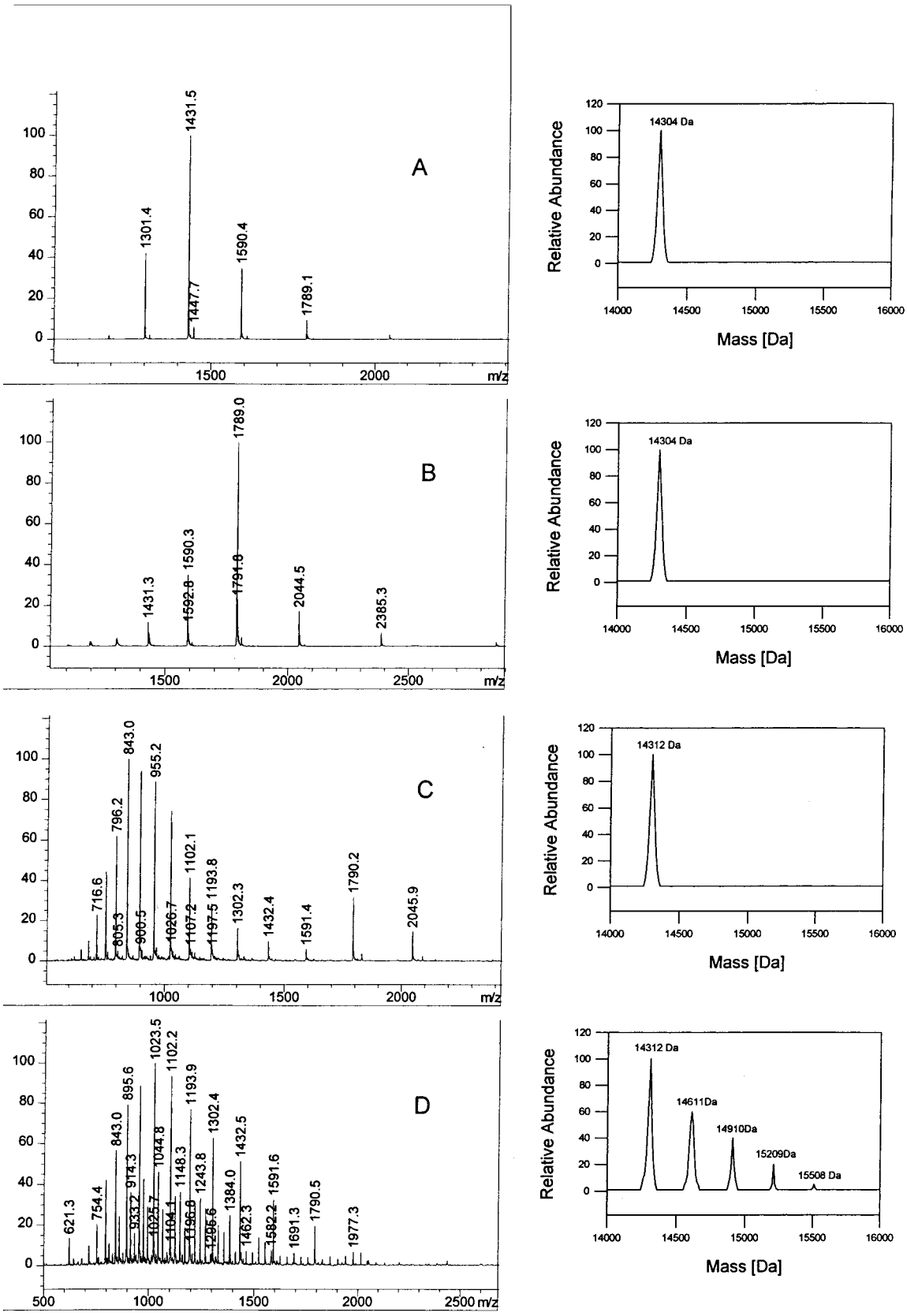

Figure 2. The top two panels indicate the electrospray mass spectrum of the oxidized form of the hen egg white lysozyme in presence of ANS at pH 2.0 (A) and pH 7.0 (B), respectively. Both yield the expected molecular weight of 14304.2 Da [inset]. No ANS molecules are seen to bind at these pH values. The bottom two panels show the electrospray mass spectrum of reduced lysozyme in the presence of ANS at pH 2.0 (C) and pH 7.0 (D). The spectrum at pH 2.0 is distinctly richer in charge states, suggesting that the lysozyme molecule is unfolded after reduction of the disulfide bonds. No ANS molecules are seen to bind at this pH value. The molecular weight of 14312 Da [inset] is in agreement with the reduction of four disulfide bridges. At pH 7.0 at least four ANS-bound species of lysozyme are seen and the molecular weights obtained after deconvolution are indicated in the inset.

binds in the sulfonate form. These correspond to species which contain from one to seven molecules of bound ANS. It is interesting to note that the charge state distribution shifts to a lower *m/z* value, suggesting that partial folding of the protein has occurred, leading to burial of some protonatable side chains. A shift in charge state distribution is also expected when the anion form of ANS binds to the protein. Indeed, ANS is known to bind at hydrophobic pockets in proteins with ion pair formation involving positively charged amino acids providing additional stability to the complex.[6] Almost no free apomyoglobin is observed at this pH, suggesting that ANS binds with great affinity to the pH 4.0 state of myoglobin. At pH 6.0, only two ANS-bound species are detectable in the mass spectrum corresponding to one and two molecules of bound ANS. Extensive studies on apomyoglobin have established that ANS can bind at the vacant heme site of apomyoglobin.[8] NMR studies on the ANS-bound form of apomyoglobin suggest ANS is predominantly bound at the vacant heme binding site, although evidence has been obtained for secondary sites.[9] Removal of heme leads to significant destabilization of apomyoglobin at near-neutral pH.[10] A well-characterized molten globule state of apomyoglobin is known to form between pH 3.5 and 4.5, conditions under which maximum ANS binding is observed.[11] The mass spectral results are in complete agreement with the conclusions from other solution studies. Similar results are observed with holomyoglobin; although at pH 6.0 no ANS binding is seen, as the heme now occupies the heme binding site and the protein is completely folded.[12]

In the case of bovine heart cytochrome c (pI = 8.0), no ANS binding is observed at pH 2.0 and 6.0. ANS-bound forms of cytochrome c can be clearly detected between pH 3.0 and 5.0. At pH 3.0, two species are seen with charge states corresponding to cytochrome c and a complex containing a single molecule of bound dye. ANS binding is maximal at pH 4.0, with as many as six ANS-bound species being seen; species with two molecules of ANS bound are the most predominant. The folding behavior of cytochrome c has been well characterized and it is known that cytochrome c forms a molten globule like state at pH 4.0.[13] Indeed, ESIMS data (not shown) demonstrate that under these conditions maximal ANS binding is observed; the predominant species has two molecules of bound ANS.

Figure 2 shows the mass spectra for both oxidized and reduced lysozyme (pI = 4.0) in the presence of ANS. It is clearly seen that for oxidized lysozyme no ANS binding is detected over the entire pH range 2.0−7.0. Lysozyme, which contains four disulfide bonds, does not unfold appreciably at low pH and is not known to form molten globule states under these conditions.[14] Even at low pH the protein exhibits relatively few charge states, suggesting that the folded conformation is retained. The reduced form of lysozyme at pH 2.0 has a mass spectrum that is rich in charge states, suggesting that the protein is unfolded upon loss of disulfide bridges. However, at this pH no ANS binding is observed. At pH 7.0 reduced lysozyme shows a bimodal charge state distribution (data not shown) with a distinct set of higher charge states, although much of the protein still appears to be unfolded. At this pH, lysozyme exhibits strong ANS binding, suggesting that the protein is now in a molten globule-like conformation. Indeed, ANS binding to reduced lysozyme at pH 7.0 has been reported previously.[15] The deconvoluted mass spectrum shows at least four distinct species of lysozyme, differing in the number of bound ANS molecules. In the course of this study, several proteins were investigated for ANS binding, including α-lactalbumin[14] and *Plasmodium falciparum* triosephosphate isomerase.[16] In all cases, ANS-bound species of protein were detected under conditions where molten globule structures had been postulated in solution in the pH range 2−7 (data not shown).

The present results suggest that there is a strong correlation between ANS binding observed in the gas phase and the nature of the protein in solution. Expanded molten globule states of proteins, which bind ANS in solution, appear to be maintained under mass spectrometric conditions. Electrospray ionization is a soft ionization method for coaxing protein molecules into the gas phase. Multiply charged protein ions in the gas phase appear to retain "conformational memory"; thus charge−state distributions are correlated to the solution state conformation.[17] Furthermore, the method is soft enough to permit non-covalent complexes to be detected in the gas phase.[5] Such complexes range from dye[18]- and drug[19]-bound protein, to multisubunit proteins.[20] 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. ESIMS provides a direct measurement of the number of ANS molecules bound per protein molecule. If the various species observed ionize with equal efficiency, then the observed intensities may, in fact, provide a direct measurement of the equilibrium distribution of different species in solution. The experiments reported here were carried out under mild ionization conditions. The interfacing conditions were made even "softer" (lower skimmer potentials) to look for additional ANS-bound species, but none were detected. This suggested that the ANS-bound forms indeed reflected the stoichiometry of binding in solution and that the instrumental conditions were not responsible for dissociating any bound ANS in the gas phase. While it has been suggested that stoichiometric ANS binding to proteins may have a direct correlation to the protein size,[4] our results demonstrate that there may be no simple relationship. This study suggests that ANS binding monitored by ESIMS may form a powerful tool to characterize non-native states of proteins in the gas phase.

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