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Aprotinin conformational distributions during reversed-phase liquid chromatography
Analysis by hydrogen-exchange mass spectrometry

Jennifer M. Sokol, Bryan W. Holmes, John P. O'Connell, Erik J. Fernandez*
Department of Chemical Engineering, 102 Engineers' Way, University of Virginia, Charlottesville, VA 22904-4741, USA

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Abstract

Hydrogen-exchange mass spectrometry analysis of the stable protein aprotinin during reversed-phase liquid chromatography shows both native and unfolded protein. The behavior is consistent with only two conformational states, a near-native state and a fully solvent-accessible state, with reversible interchange of species within and between the mobile and stationary phases. The amount of unfolded form is greater on C_{18} relative to C_4 alkyl modified silica surfaces. The addition of $(NH_4)_2SO_4$, Na_2SO_4, NaCl, or NaSCN to the mobile phase stabilized native conformation on the chromatographic surface, especially on the C_4 media. Finally, the retention and the proportion of denatured form increases with added salts in an order consistent with the lyotropic series, but reversed from that observed for small molecules.

Keywords: Salt effects; Mass spectrometry; Aprotinin; Proteins

1. Introduction

Reversed-phase liquid chromatography (RPLC) is a powerful tool for separating and purifying proteins, particularly protein variants. It has been shown that proteins with as little as a single amino acid difference can be effectively separated by RPLC [1]. For example, Oroszlan et al. [2] have shown that variants of recombinant human growth hormone can be efficiently separated. Commercially, preparative RPLC is considered most valuable for target proteins that are small and relatively stable. One prominent example is the isolation of recombinant insulin from variants [3] which is a key issue in the commercial insulin process [4]. However, the value of preparative RPLC can be limited by several factors, including safety and environmental problems associated with the use of organic solvents, low recoveries from the denaturing mobile and stationary phases involved, and the appearance of several kinds of complex chromatographic behavior.

One class of complex behavior is the elution of broad, shouldered, or multiple peaks, even when a pure protein sample has been injected [5,6]. This is probably due to protein conformers being separated [2,7]. Wu et al. [5] used activity analysis of two peaks to show that an early eluting species was native protein while a later eluting species had been

*Corresponding author. Tel.: +1-434-924-1351; fax: +1-434-982-2658.
E-mail address: erik@virginia.edu (E.J. Fernandez).

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reversibly denatured. This elution order is likely to be due to increased stationary-phase exposure of hydrophobic residues in the unfolded protein [5,8]. The analysis of Melander et al. [9] showed that complexities can arise by interconversion of different species if its rate is comparable to the chromatographic time scale, a view which can be applied to interconverting protein conformations.

Added salts have been shown to significantly alter the chromatographic behavior of proteins during RPLC. Early in the development of protein RPLC, the use of various salts as ''ion-pairing'' agents was shown to significantly alter retention of small molecules, peptides, and proteins. Both hydrophobic [10] and hydrophilic salts [11] were shown to be effective for this purpose. Relatively high concentrations of salts can be used to modulate protein RPLC selectivity [12] and peak shape [13].

In general, the effects of salt on protein adsorption, conformation, and chromatographic behavior in RPLC have not been extensively studied. In contrast, salt effects on the retention of small molecules have even been correlated such as with molal surface tension increments and lyotropic series position for both hydrophobic interaction chromatography (HIC) and RPLC [14–16]. For proteins, salts can influence stability as well as adsorption, and it is well known that salts with greater molal surface tension increments (e.g. SO_4 salts) stabilize proteins in solution [17]. It is appropriate to consider if the combined effects of salts on protein stability and adsorption may explain some of the complex behaviors of RPLC and exceptions to the lyotropic series ordering in HIC [18,19].

Several methods have been used by previous investigators to investigate protein conformational changes on RPLC surfaces. With Raman and FTIR spectroscopy, Sane et al. detected changes in protein secondary structure and identify a late eluting peak as non-native [20]. Fluorescence has also been used as a sensitive measure of tertiary structure after elution [21] and during adsorption [22]. Recently, hydrogen–deuterium isotope exchange (HX) detected by nuclear magnetic resonance (NMR) has been used as a sensitive measure of changes in solvent accessibility [8,12,13]. This approach can be applied to any stationary phase and can reveal structural changes at many sites within a protein. However, one significant limitation of these previous approaches is an inability to give the distribution of conformers.

For modeling purposes, it is important to know how many protein species are present, and what the adsorption–desorption properties of such species are. Fortunately, proteins often tend to change conformation in a cooperative manner, and models of protein denaturation as a two-state process have been used productively to determine protein stability [23]. Models of simultaneous chromatography and interconversion of two species have also been developed [9]. Thus, it is reasonable to begin analysis of denaturation during RPLC with the four-state model shown in Fig. 1. Here, protein in the mobile phase exists as either native protein, or as a single, unfolded protein species. Our previous studies of lysozyme during RPLC show that the unfolded protein species may be completely denatured, or only partly unfolded [8]. The distinction of form is immaterial as long as only one non-native protein species is present in significant amounts along with the native species. Both of these forms can be adsorbed on the chromatographic surface so the four species can reversibly interchange according to Fig. 1. While this approach may not be strictly or generally true, NMR has shown that aprotinin behavior in RPLC columns can be described as reversible [12,13].

In the present work, we have investigated whether a small number of conformational states adequately describes protein adsorption and conformational change on RPLC surfaces. Changes in chromatographic behavior and the distribution of conformational states have been explored as a function of

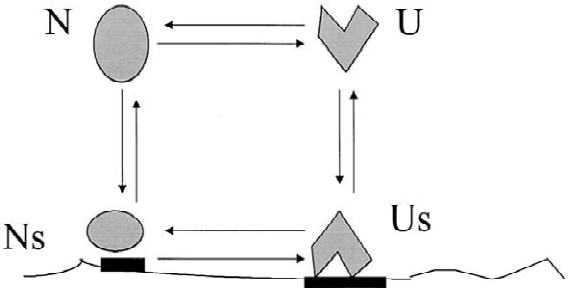

Fig. 1. A simple four-state model of protein unfolding and adsorption.

stationary phase chemistry (C_4 and C_{18} ligands) and the use of added salts spanning the lyotropic series. Hydrogen–deuterium exchange detected by mass spectrometry (HX-MS) was used to reveal the conformational distribution of a model protein, aprotinin, adsorbed to, and eluted from, an RPLC surface. Aprotinin was considered relevant to preparative RPLC because of its low molecular mass and its relatively high stability arising from two disulfide bonds.

2. Experimental

2.1. Chromatography materials

All chromatography was performed on an Akta Explorer provided by Amersham Biosciences (Uppsala, Sweden) at ambient temperature. Prepacked C_4 and C_{18} reversed-phase columns (25 cm×4 mm) and aprotinin were gifts of Novo Nordisk (Gentofte, Denmark). Deuterium oxide (^2H_2O) was obtained from Cambridge Isotopes (Andover, MA, USA). Acetic acid, ammonium sulfate, sodium acetate, and sodium sulfate were obtained from Fisher Scientific (Fairlawn, NJ, USA), potassium phosphate, sodium chloride, ethanol, and 2-propanol were obtained from Sigma (St. Louis, MO, USA), and sodium thiocyanate was obtained from Mallinckrodt (Paris, KY, USA).

Loading and labeling buffers were prepared as 10 m*M* phosphate in either H_2O or ^2H_2O. The pH was adjusted to 7.0 for protonated solutions and 6.6 for deuterated solutions to account for isotope effects [24]. Quench buffers were prepared with 10 m*M* sodium acetate in H_2O or ^2H_2O and the pH was adjusted with acetic acid to 3.1 and 2.8, respectively. The elution buffers with 2-propanol were prepared as 30% (w/w) in quench buffer (step elution) or load buffer (gradient elution). We studied the effect of added salt, by addition of ammonium sulfate, sodium sulfate, sodium chloride, and sodium thiocyanate to all buffers at an ionic strength (*I*) of 0.375. This ionic strength was used to have a significant effect on conformation and retention while being below the solubility limit. For example, $I=0.375$ for the sulfate salts was found to be near the solubility limit in the 30% propanol mobile phase. It also allowed comparison with our prior work [8,12,13]. Columns were regenerated with a solution of 70% ethanol in 1 *M* acetic acid.

2.2. Mass spectrometry materials

Mass spectra were obtained using an electrospray ionization mass spectrometer (LCQDuo, ThermoFinnigan, San Jose, CA, USA). Protein samples were desalted and concentrated using a Vydac C_{18} guard column with a Spectra System P4000 pump system and UV 2000 detector (ThermoQuest, San Jose, CA, USA). The desalting buffers were A: 0.5% (v/v) acetic acid in ^2H_2O and B: 0.5% (v/v) acetic acid in acetonitrile (Fisher).

2.3. Step elution methods

Pre-equilibration with the loading buffer was performed until a new baseline was reached. At this point, two column volumes of load buffer were used to equilibrate the column followed by injection of aprotinin (50 mg/ml in H_2O) onto the column using a 1-ml sample loop. The column was washed with one column volume of load buffer to remove unbound protein. The sample was held on the column for 5 min before the labeling buffer (load buffer prepared in ^2H_2O) was injected for 5.25 min. The column was then washed with the appropriate quench buffer for 5.25 min. Elution buffer (see Section 2.1) was used to desorb protein into an iced test tube containing quench buffer. The column was then regenerated with three column volumes of cleaning buffer (see Section 2.1). The eluted protein was diluted with quench buffer to about 0.5 mg/ml, desalted and then analyzed by MS. Step elution was performed with no added salt, as well as with each of the above listed salts. A flow-rate of 2 ml/min was used throughout the protocol. After regeneration and prior to reuse, blank injections were used to ensure there was no carryover among runs.

2.4. Gradient elution methods

Gradient elution experiments were performed in a similar manner to step elutions though a flow-rate of 1 ml/min was used. The column was regenerated and equilibrated as above. Protein was injected using

a 10-ml sample loop and the column was rinsed and held as above. The gradient was started with buffers prepared in H_2O. The protein was loaded and the gradient was initiated in (load) buffer A. The total gradient was from 0 to 70% (elution) buffer B. When the gradient reached 25% B, the column was taken off line and the pump was rinsed with the deuterated analogs of the A and B buffers (prepared with same solutes, but in ^2H_2O) for 1 min at a flow-rate of 6 ml/min. The gradient continued with the deuterated buffers. The eluting protein was divided into three 1.5-ml fractions, collected into test tubes prefilled with 1.5 ml quench buffer and prechilled in an ice bath. After the samples were collected, the tubes were replaced in the ice bath and MS analysis was performed.

2.5. Mass spectrometry methods

Samples (100 μl) were desalted and concentrated by RPLC (Vydac C_{18} guard column) in-line, just prior to MS analysis. A segmented gradient, where the percentage of B is increased, held steady, then increased again, was used at a flow-rate of 0.5 ml/min. To minimize exchange during these steps, the sample loop and guard column were packed in ice. A splitter was used to keep the MS off line until the salt had passed through the system, as detected by UV. The +6 charge state was converted to molecular mass to obtain the mass spectra shown. No changes in the charge state envelopes were observed under different conditions. This is consistent with the idea that during analysis the protein had already returned to the same conformation for all experiments after quenching and prior to MS analysis. The spectra obtained during protein elution were averaged and analyzed to determine the molecular mass of the protein species. All chromatographic and mass spectrometry experiments were replicated at least once to ensure reproducibility.

3. Results and discussion

3.1. Step elution chromatography

Aprotinin was subjected to both step and gradient elution chromatography with hydrogen-exchange labeling performed as described above. For step elution chromatography, a single chromatographic peak was observed under all conditions (data not shown). After the protein was eluted, it was analyzed as a single pool by MS as described below. A step from 0% propanol to 30% propanol was chosen to ensure that essentially all of the protein would be eluted quickly, limiting the time of unwanted hydrogen exchange.

3.2. Gradient elution chromatography

Gradient elution experiments were also performed using C_4 and C_{18} stationary phases and a range of salts spanning the lyotropic series. As shown in Fig. 2, the shapes of the chromatographic peaks observed under various salt conditions contained shoulders, complex tailing behavior, and peak broadening, suggesting that there was an imperfect separation of different conformers. This behavior was more pronounced for the C_4 column. Chromatographic peaks on the C_{18} column were generally more symmetric and narrower. As expected, retention was always greater on the C_{18} media at the same mobile phase

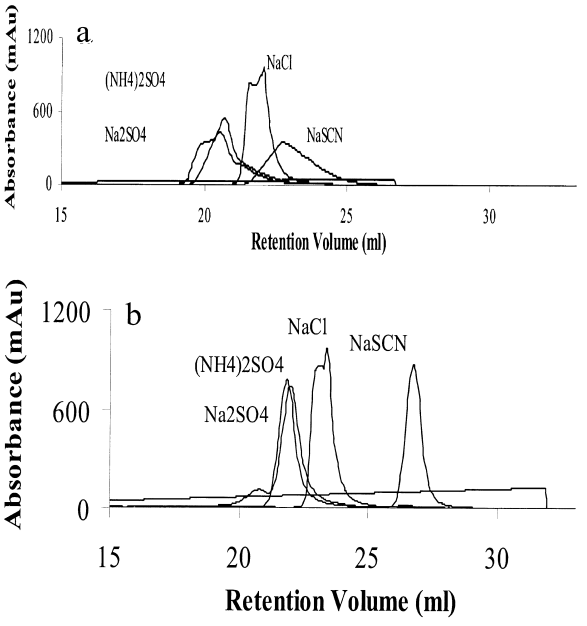

Fig. 2. Chromatograms obtained in gradient chromatography under various salt conditions for (a) C_4 column and (b) C_{18} column.

Table 1
Aprotinin recovery as a function of stationary and mobile phase conditions. Recovery is defined as the mass ratio of eluted protein to injected protein

Salt	Stationary phase	
	C_4	C_{18}
$(NH_4)_2SO_4$	98	92
Na_2SO_4	98	91
NaCl	97	91
NaSCN	95	91

condition. The elution order with different salts was the same on each surface.

The recovery of aprotinin also varied with mobile and stationary phase conditions. For these purposes, recovery was defined as the mass ratio of eluted protein to injected protein. More protein was recovered from the C_4 surface than from the C_{18} surface, as shown in Table 1. The kosmotropic sulfate salts yielded the highest recoveries and the chaotropic NaSCN the lowest. Note that the recovery was almost identical for all salts on the C_{18} surface, as was the peak shape, indicating that the choice of surface is probably more important than the choice of salt.

3.3. HX-MS analysis of step elution data

Hydrogen–deuterium exchange detected by mass spectrometry was used to investigate the conformations of species present on the chromatographic surface during step-elution experiments. Because MS provides the distribution of labeled masses, the distribution of conformational states could be determined. The labeling protocols used to investigate different contributions to the unfolding are shown in Fig. 3. For the labeling experiments involving adsorption, protein was adsorbed on the column in protonated buffer and held for 5 min before elution. During the surface labeled experiment, the protonated buffer was switched to a deuterated buffer after 5 min for labeling particles. A pH 3 buffer was then injected for 5.25 min to partially quench exchange. The protein was eluted into an ice bath to better quench exchange, and collected for RPLC desalting and MS analysis. A simple labeling experiment involving 5 min of labeling after step elution showed no significant difference in labeling compared with protein labeled for the same time without prior exposure to the surface under native conditions. This suggests that the protein refolds quickly after elution.

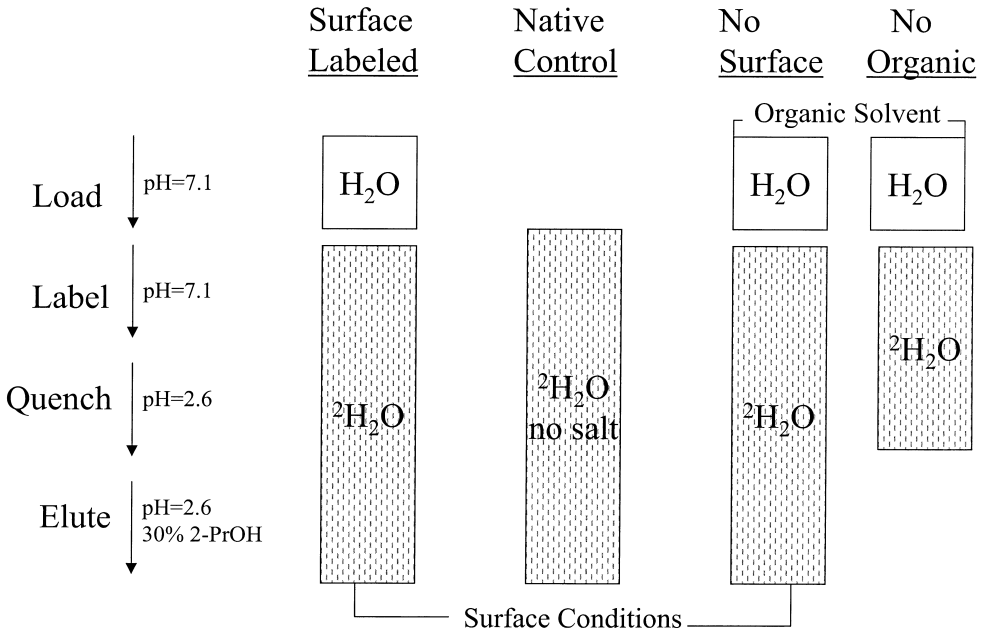

Fig. 3. Exchange conditions for step elution and control experiments.

This is consistent with our prior observations that, under a variety of RPLC conditions, aprotinin gives native chemical shift values throughout the two-dimensional NMR spectrum after elution [12].

The native state was initially examined using the protocol shown in Fig. 3. The protein was dissolved in deuterated water and underwent exchange for 10.5 min before being quenched. A single peak with a molecular mass of 6582 was obtained (see Fig. 4(a)). This value is 69 u greater than the natural abundance mass, consistent with deuterium labeling of certain rapidly exchanging side chain and amide backbone hydrogens (approximately one per residue). To determine the degree of labeling observed for fully exposed protein, a ''fully solvent exposed'' experiment was performed under the same conditions as the native experiment, except for the addition of 20% β-mercaptoethanol (BME) as a reducing agent, 4 *M* guanadinium hydrochloride (GdnHCl) as a denaturant, and heating to 100 °C for 5 min followed by immediate cooling. These conditions were chosen because they have been shown to result in fully denatured aprotinin [25]. The measured mass for the fully solvent exposed labeling experiment was 6625, 112 u greater than the native molecular mass (see Fig. 4(b)). The protection in solution of 43 amides represents ~74% of the amide backbone, consistent with the presence of a very stable hydrophobic core. This is slightly higher than the number of protected reporter groups (29 groups) used in our previous NMR studies of aprotinin [13]. For the labeling experiments involving adsorption, protein was adsorbed on the column in protonated buffer and held for 5 min before elution. During the surface-labeled experiment, the buffer was switched to a deuterated buffer after 5 min. Exchange was then quenched by lowering the pH of the buffer. The protein was eluted into an ice bath, and collected for RPLC desalting and MS analysis.

To distinguish the denaturing effects of the surface from any undesired hydrogen exchange caused by the 2-propanol used for elution, two labeling experiments were performed without the presence of the chromatographic surface. Firstly, in a ''no surface'' experiment, the protein was exposed to all of the same mobile phase conditions as in the adsorption experiments, without being adsorbed on the surface. Any labeling difference between this experiment and the one incorporating the chromatographic surface would reveal unfolding that occurred during the labeling period while the protein was adsorbed. Secondly, a ''no organic'' labeling experiment was performed under the same conditions as the ''no surface'' experiment, but without organic cosolvent. This experiment was performed to determine whether any unfolding which might occur in the presence of the eluting solvent could result in significant exchange, despite the quenching conditions used. The labeling times for both experiments matched the surface exchange experiment. Both the

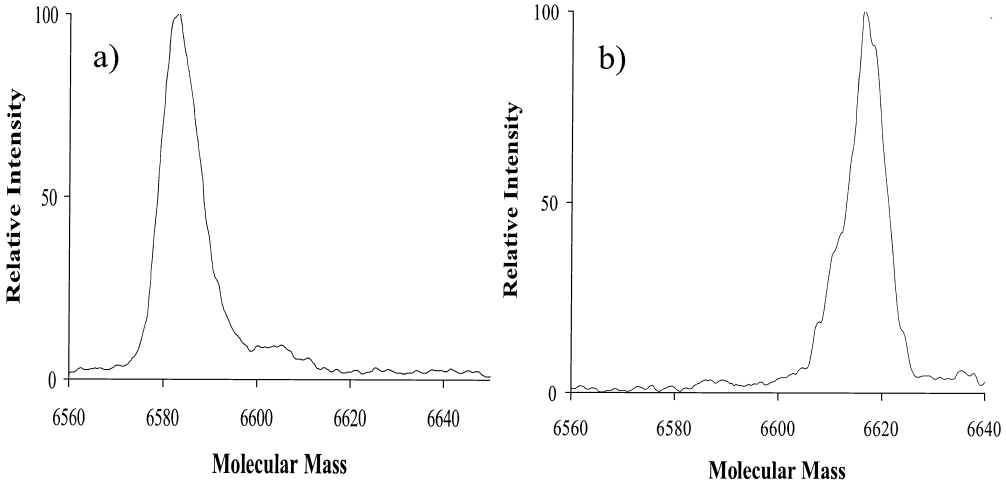

Fig. 4. Mass spectra from (a) native and (b) fully solvent exposed controls.

''no surface'' and ''no organic'' experiments yielded mass spectra (data not shown) containing single peaks with centroids quite consistent with the control experiment under native conditions, as shown in Fig. 4(a). Thus, it can be concluded that the mobile phase conditions during adsorption did not cause denaturing, and that there was no undesired labeling due to the presence of organic in the elution step. A single peak in the hydrogen-exchange mass spectrum was observed in the presence of all four salts examined (data not shown). This is not unexpected for such a stable protein [25].

Fig. 5 shows the mass of the protein species eluted under all mobile and stationary phase conditions. The ''no organic'' and ''no surface'' experiments yielded molecular masses that were very similar to each other and to the native, regardless of the salt used. Thus, the mobile phase conditions, even including organic modifier did not dramatically increase hydrogen exchange. This indicates that the solvent accessibility and the protein conformation are native under all these conditions.

Protein samples which were allowed to exchange while adsorbed to the C_4 and C_{18} media (''surface labeled'' protocol in Fig. 3) produced altered mass spectra as shown in Fig. 6. At high salt concentrations, two mass peaks were observed. The widths of the peaks are ~20 u, consistent with those observed for the native protein (see Fig. 4(a)). The peak width in the native condition is due mainly to

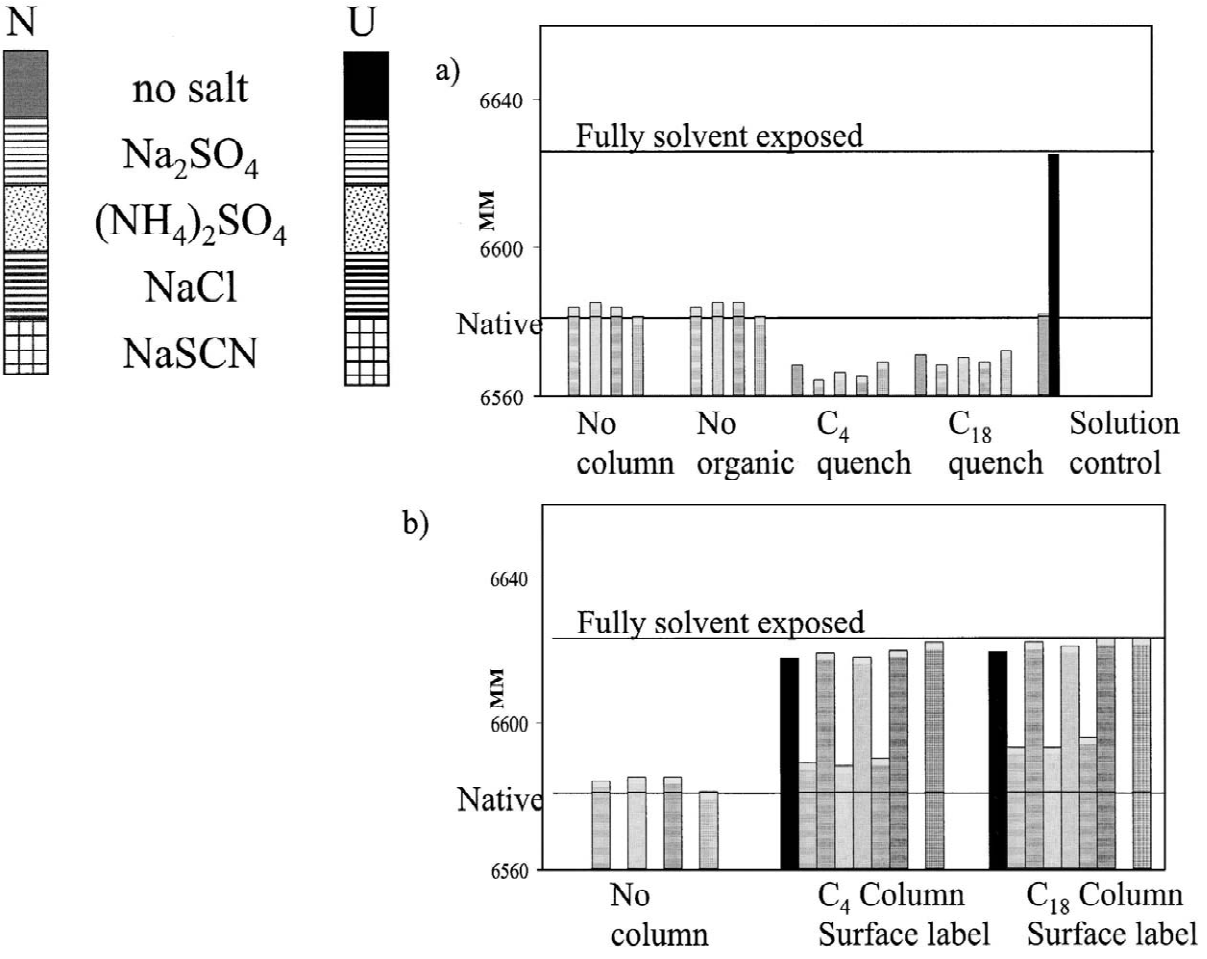

Fig. 5. Molecular mass of protein found during step elution, (a) no surface exposure and (b) surface exposure conditions.

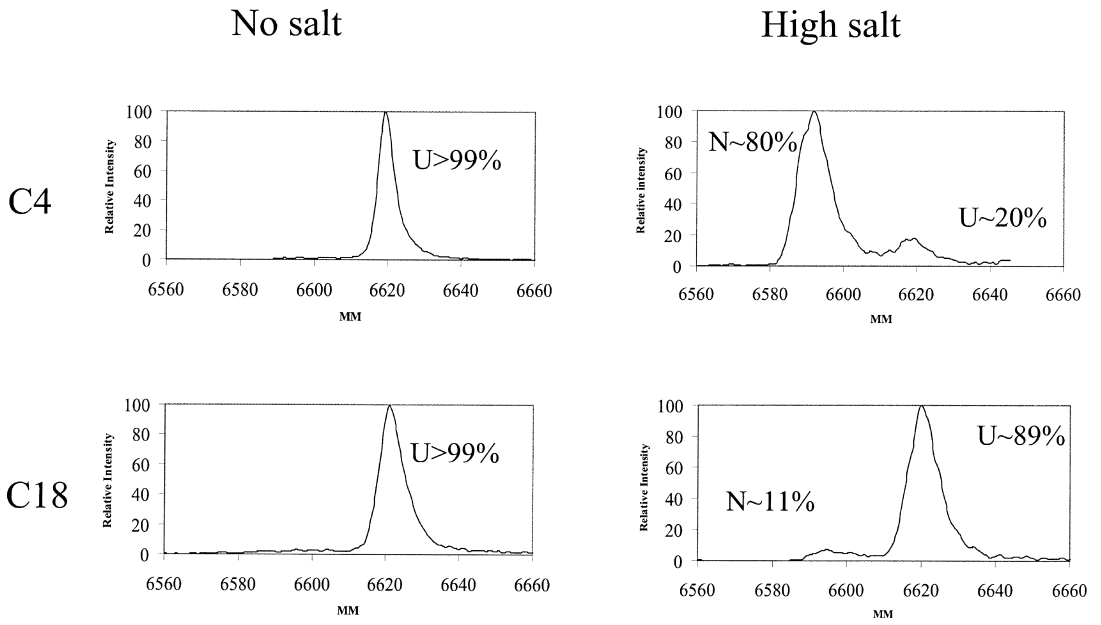

Fig. 6. Step-elution mass spectra for most stable and most denaturing conditions. Added salt is Na_2SO_4. Percent unfolded protein is also shown.

the natural abundance isotope distribution (e.g. ^{13}C and ^{15}N vs. ^{12}C and ^{14}N), with a small contribution arising from the instrument. Since no intermediate peaks nor broader distributions were observed in the mass spectra, aprotinin unfolding appears to have remained cooperative on the chromatographic surface, with only two species being present. The low mass peaks in the spectra of Fig. 6 have molecular masses consistent with native aprotinin and the high mass peaks are consistent with fully solvent exposed protein. While intermediates have been observed in the folding of many proteins [26], the folding of aprotinin is two-state in solution [27]. In prior HX-MS studies, intermediate peaks and broad distributions have been observed for proteins that have kinetic folding intermediates [28]. While we have not performed a detailed pulsed-labeling study here, there is no evidence for significant amounts of any such intermediates in our mass spectra. This suggests that, for aprotinin, we need consider only two conformational species on the surface and in the solution: native and fully solvent exposed.

Fig. 5 also shows the molecular masses for the surface labeling experiments on the C_4 and C_{18} RPLC media. Comparison of M_r observed under C_4 conditions with those under C_{18} conditions shows that the fully solvent-exposed species has the same degree of solvent exposure on both surfaces, while the native species shows a slight increase in exchange on the C_{18} surface. This may arise from an increase in local unfolding/refolding behavior of the protein on the C_{18} surface, allowing increased solvent exposure time to a few residues, though keeping the global structure intact. It is also possible that undesired labeling of the protein during the quench and elution steps could be enhanced on the C_{18} surface due to stronger binding and/or more unfolding upon exposure to elution conditions. Another explanation is that the native structure may be slightly different on the C_4 and C_{18} surfaces. We are unable to distinguish among these possibilities. Fig. 5 also shows that varying salt type did not have a significant effect on the masses of the peaks from either the C_4 or C_{18} media. Thus, while changes in salt may change the amount of each species, they do not significantly alter their structures.

It is interesting to note that even a protein as stable as aprotinin can become fully solvent-exposed in an RPLC environment. Aprotinin melts at greater than 100 °C and 4 *M* GdnHCl [25]. However, Fig. 6

shows that in the absence of salt both the C_4 and C_{18} hydrophobic RPLC surfaces produce only a single, high mass peak associated with essentially the fully labeled species—i.e. completely solvent-accessible aprotinin. It should be noted that because the quench step in the labeling protocol is applied before elution, the labeling reflects conformation during the adsorption step and *before* elution. Thus, this experiment demonstrates that both the C_4 and C_{18} surfaces alone can render fully solvent-accessible this highly-stable protein. Certainly the disulfide bonds of aprotinin provide much of its stability. Others have noted that complete unfolding of the protein produces predictable behavior, such as its estimated contact area (*Z* parameter) being proportional vs. molecular mass [29,30]. Consequently, reducing the disulfides should modify chromatographic behavior. Nonetheless, Fig. 6 also shows that added salt (I= 0.375) can increase the fraction of protein with native-like exchange solvent accessibility on an RPLC surface. Further, while only about 10% of the protein is native in the presence of salt on the C_{18} media, 80% of the protein is native on the C_4 surface under the same mobile phase conditions. Thus, hydrophobicity of the C_{18} surface appears to dominate over the stabilizing effect of salt, while the stabilizing effect of the salt dominates on the C_4 surface.

3.4. Salt effects during gradient elution

The hydrogen exchange labeling protocols for gradient elution (Fig. 7) were similar to those for step elution (Fig. 3). In the case of the gradient elution, the switch to the deuterated buffer was performed in the middle of the gradient, before any elution of protein, as indicated in the gradient elution chromatogram and diagram of Fig. 8. Because the gradient elution chromatography showed complex peak shapes (Fig. 2), we collected the eluting protein in three separate and equal volume fractions to determine whether the peak shapes were caused by partial resolution of multiple conformers. As an example, the gradient elution chromatogram and fractionation are shown in Fig. 8 for the ammonium sulfate case.

The conformational composition of each fraction was determined by HX-MS. Fig. 9 shows the resulting mass spectra for the protein eluted from the C_4 column in the presence of $(NH_4)_2SO_4$. For these and all other conditions analyzed, the recovered protein was either native or unfolded (Fig. 9 and data

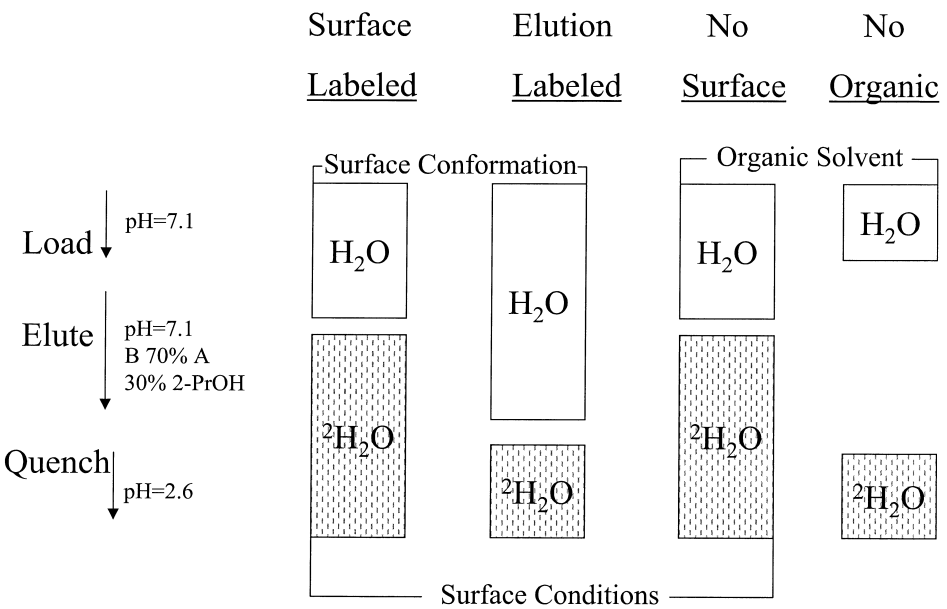

Fig. 7. Exchange conditions for gradient-elution and control experiments.

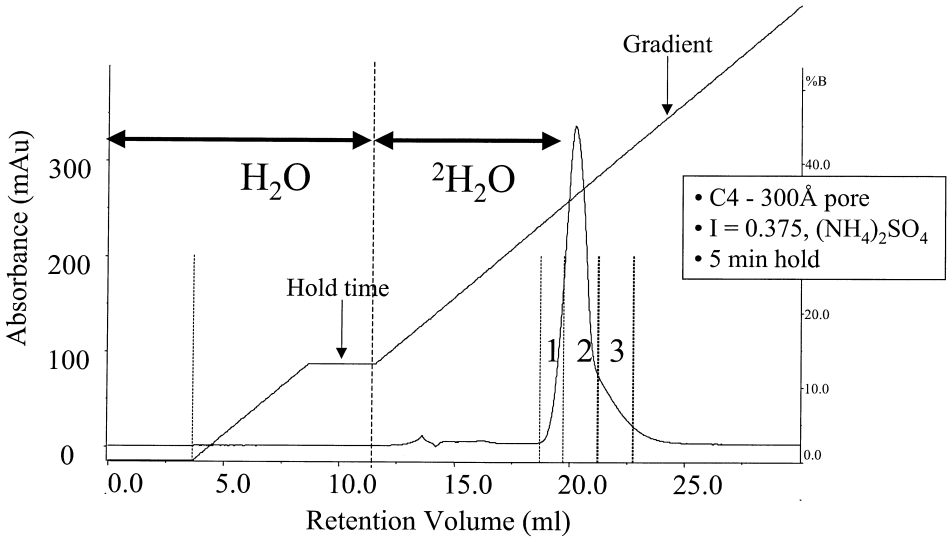

Fig. 8. A sample gradient-elution chromatogram showing when fractions 1, 2, and 3 are collected, and where the buffer switch occurs.

not shown), as with the earlier step-exchange experiments. The proportion of native protein in each fraction is indicated in Fig. 10 for each of the conditions studied. The composition of the fractions was clearly different, with the earlier fractions consistently containing more native protein. The percentage of native protein on the surface in each fraction decreased following the lyotropic series, with the most chaotropic salt, NaSCN, producing the most denaturation.

The chromatographic results (Fig. 2) also show that retention order follows the lyotropic series, with the most chaotropic salts producing the greatest retention. Interestingly, this trend is opposite to that observed for small molecules in RPLC, as Melander and Horvath [14] showed that salts with lower molal surface tension increments (more chaotropic salts) decrease the retention of small hydrophobic solutes on RPLC media. Of course, any specific interactions of salts with salts or additives by ion-pairing [10,11] could also influence the strength of adsorption. Our opposite trend with aprotinin coincides with greater unfolding of the molecule, which would be expected to increase the strength of protein adsorption on a hydrophobic surface. Thus, salts cause competing effects on adsorption and stability, resulting in complex peak shapes when the time scale of the folding/unfolding reaction is comparable to that of the chromatography. In this particular case, the destabilizing effect of the salts dominates, producing a salt type–retention relationship opposite to that observed for small molecules.

It should be noted that although the chromatograms for both surfaces show single peaks with somewhat complex shapes, the mass spectra are considerably different. Further, while different salts (such as Na_2SO_4 and NaSCN) yield to somewhat different solution conditions, there are apparent consequences on the equilibrium constants for adsorption and elution, even on the same surface. Finally, as predicted by lyotropic series, the two sulfate salts have similar retentions and peak compositions; the cation has little effect.

In addition to losing less protein on the C_4 column than on the C_{18} column, the fraction of eluted protein that remained native on the surface was greater for the C_4 column (Fig. 10) for all salt conditions studied. The greatest native fraction on the C_{18} surface was less than the lowest native fraction on the C_4 media. The more hydrophobic surface always had a greater percentage of solvent-exposed protein.

Previous work in this lab using hydrogen exchange detected by NMR yielded a residue level analysis of this system [12,13]. Although information about conformational distributions could not be obtained in those studies, distinct types of exchange

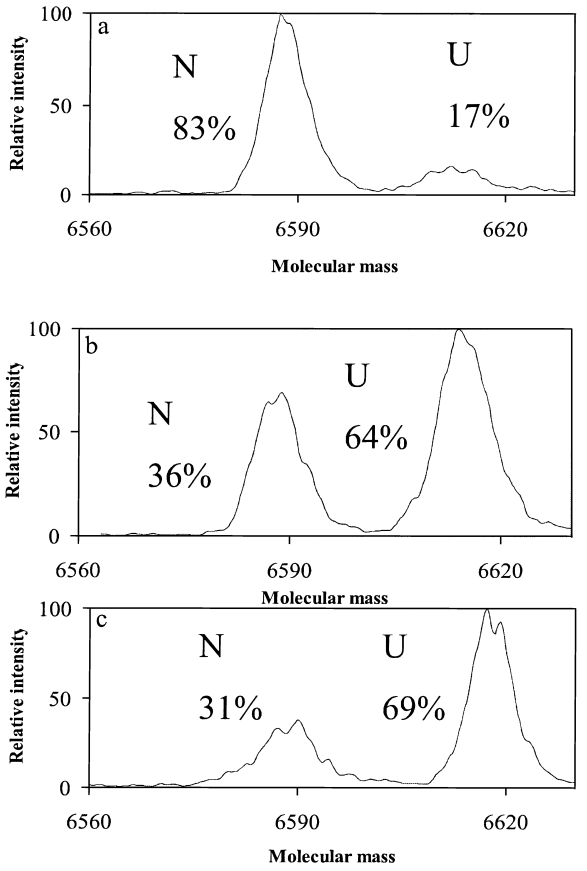

Fig. 9. C_4 column $(NH_4)_2SO_4$ MS spectra and percent native vs. percent unfolded protein for fraction (a) 1, (b) 2, and (c) 3, with percentages of native and unfolded protein indicated.

patterns along the polypeptide chain were observed. Under some conditions, the amount of exchange was similar for many residues, while for other conditions, the exchange showed a pattern of protection consistent with retention of native, particularly beta-sheet, structure. With NMR, the conditions showing the greatest contribution of structured component were the same as the conditions of smallest unfolding in this study (C_4, Na_2SO_4) [12]. The conditions which showed the least protection by HX-NMR are consistent with those having no native species in this study (C_{18}, no salt) [13].

NMR showed some profiles with a native protection pattern of smaller amplitude. There are two possible explanations for this. One possibility is that a mixture of species is present; the other is that there is a partially unfolded protein [31,32]. For the reduced amplitude patterns observed by McNay, no distinction between these possibilities could be made. The NMR conditions that led to these intermediate profiles (C_4, NaCl) [12], were the same as those for this study with a mixture of two species. In the present study, these conditions clearly lead to a mixture of native and fully unfolded species (Fig. 6). Thus, we find that NMR and MS are both valuable analysis tools, yielding complementary information.

4. Conclusions

Aprotinin, though a stable protein in solution, can

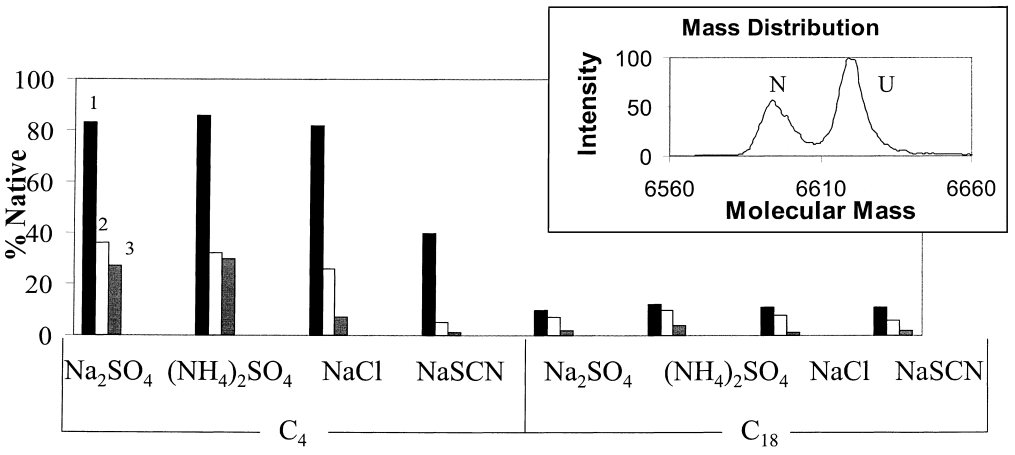

Fig. 10. Percent native for each fraction under all gradient-elution conditions analyzed by MS peak areas.

become fully solvent-exposed when adsorbed on a hydrophobic surface, such as in RPLC. However, unfolding remains cooperative in the presence of such a denaturing surface, so that only native-like and unfolded protein species are detected. This means that a four-state model, as shown in Fig. 1, should be sufficient to characterize this system. The addition of salt to the mobile phase can significantly stabilize native protein conformation on chromatographic surfaces, particularly on media with less hydrophobicity. Salt type affects protein retention time by changing the degree of protein unfolding on the surface. While we report here results only for aprotinin, it is expected that variations of retention and fraction of unfolded form may occur for other proteins. This could promote increased separation in RPLC. HX-MS is a useful technique for studying protein behavior in the presence of RPLC surfaces, avoiding column packing interference on optical techniques and being both complementary and much more rapid than NMR.

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