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Journal of Chromatography A, 849 (1999) 149–159

JOURNAL OF CHROMATOGRAPHY A

Protein structure perturbations on chromatographic surfaces

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Received 10 February 1999; received in revised form 21 April 1999; accepted 21 April 1999

Abstract

Amide I band Raman spectroscopy was used to quantify the secondary structure contents of proteins adsorbed on ion-exchange and reversed-phase materials. Neither ribonuclease A, a rigid protein, nor α-lactalbumin, a flexible protein, exhibited any significant secondary structural change on adsorption to an agarose-based cation-exchange support. On reversed-phase supports, however, lysozyme demonstrated a significant perturbation in secondary structure in the adsorbed state as compared to its structure in solution. For a constant concentration of adsorbed protein, the perturbed structure of adsorbed lysozyme was relatively insensitive to mobile phase conditions. However, the extent of structural change decreased as the concentration of adsorbed protein decreased. In agreement with the Raman spectroscopic characterization, reversed-phase linear gradient elution of lysozyme produced two peaks: a weakly binding peak corresponding to the native state and a strongly binding peak corresponding to the denatured state. The results presented in this paper demonstrate the utility of the Raman spectroscopic technique for in-situ characterization of protein secondary structures and their use in the molecular-level interpretation of protein retention behavior.

Keywords: Raman spectrometry; Protein folding; Stationary phases, LC; Proteins; Ribonuclease; Lactalbumin; Lysozyme

1. Introduction

Proteins are known to undergo structural changes during certain chromatographic separations [1–4]. These changes in protein structure may occur in all types of chromatographic columns, though the phenomenon is most prevalent in reversed-phase liquid chromatography [5–7]. Reversed-phase liquid chromatography uses a nonpolar stationary phase, an organic solvent as a mobile phase modifier, and an acidic mobile phase, each of which is potentially denaturing. Structural changes have also been observed in hydrophobic interaction chromatography (HIC) [8]. This technique employs a mildly hydrophobic stationary phase and high salt concentrations in the mobile phase to drive the adsorption of proteins onto the chromatographic surface. In ion-exchange chromatography there is no direct evidence of surface-induced structural changes. However, ion-exchange surfaces have been found to accelerate thermal or urea-induced denaturation processes [9,10].

In denaturing systems, a protein may become distributed among multiple conformational states during its passage through the column. These conformers may have distinct adsorption behaviors leading

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0021-9673/99/$ – see front matter
PII: S0021-9673(99)00554-3

to band broadening and/or multiple peaks in the elution profile [1,11,12]. Such conformational changes in chromatographic systems have several implications. In preparative-scale chromatography, if the protein of interest denatures irreversibly, the yield of active protein for the process step will be decreased. On the analytical scale, conformational effects could lead to errors in parameter estimation for adsorption/desorption models and in sample purity analyses. On a more fundamental level, conformational changes, if ignored, could lead to misinterpretation of adsorption mechanisms. Hence it is important to understand how and why denaturation occurs and what effects denaturation can have on chromatographic performance.

Researchers have employed a range of approaches to characterize proteins in chromatographic systems. The majority of these investigations involved simple isocratic or linear gradient elution studies to obtain information on protein denaturation kinetics and thermodynamics [12,13]. In those studies, changes in the retention times and *z*-numbers (the slopes of log–log plots of protein dynamic capacity factors versus mobile phase modifier concentrations) of a variety of proteins were studied as a function of chromatographic conditions. While these studies yielded qualitative information, little quantitative information on the structural states of proteins adsorbed on stationary phases was obtained. Accordingly, it is difficult to compare the extent of denaturation from one experiment to another. Furthermore, this approach does not permit adsorption behavior to be related to adsorbed protein structure.

Circular dichroism (CD) [14–16] and Fourier transform infrared spectroscopy (FT-IR) [4] have been used to characterize protein structures in solution before and after elution from a chromatographic column. However, spontaneous refolding of proteins after desorption complicates interpretation of these results. The post-exposure conformation may be an inadequate representation of the state of the protein when it is actually interacting with the media.

Several previous investigators have attempted to characterize the structures of proteins in the adsorbed state. Kondo and Mihara [17] used CD to estimate the secondary structure contents of proteins adsorbed on silica particles. However to minimize light-scattering effects, these experiments were limited to unfunctionalized nano-particulate materials. Boulkanz et al. [7] used FT-IR to characterize the structure of human serum albumin adsorbed on a reversed-phase support. The secondary structural changes reported by the authors were, however, within the structural estimation errors typically encountered in FT-IR measurements. In addition, the large background contribution of water to the amide I infrared signal often makes reliable estimation of protein structure in the adsorbed state difficult. To circumvent this problem, Katzenstein et al. [6] used deuterated mobile phases during FT-IR spectroscopic characterization of proteins on C_8-silica bonded silica beads. While these authors observed significant changes in the amide I absorbance spectra, they could not quantify the structure of the adsorbed proteins.

Fluorescence spectroscopy has been used to characterize the tertiary structures of proteins adsorbed on flat surfaces [18,19], nano-particles [20] and HIC and reversed-phase supports [6,8]. This technique, while very sensitive, provides information only about the microenvironment of tryptophan and tyrosine residues in the protein. Also, in the case of a protein containing multiple tryptophan or tyrosine residues, interpretation of steady state fluorescence spectra becomes difficult.

Recently, McNay and Fernandez [21] have used nuclear magnetic resonance (NMR) spectroscopy and amide proton–deuteron exchange techniques to study the unfolding of lysozyme adsorbed on reversed-phase supports. The great strength of this technique is that individual residues that have amide protons that are exchangeable in the adsorbed state and subsequently protected from exchange upon desorption may be identified; that is, residues that participate in transient unfolding phenomena on the surface can be identified. The limitations of this technique are that amide protons which are exchangeable in the native state cannot function as reporter groups and the structure of the protein in the adsorbed state is indeterminate.

Amide I band Raman spectroscopy is an alternative, quantitative technique for the characterization of the secondary structures of proteins. Some of the advantages of Raman spectroscopy include its suitability for protein samples in solid, solution, suspension, immobilized and adsorbed states with

minimal preparation. We have recently developed a new structure estimation algorithm that accurately accounts for solvent, stationary phase and fluorescence contributions to sample spectra and reliably estimates the secondary structure contents by a deconvolution of the underlying amide I band signal [22].

In this investigation, amide I band Raman spectroscopy was used to characterize proteins adsorbed on standard chromatographic materials. Quantitative secondary structure estimates were obtained for adsorbed proteins under different stationary phase and mobile phase conditions. Finally, linear gradient elution experiments were performed to correlate the observed secondary structural changes with the retention behavior in a chromatographic column.

2. Experimental

2.1. Equipment

All Raman spectra were collected on a Jobin Yvon T64000 Raman system (ISA, Edison, NJ, USA) with a triple monochromator operated in additive mode. The excitation source was an argon ion laser operated at 514.5 nm (Model 95, Lexel Laser, Fremont, CA, USA). The laser power at the source ranged from 33 to 100 mW, depending on the sample. A liquid nitrogen cooled CCD with a chip size of 1 in.×1 in. was used as a detector (1 in.=2.54 cm). Spectral resolution was ~0.6 cm^{-1}. All linear gradient elution experiments were performed on an Akta Explorer integrated chromatography system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.2. Chemicals

S-Sepharose (agarose-based cation-exchange media) and Sephasil-4 (silica-based C_4-ligated reversed-phase media) bulk stationary phases were obtained from Amersham Pharmacia Biotech. A C_4-silica column (150×4.6 mm) and C_{18}-silica bulk media were obtained from Vydac (Hesperia, CA, USA). HPLC-grade ethanol was obtained from Fisher Scientific (Pittsburgh, PA, USA). HPLC-grade trifluoroacetic acid (TFA) and hydrochloric acid as well as the salts used were obtained from Aldrich (Milwaukee, WI, USA). Lysozyme from chicken egg white (L-7001), ribonuclease A from bovine pancreas (R-5000) and α-lactalbumin from bovine milk (L-5385) were obtained from Sigma (St. Louis, MO, USA). Water with a resistivity of greater than 18 MΩ/cm, prepared by serial treatment of tap water via microfiltration, passage through activated carbon and Purolite NRW-37SC mixed ion-exchange resin beds, reverse osmosis filtration, 185- and 254-nm ultraviolet sterilizers, and ultrafiltration through an M_r 50 000 membrane, was used throughout.

2.3. Procedures

2.3.1. Batch adsorption

For in-situ structure characterization, small batches (10 to 20 mg) of chromatographic media were first equilibrated with an appropriate mobile phase (S-Sepharose: 20 m*M* phosphate buffer with 50 m*M* Na^+, pH 6.0; Sephasil-4 and C_{18}-silica: deionized water with either a trace of HCl for pH adjustment or a mixture of 0.1% TFA and 5% to 10%, v/v, ethanol). In experiments with reversed-phase media, a small amount of salt (NaCl, NaI or KBr; 10 m*M* to 100 m*M*) was also added to the mobile phase. The salt helped quench the intense fluorescence from the stationary phase. Proteins were dissolved in the equilibrating mobile phase to make solutions with a concentration of 10 mg/ml. The stationary phase slurry was then mixed with the protein solution. Depending on the adsorbed protein concentration that was desired, the volume ratio of stationary phase slurry to protein solution ranged from 4:1 to 1:2. The samples were slowly mixed on an end-over-end rotator for 1 to 2 h. The slurries were then loaded into capillary tubes for protein secondary structure characterization. In some cases, the concentration of protein in the supernatant was determined using absorption at 280 nm. The adsorbed protein concentration was then estimated using a mass balance.

2.3.2. Raman spectroscopy

The secondary structures of proteins in adsorbed and solution states were characterized using Raman spectroscopy. The spectrometer and the data acquisition procedure have been described in detail elsewhere [22]. Capillary tubes filled with about 20 μl of

sample were mounted on the macro-sampling accessory of the spectrometer. For adsorbed protein samples, the stationary phase particles were allowed to settle to the bottom of the capillaries. Raman spectra of the settled bed of stationary phase particles and of the clear liquid supernatant were then collected. Background spectra were collected under identical conditions, but without protein. To characterize the effects of the mobile phases alone, proteins were dissolved in the mobile phases and analyzed. Background spectra of each mobile phase alone were also collected. The collection time per sample varied from 20 to 60 min per replicate. All spectra collections were performed in triplicate.

Amide I band Raman spectra in the 1500 to 1800 cm^{-1} region were analyzed for secondary structure. This procedure is based on the reference spectra approach. First Raman spectra for several well characterized crystalline proteins were collected. By correlating the spectra of these proteins in the conformationally sensitive amide I region with their secondary structure determinations from X-ray crystallography [23], amide I region reference spectra for pure secondary structure types were calculated. To analyze adsorbed or solution protein spectra, the amide I band regions were fit as linear combinations of the pure structure reference spectra and corresponding adsorbent and/or solvent background spectra. The secondary structure estimates for each protein sample were then calculated from the superposition coefficients. In this procedure, we parse secondary structure contents into five categories: (1) ordered helix, including all the α-helical residues except for two residues at either end of an α-helix; (2) unordered helix, including the two residues at each end of an α-helix as well as 3_{10} helices; (3) sheet, including all residues participating in parallel and anti-parallel β-sheet strands; (4) turn, including all the residues participating in β-turns; and (5) unordered, including all the residues not assigned to any of the first four ordered structure types. Since the amide I band spectra of unordered helix and sheet residues are very similar, it is not possible to separately estimate their fractional content in a protein of unknown structure. Accordingly contents of only four secondary structural categories (ordered helix, unordered helix+sheet, turn, unordered) are reported for each protein sample. The structure estimation procedure is fully automated and does not require any intervention or judgement on the operator's part. The full details of the spectral interpretation procedure have been described elsewhere [22]. Validation studies using model proteins with well characterized three-dimensional structures were performed: the overall averaged error in our Raman-based secondary structure estimates relative to reported X-ray structure data is about 4 structure percent [22].

2.3.3. Linear gradient elution chromatography

Experiments were conducted with reversed-phase media only. The composition of mobile phase A was water–ethanol (90:10, v/v), 0.1% TFA and 100 m*M* sodium chloride while that of the mobile phase B was water–ethanol (10:90, v/v), 0.1% TFA and 100 m*M* sodium chloride. Experiments were carried out at different mobile phase compositions (0% B to 40% B) during loading. In each gradient experiment, a final mobile phase composition of 100% B was used. The column was initially equilibrated for 10 column volumes with the appropriate starting mobile phase. Fifty microliters of a 1 mg/ml protein solution in mobile phase A was loaded onto the column and eluted using linear gradients of varying lengths. A dual wavelength UV diode detector (λ=280 nm and 215 nm) and a conductivity meter were used to monitor the protein concentration and the ethanol concentration at the column exit.

3. Results and discussion

The secondary structure analyses for ribonuclease A and α-lactalbumin adsorbed on the S-Sepharose cation-exchange gel are presented in Fig. 1. In the figure, the estimates for percent ordered helix (A), unordered helix+sheet (B), turn (C) and unordered (D) contents for both adsorbed and solution states are shown with the associated standard deviations resulting from triplicate analyses of spectra. Evidently, there is little perturbation of secondary structure of the examined proteins on adsorption. Ribonuclease A and α-lactalbumin span a broad range of backbone flexibility, with adiabatic compressibilities of $1.12 \cdot 10^{-12}$ and $8.27 \cdot 10^{-12}$ cm^2/dyn, respectively [23]. However, for these two proteins, the relative

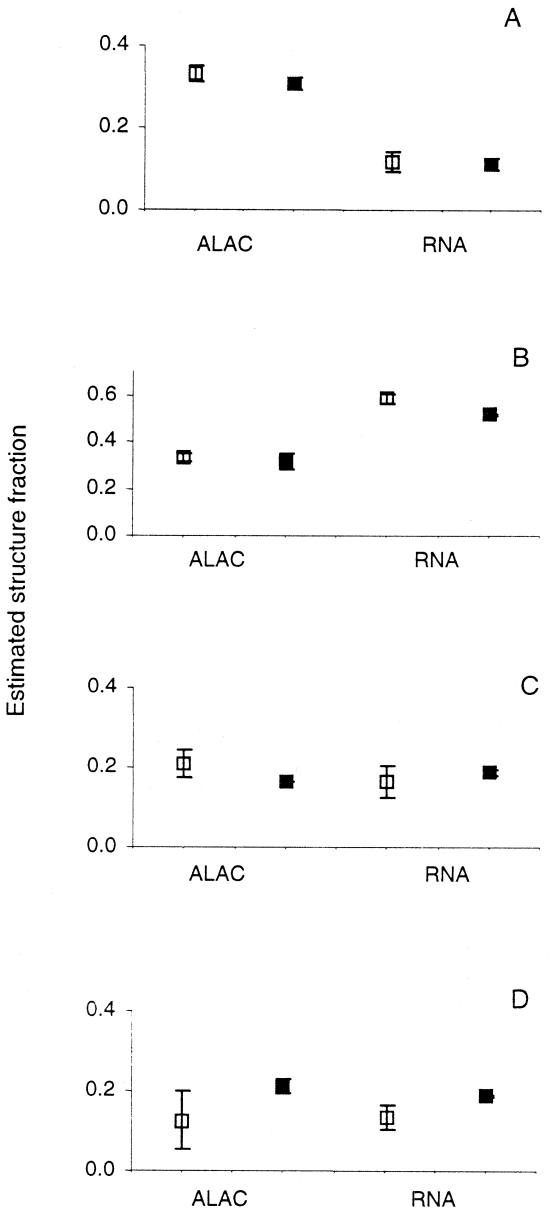

Fig. 1. Secondary structure estimates for proteins adsorbed on S-Sepharose. Individual secondary structure elements shown: (A) ordered helix; (B) unordered helix+sheet; (C) turns; and (D) unordered. Abbreviations: α-lactalbumin (ALAC) and ribonuclease A (RNA) Adsorption conditions: 20 m*M* phosphate buffer, 50 m*M* Na, pH 6.0. Symbols: □, protein in solution; ■, adsorbed protein. Error bars represent ±one standard deviation of estimates from analyses of triplicate spectra.

rigidity of the protein backbone played no apparent role in the structural response to adsorption. This result confirms the expected benign nature of the highly hydrophilic agarose-based ion-exchange gels and demonstrates the reliability of the Raman spectroscopic analysis technique as applied to proteins adsorbed on particulate surfaces.

In contrast to ion-exchange chromatography, proteins are known to be prone to structural perturbations in reversed-phase chromatography [5–7]. Experiments were conducted in reversed-phase systems using ethanol and small concentrations of salt (10–100 m*M*) as mobile phase modifiers. The secondary structure analyses of proteins adsorbed on silica-based reversed-phase media under a variety of conditions are shown in Figs. 2 and 3. In these figures, the Raman estimates for secondary structure contents (symbols) are given along with the crystallographically determined secondary structure contents (solid lines) for each experimental condition. As a control for the Raman analysis technique, the secondary structure contents of lysozyme in solution were estimated independently (data not shown) and found to be almost identical to the crystallographically assigned structure [24]. As seen in the figures, adsorbed lysozyme exhibits considerable secondary structure perturbation from its solution structure.

The effect of the mobile phase pH and the type of acid on adsorbed protein structure is examined in Fig. 2. At pH 2.1, with TFA or HCl, as well as at pH 6.5, lysozyme adsorbed on C_4-silica exhibited significant losses in ordered helix and unordered contents with corresponding gains in unordered helix+sheet content. Our estimates indicate that over 25% of the lysozyme backbone was perturbed due to adsorption on the C_4 material. The relative insensitivity of adsorbed protein structure to mobile phase pH, as seen in Fig. 2, implies that the observed structural changes were not due to acid-induced unfolding phenomena [25] at the low pH typically employed in reversed-phase chromatography, but rather due to the adsorption process itself. This is not a surprising result considering the known structural stability of lysozyme in solution below pH 2 [26].

Fig. 2 also shows the structure of lysozyme adsorbed on a silica based *n*-C_{18} reversed-phase material in the presence of the pH 2, TFA, 10% ethanol solution. Interestingly, the secondary struc-

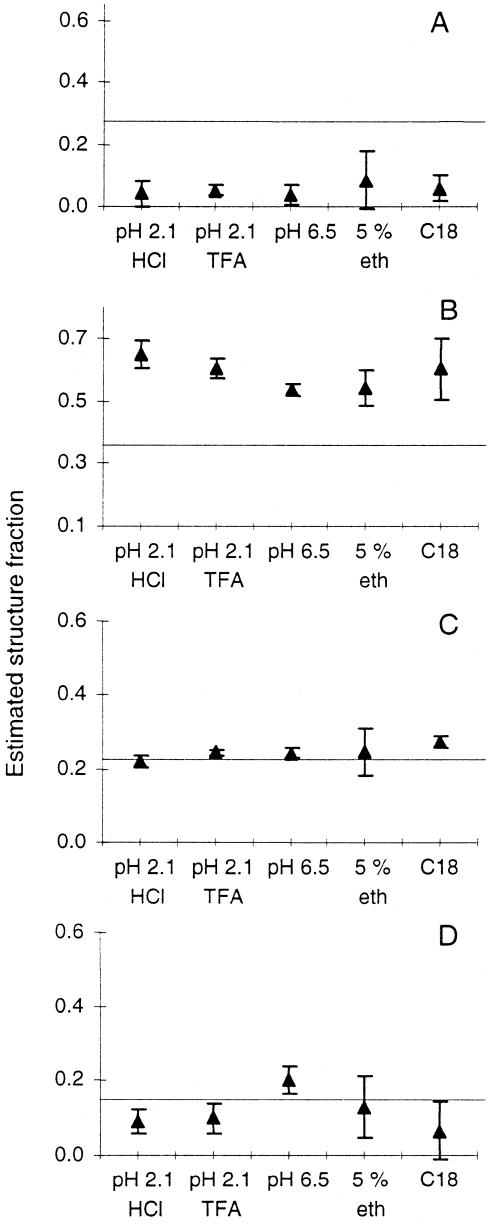

Fig. 2. Effect of mobile phase and stationary phase conditions on the secondary structure estimates for lysozyme adsorbed on reversed-phase materials. Individual secondary structure elements shown: (A) ordered helix; (B) unordered helix+sheet; (C) turns; and (D) unordered. Symbols and lines: ▲, adsorbed protein; —, X-ray structure. Base case adsorption conditions: mobile phase consists of deionized water+0.1% TFA, pH 2.1, 10% (v/v) ethanol, and 100 m*M* sodium chloride; the stationary phase was C_4-silica. Changes from the base case conditions are indicated on the *x*-axis. Error bars represent ±one standard deviation of estimates from analyses of triplicate spectra.

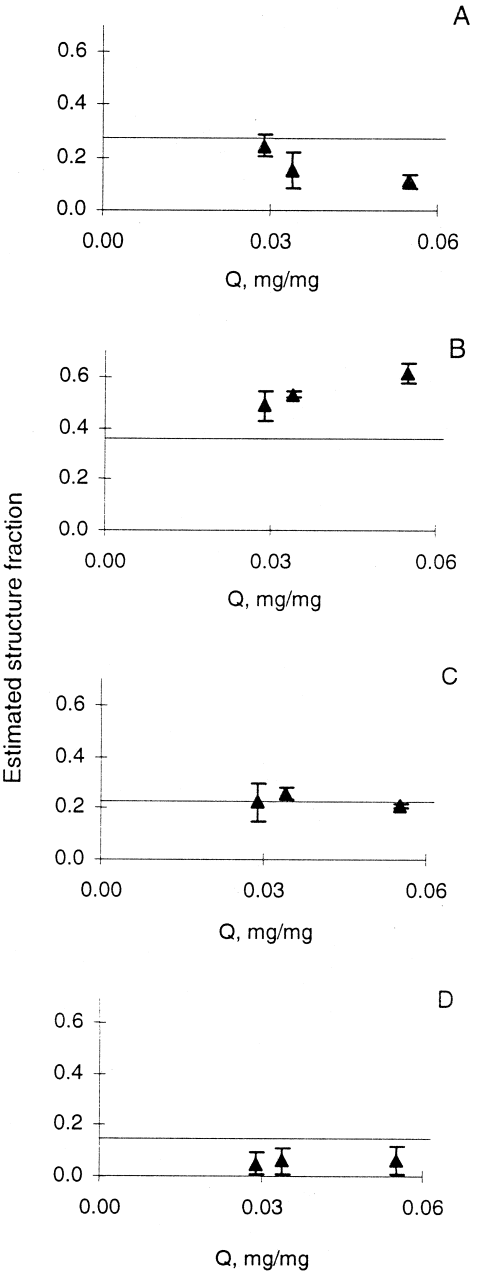

Fig. 3. Effect of surface concentration (*Q*) on the secondary structure estimates for lysozyme adsorbed on C_4-silica reversed-phase media. Individual secondary structure elements shown: (A) ordered helix; (B) unordered helix+sheet; (C) turns; and (D) unordered. Symbols and lines: ▲, adsorbed protein; —, X-ray structure. Adsorption conditions: deionized water with 0.1% TFA, pH 2.1 and ethanol and sodium chloride. Error bars represent ±one standard deviation of estimates from analyses of triplicate spectra.

ture of the adsorbed lysozyme is perturbed in a similar fashion to that for the corresponding *n*-C_4 media case. Thus, it appears that for lysozyme, the secondary structure in the adsorbed state is relatively insensitive to the surface hydrophobicity. The effect of the ethanol concentration in the mobile phase on the structure of lysozyme adsorbed on the C_4-based media is also shown in Fig. 2. In the range of ethanol concentrations studied, 5% to 10% (v/v), the extent of structural change in lysozyme on adsorption appears to be independent of ethanol concentration.

The experiments shown in Fig. 2 were conducted at an adsorbed protein concentration of approximately 50 mg protein per gram of dry stationary phase. The effect of adsorbed protein concentration on the secondary structure of lysozyme in the adsorbed state, holding mobile phase conditions constant, was also examined. Different adsorbed protein concentrations were achieved via employing various stationary phase–protein mass ratios during batch adsorption. As seen in Fig. 3, the structural changes for adsorbed protein at a concentration of 0.055 mg protein/mg media were comparable to those observed in Figs. 2. However, the extent of the structural changes significantly decreased as the adsorbed protein concentration decreased.

In order to estimate the saturation capacity of the C_4-silica, the isotherms for lysozyme adsorption on this material under two different salt concentrations and at 10% (v/v) ethanol were determined. As seen in Fig. 4, at 10 m*M* sodium iodide, the lysozyme adsorption isotherm is considerably depressed as compared to the isotherm at 100 m*M* sodium iodide. The maximum adsorption capacity of the C_4-silica material for lysozyme appears to be around 0.055 mg protein/mg media. Thus, at this adsorbed protein concentration in Fig. 3, the stationary phase surface was saturated. Since, from Fig. 3, the extent of structural changes in adsorbed lysozyme increase as the surface approaches saturation, lateral interactions must play a significant role in determining the extent of structural perturbation. At saturation, there must be considerable intermolecular contact and possibly even aggregation. Accordingly, we may speculate that the increased unordered helix+sheet content for lysozyme adsorbed under high surface concentration conditions is due, at least in part, to intermolecular β-sheet formation. Intermolecular β-sheet formation is a common signature of protein aggregate formation [27]. At lower adsorbed protein concentrations, in the range of 25 to 30 mg protein/g media, which were equivalent to about 50% of the saturation capacity, there were fewer intermolecular interactions leading to a lower overall unordered helix+sheet content in the adsorbed state. Adsorbed protein structural characterization results (data not shown) from similar experiments using potassium bromide led to similar results.

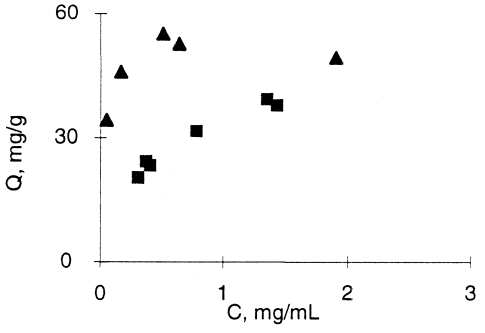

Fig. 4. Adsorption isotherms for lysozyme adsorbed on C_4-silica at two different salt concentrations. Symbols and lines: ■, 10 m*M* sodium iodide; ▲, 100 m*M* sodium iodide. Adsorption conditions: deionized water with 0.1% TFA, pH 2.1 and ethanol and sodium iodide.

It has been shown that during reversed-phase chromatography, significant quantities of organic phase may become extracted into the stationary phase [28]. As a result of such partitioning the adsorbed protein may experience a higher organic modifier concentration as compared to the protein in the mobile phase. Some researchers have suggested that this lower dielectric microenvironment may be responsible for structural changes in the adsorbed protein. To explore such a possibility, the structure of lysozyme was characterized in solutions containing varying fractions of ethanol. Results are shown in Fig. 5. Clearly, even at an ethanol concentration of 50% (v/v) with an approximate dielectric constant of 50, lysozyme exhibited minimal structural perturbation from its X-ray structure. This indicates that the structural changes observed in Figs. 2 and 3 were not due to solvent effects in the stationary phase microenvironment.

Chromatographic experiments were also carried out to provide additional insight into the behavior of

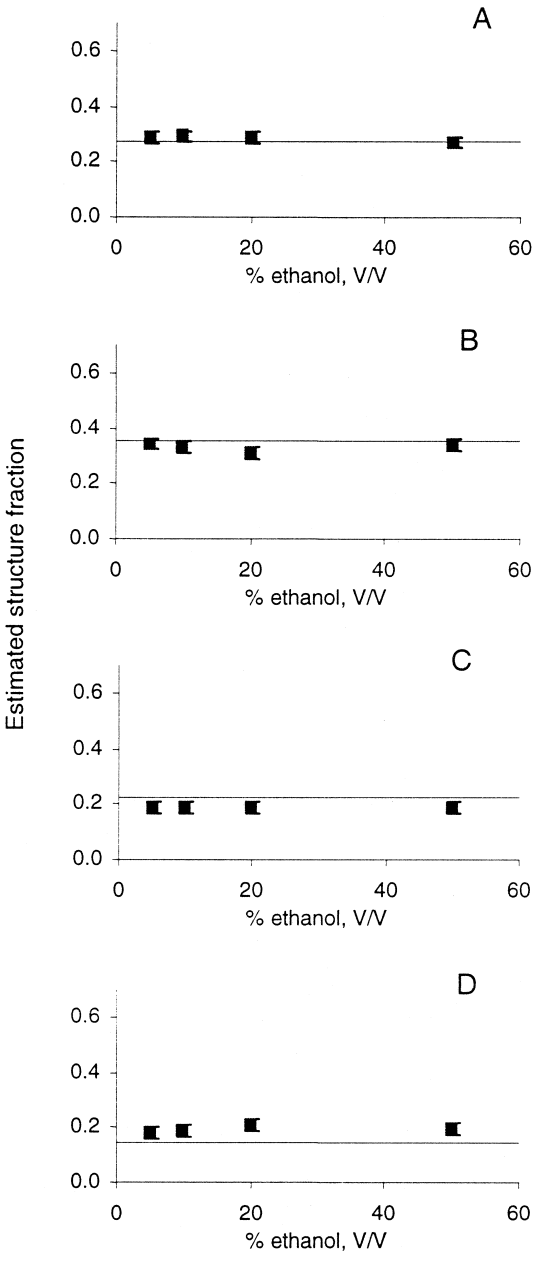

Fig. 5. Effect of mobile phase ethanol concentration on the secondary structure of lysozyme in solution. Individual secondary structure elements shown: (A) ordered helix; (B) unordered helix+sheet; (C), turns; and (D) unordered. Symbols and lines: ■, protein in solution; —, X-ray structure. Solution conditions: deionized water with 0.1% TFA, pH 2.1 and ethanol and sodium chloride. Error bars represent ±one standard deviation of estimates from analyses of triplicate spectra.

lysozyme in these reversed-phase systems. Lysozyme was loaded onto an analytical scale C_4 column at different ethanol concentrations and was then eluted from the column using linear gradients of different slopes to 90% (v/v) ethanol. Fig. 6 shows chromatograms at two different initial ethanol concentrations. In both cases, there is a major peak (peak 2) that elutes at around 42% (v/v) ethanol. However, an additional peak (peak 1) near the dead volume of the column was only seen for the 26% (v/v) initial ethanol concentration experiment (Fig. 6B). Peak 1 does not appear when lysozyme is loaded at or below 18% (v/v) ethanol. One possible explanation for the appearance of the second peak is adsorption-induced structural changes. To test this hypothesis, peaks 1 and 2 from Fig. 6B were

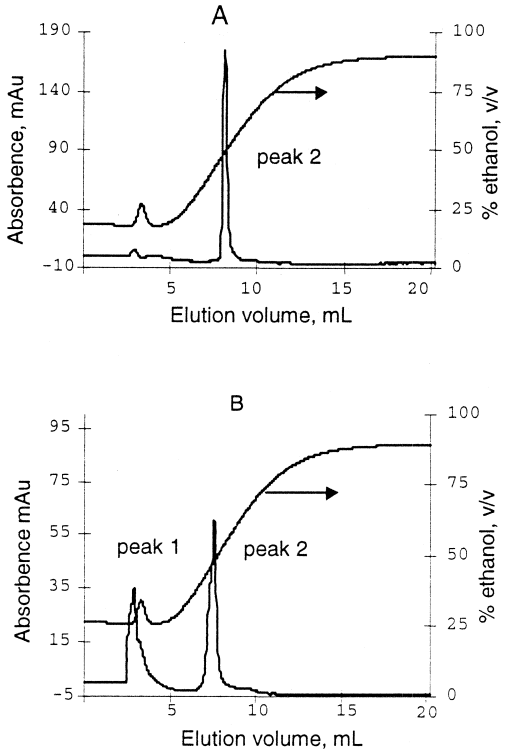

Fig. 6. Linear gradient elution profiles for lysozyme. (A) Linear gradient, 18–90% ethanol; (B) linear gradient, 26–90% ethanol. Chromatographic conditions: flow, 0.6 ml/min; column, 150×4.6 mm; mobile phase, deionized water with 0.1% TFA, pH 2.1 and ethanol and 100 m*M* sodium chloride. Chromatogram detected by optical absorbance at 280 nm; ethanol gradient based on conductivity trace.

collected and re-chromatographed under the same conditions. Interestingly, both fractions, despite their initial homogeneity, elute as two peaks as shown in Fig. 7. The retention times for the two peaks in each case match closely with those of the original peaks 1 and 2 (Fig. 6). From these data it appears that lysozyme undergoes reversible unfolding on the C_4 column. Lysozyme is a relatively hydrophilic molecule [29] and in the native state should be weakly retained on a hydrophobic surface (peak 1). However, once it unfolds on the surface its hydrophobic interior is exposed, allowing it to interact to a greater extent with the stationary phase ligands. Consequently, the strongly retained peak 2 probably corresponds to the denatured state which was seen to exhibit increased unordered helix+sheet content via Raman spectroscopy.

Thus, in the current investigation we have quantitatively characterized lysozyme adsorbed on C_4- and C_{18}-silica under a variety of conditions. Quantitative secondary structure estimation has allowed us to identify a possible mechanism for the denaturation of adsorbed lysozyme; a significant component of the observed denaturation seems to be attributable to aggregation and subsequent intermolecular β-sheet formation among adsorbed molecules. To our knowledge, this is the first evidence of aggregation of lysozyme on reversed-phase supports. A detailed interpretation of this sort was not possible in previous studies [19,30–32] because of their mostly qualitative nature.

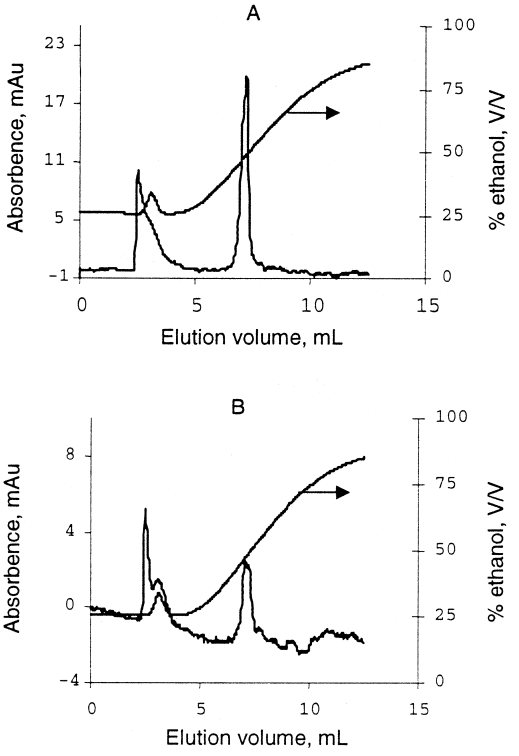

Fig. 7. Linear gradient elution of re-injected peaks 1 and 2 from Fig. 6. (A) Re-injected peak 1; (B) re-injected peak 2. For both chromatograms, fractions were collected from the elution experiment shown in Fig. 6B. All chromatographic conditions identical to those of Fig. 6.

Several previous results in the literature provide indirect support of our findings. Robeson and Tilton [19] used attenuated total reflection (ATR) with FT-IR to characterize lysozyme adsorbed on silica. As the adsorbed protein concentration increased, they observed spontaneous reorganization of adsorbed layer. The authors concluded that the reorganization was due to lateral interactions between the adsorbed molecules. Using a similar approach, Ball and Jones [32] characterized temperature induced structural changes in lysozyme adsorbed on silicone. Above a certain transition temperature, they observed appearance of a new peak around 1621 cm^{-1} which is indicative of β-sheet formation. Furthermore, the transition temperature decreased as the surface coverage increased. The results, in agreement with the current investigation, suggest that lysozyme adsorbed on a hydrophobic surface becomes increasingly susceptible to β-sheet formation as the adsorbed protein concentration increases.

During linear gradient elution experiments with pure lysozyme, Ingraham et al. [30] observed a lysozyme peak that eluted close to the dead volume of the column. Under more denaturing conditions, a portion of the lysozyme eluted much later during the gradient. This later eluting component could be similar to the denatured state of lysozyme that we have characterized in this study. Lu et al. [31] attempted to characterize lysozyme adsorbed on *n*-butyltriethoxy silane bonded silica using fluorescence. Lysozyme, which was loaded under strongly binding conditions, eluted as two peaks: one with a short residence time and the second with a much longer residence time. They interpreted their fluorescence data on the kinetics of the structural adaptation

following adsorption using a two-step mechanism. In the first step, which occurs almost instantaneously, tryptophans in the protein move to a more hydrophilic environment indicating partial unfolding of lysozyme on the surface. In the second step, which occurs over a few minutes, tryptophans move to a more hydrophobic environment. This later step could be due to aggregation among lysozyme molecules on the surface as suggested in the current investigation. Aggregation may trap previously exposed tryptophans within the contact regions between aggregating molecules, in effect moving them to a more hydrophobic environment. Alternatively, the second step may involve association of the tryptophan residues with the hydrophobic surface itself.

The NMR-based work of McNay and Fernandez [21] with lysozyme adsorbed on C_4- and C_{18}-based reversed-phase media is particularly cogent to this study, providing the identity of specific residues involved in structure-disrupting interactions with these surfaces. Our findings are similar in that we both find that denaturing interactions are extensive. The NMR study demonstrated the participation of specific residues from β-sheet segments and, to a smaller extent, α-helix segments; this is consistent with our finding that the adsorbed state is typically depleted in helix content and enriched in sheet content. The results of our studies differ in that McNay and Fernandez saw evidence for greater structural perturbation on the C_{18} media relative to the C_4 media; our structure estimates for the adsorbed states on both media were similar. A complicating factor in a comparative analysis of our results is that the protein loadings were different. Loadings in the NMR study were done in the absence of a mobile phase modifier such as ethanol and are likely much greater than those we report; we find that loading, and presumably lateral protein–protein interactions, significantly impact adsorbed protein structure.

Thus, the results presented in this paper are in agreement with several direct and indirect observations from the previous literature. At high adsorbed protein concentrations, lysozyme undergoes a structural transition to a high unordered helix+sheet containing state. Intermolecular interactions appear to play a significant role in driving these structural changes. Spectroscopic work of other investigators suggests, as discussed above, that structural transitions of this type may not be limited to reversed-phase materials, but may also be observed on other hydrophobic surfaces.

4. Conclusions

In this study, the secondary structure of lysozyme adsorbed on reversed-phase supports under a variety of conditions was characterized quantitatively. The surface concentration of the adsorbed protein was observed to play a significant role in driving structural changes in adsorbed lysozyme. The findings presented in this paper has several implications pertinent to the reversed-phase liquid chromatography of proteins. First, lysozyme adsorption behavior indicates that secondary structures of proteins can be significantly perturbed by adsorption on reversed-phase materials. These structural changes could have a dramatic impact on the corresponding retention behavior. As we observed, in linear gradient experiments, the native state of lysozyme exhibits minimal retention on a C_4 material while the perturbed state of lysozyme elutes at around 45% (v/v) ethanol concentration. This is consistent with the expectation that the perturbed state results either in the exposure of a greater number of hydrophobic residues to the media or in a greater number of lateral protein interactions which must be disrupted to permit desorption.

Second, for relatively hydrophilic molecules such as lysozyme, structural perturbation could be a necessary requirement for retention on a reversed-phase column. In reversed-phase liquid chromatography, hydrophobic interactions with the stationary phase ligand are primarily responsible for binding of protein to the column. For stability in aqueous environments, hydrophilic residues in a protein are concentrated on its surface while the hydrophobic residues are typically buried in the protein's interior. For a protein with low overall hydrophobicity, the surface hydrophobicity in the native state is likely to be even lower. Accordingly, strong protein–surface binding would necessitate partial exposure of the protein's hydrophobic interior. The rearrangement of the molecule required to achieve optimum interaction

between the hydrophobic residues and the stationary phase ligands would ultimately determine the extent of adsorption-induced structural perturbation.

Third, this investigation also points out the importance of loading effects in determining the extent of structural perturbation. The extent of lysozyme denaturation increases significantly with increasing adsorbed protein concentration. This suggests that in addition to equilibrium and transport effects, intermolecular interactions may also effect elution behavior in non-linear chromatography

This work clearly demonstrates that the conditions used in reversed-phase chromatography may induce significant conformational changes in a protein and that these structural changes may play a key role in determining retention behavior in a chromatographic column. A caveat: the results from this study are quite possibly specific to lysozyme. We are currently seeking a unified interpretation of lysozyme behavior on reversed-phase media jointly with the Fernandez group at the University of Virginia. We also intend to employ Raman spectroscopy to investigate the impact of adsorption in chromatographic systems on the structures of several other proteins and to identify the feasible operating parameter space for preparative scale reversed-phase chromatography that minimizes irreversible losses of active protein.

Acknowledgements

We thank J. McNay and E. Fernandez of the University of Virginia for providing us with an advance copy of their manuscript and for several interesting discussions. This work was supported, in part, by a National Science Foundation Career Award to T.M.P. (T.M.P., grant No. BES-9502184) and by Amersham Pharmacia Biotech.

References

[1] M.T.W. Hearn, M.I. Aguilar, J. Chromatogr. 397 (1987) 47.
[2] F.E. Regnier, Science 238 (1987) 319.
[3] S. Lin, B.L. Karger, J. Chromatogr. 499 (1990) 89.
[4] V.E. Turula, J.A. de Haseth, Anal. Chem. 68 (1996) 629.
[5] S.A. Cohen, K. Benedek, S. Dong, Y. Tapuchi, B.L. Karger, Anal. Chem. 56 (1984) 217.
[6] G.E. Katzenstein, S.A. Vrona, R.J. Wechsler, B.L. Steadman, R.V. Lewis, C.R. Middaugh, Proc. Natl. Acad. Sci. USA 83 (1986) 4268.
[7] L. Boulkanz, N. Balcar, M.-H. Baron, Appl. Spectrosc. 49 (1995) 1737.
[8] P. Oroszlan, R. Blanco, X.-M. Lu, D. Yarmush, B.L. Karger, J. Chromatogr. 500 (1990) 481.
[9] E.S. Parente, D.B. Wetlaufer, J. Chromatogr. 288 (1984) 389.
[10] E.S. Parente, D.B. Wetlaufer, J. Chromatogr. 314 (1984) 337.
[11] M.J. O'Hare, E.C. Nice, J. Chromatogr. 171 (1979) 209.
[12] K.S. Benedek, S. Dong, B.L. Karger, J. Chromatogr. 317 (1984) 227.
[13] A.W. Purcell, M.I. Aguilar, M.T.W. Hearn, J. Chromatogr. A 711 (1995) 61.
[14] A.F. Drake, M. Fung, C.F. Simpson, J. Chromatogr. 476 (1989) 159.
[15] Y. Kurosu, T. Sasaki, T. Takakuwa, N. Sakayanagi, K. Hibi, M. Senda, J. Chromatogr. 515 (1990) 407.
[16] L. Boulkanz, C. Vidal-Madjar, N. Belcar, M.-H. Baron, J. Colloid Interface Sci. 188 (1997) 58.
[17] A. Kondo, J. Mihara, J. Colloid Interface Sci. 174 (1996) 214.
[18] A.N. Asanov, L.J. DeLucas, P.B. Oldham, W.W. Wilson, J. Colloid Interface Sci. 196 (1997) 62.
[19] J.L. Robeson, R.D. Tilton, Langmuir 12 (1996) 6104.
[20] M.C.L. Maste, E.W. Pap, A. van Hoek, W. Norde, A.J.W.G. Visser, J. Colloid Interface Sci. 180 (1996) 632.
[21] J.L. McNay, E.J. Fernandez, J. Chromatogr. A 849 (1999) 135.
[22] S.U. Sane, S.M. Cramer, T.M. Przybycien, Anal. Biochem. 269 (1999) 255.
[23] K. Gekko, Y. Hasegawa, Biochemistry 25 (1986) 6563.
[24] M. Levitt, J. Greer, J. Mol. Biol. 114 (1977) 181.
[25] Y. Goto, L.J. Calciano, A.L. Fink, Proc. Natl. Acad. Sci. USA 87 (1990) 573.
[26] K. Hamaguchi, K. Hayashi, in: M. Funatsu, K. Hirami, K. Imahori, T. Murachi, K. Narita (Eds.), Proteins – Structure and Function, Vol. 1, Wiley, New York, 1972, p. 85.
[27] S.Y. Patro, T.M. Przybycien, Biotechnol. Bioeng. 52 (1996) 193.
[28] R.M. McCormick, B.L. Karger, Anal. Chem. 52 (1980) 2249.
[29] C.C. Bigelow, J. Theoret. Biol. 16 (1967) 187.
[30] R.H. Ingraham, S.Y.M. Lau, A.K. Taneja, R.S. Hodges, J. Chromatogr. 527 (1985) 77.
[31] X.-M. Lu, A. Figueroa, B.L. Karger, J. Am. Chem. Soc. 110 (1988) 1978.
[32] A. Ball, R.A.L. Jones, Langmuir 11 (1995) 3542.