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How does a protein unfold on a reversed-phase liquid chromatography surface?☆

Jennifer L. McNay, Erik J. Fernandez*

Department of Chemical Engineering, University of Virginia, Charlottesville, VA 22903-2442, USA

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Abstract

Nuclear magnetic resonance and isotope-exchange techniques were used to study unfolding of lysozyme adsorbed to reversed-phase liquid chromatography surfaces. All surfaces resulted in significant amide exchange, indicating solvent exposure and some loss of native structure. However, none of the surfaces resulted in complete exchange. The greatest amount of structure was preserved on the C_4 silica, with the most protection in the α-helix domain. C_{18} silica and Source RPLC resulted in much greater solvent exposure. No simple correlation was found between chromatographic retention and degree of surface unfolding. Variations in residual conformation may explain the complex retention behavior of proteins vs. small molecules. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Chromatography is an integral part of the separation and purification of proteins and enjoys widespread use in pharmaceutical processing. Reversed-phase liquid chromatography (RPLC) is a method of choice for analytical separations of complex peptide and protein mixtures because of its excellent resolution. Protein variants that differ by only one residue have been separated by RPLC [1], and conformational isomers of a 37-residue peptide have been resolved [2]. Thus, RPLC has the potential to be an extremely powerful purification tool. However, RPLC has found limited preparative commercial application because of the inability to accurately predict protein retention, protein denaturation, and the use of organic solvents. An improved understanding of protein conformational changes induced by RPLC may help address the first two issues.

Conformational changes in proteins interacting with RPLC surfaces have been well documented [1,3–6]. This behavior can lead to decreased production yields if the protein does not refold to the native structure following elution [6]. However, in some circumstances, conformational changes due to surface interaction can be beneficial. For example, increases in adsorption capacity of RPLC surfaces have been attributed to protein conformational changes [7]. Furthermore, the adsorption coefficient

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*Corresponding author. Tel.: +1-804-296-1524; fax: +1-804-982-2658.

E-mail address: erik@virginia.edu (E.J. Fernandez)

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of a protein may change by as much as 10^{10} between the native and denatured states [5]. However, if one is unaware of such phenomena and their kinetics [8], the resulting shouldered or multiple peaks may be incorrectly interpreted as sample impurities [5] or poor column performance. Thus, it is important to understand how proteins interact with surfaces to rationally design and operate RPLC columns.

Protein conformational changes due to surface interactions have been related to experimentally measurable changes in thermodynamic and chromatographic properties. Differential scanning calorimetry (DSC) studies have shown that proteins adsorbed to hydrophobic surfaces exhibit lower melting temperatures than in solution [9]. Chromatographic methods have also been used directly to examine protein–surface interactions. Purcell et al. [4] measured the retention for several peptides on RPLC surfaces as a function of temperature. Increases in retention were interpreted as increases in hydrophobic contact area between the solute and the RPLC surface, implying that the peptide unfolded on the surface. A sharp increase in retention was found at a specific temperature which was different for different peptides, which enabled conclusions to be made about the relative stability of different peptides on the RPLC surface. Adsorption isotherms have also provided indications of surface induced conformational changes via a protein chemical modification techniques [10].

Spectroscopic methods have provided more detailed information on which parts of the molecule are involved in surface-related conformational changes than is possible with the techniques described above. Fourier transform infrared spectroscopy (FT-IR) has been used to detect changes in the overall secondary structure in proteins on RPLC surfaces via the amide I region [9]. Circular dichroism (CD) is sensitive mainly to secondary structure changes in solution and has been used to detect irreversible secondary structure changes induced by RPLC surfaces [11,12]. Under specialized conditions, CD observations of protein under adsorbed conditions have also been made [13,14]. Recently, Raman spectroscopy has also been employed to detect secondary structure changes on RPLC surfaces [15]. However, the above techniques cannot identify specific residues which are involved in conformational changes. Further, they are limited to analysis of certain kinds of surfaces. In some instances, the protein of interest is known to contain a single fluorescent residue, such as tryptophan, or has a fluorescence spectrum which is highly sensitive to tertiary structural changes. Comparison of fluorescence spectra of proteins in bulk solution to spectra of proteins adsorbed to surfaces can then be used to detect surface-induced conformational changes [5,16]. Oroszlan et al. used this method to show that conformational changes can be correlated with chromatographic retention. This method provides limited information about which parts of the molecule are involved in the structural changes since only one reporter group is used [5].

NMR spectroscopy is a complementary spectroscopic tool which has been used to investigate protein structure [17], folding [18], denatured states [19], and protein–ligand interactions [20]. Hydrogen–deuterium isotope exchange techniques and ^{1}H NMR can be used to label and detect specific sites of protein unfolding and increased solvent exposure. This method has been used to study the structure of lysozyme denatured by cosolvents [21], conformational changes in lysozyme caused by precipitation [22], lyophilized protein in organic solvents [23], and identify the adsorbing site of lysozyme on hydroxyapatite [24].

Hydrogen–deuterium exchange techniques are based on the ability of an amino acid amide proton to exchange with solvent protons. Amino acids on the surface of the molecule are known to have exchange rate constants up to 10^{8} greater than those for amino acids inside the protein [25]. Since a proton will produce a peak in a ^{1}H NMR spectrum but a deuteron will not produce a peak, amino acids can be labeled with protons. In deuterated solvent, residues directly exposed to the solvent will exchange protons for deuterons as these residues are unprotected from exchange. Residues buried inside the molecule, which are protected from exchange, will remain protonated. Thus, changes in the hydrogen-exchange behavior of a particular amino acid can show whether the residue has moved from the interior of the molecule to the outside, or vice versa. Furthermore, the exchange reaction is a strong function of pH, with the minimum exchange rate at ~pH 3 [26,27]. As the pH either increases or decreases from the minimum, the hydrogen exchange rate increases

approximately one order of magnitude for each pH unit change. Thus, changing pH is a useful method of quenching the exchange reaction and preserving a specific exchange-labeled state. Because two-dimensional (2D) NMR can identify individual amide hydrogens, the structural details of exchange-labeled states can be elucidated [28]. Residues with the fastest exchange rates, which are located primarily on the surface of the molecule and are thus unprotected even in the native state, are not generally visible in the 2D NMR spectrum. However, the remaining set of residues which have exchange rates slow enough to be visible in the 2D NMR spectrum form a useful set of reporter groups.

The goal of the current investigation was to use ^{1}H NMR and hydrogen–deuterium exchange to directly measure conformational changes at the residue level in hen egg white lysozyme (HEWL) caused by interaction with an RPLC surface. The relationship between surface unfolding and chromatographic retention was also explored. Silica modified with attached C_4 and C_{18} alkyl chains and a polystyrene–divinylbenzene surface (Source 15 resin) were investigated. These surfaces are representative of the major surface types used in RPLC [29]. Based on small molecule retention data C_4 and Source 15 surfaces are of comparable hydrophobicity, while the C_{18} surface is more hydrophobic than the other two surfaces (unpublished data). HEWL was selected for this investigation because it is a small protein suitable for NMR analysis, and has a well characterized structure and folding pathway [30].

2. Methods

2.1. Chromatography

RPLC columns used in this study were Sephasil C_4, Sephasil C_{18}, and Source 15 (Amersham Pharmacia Biotech, Uppsala, Sweden). The size-exclusion (SEC) column was a Sephadex Hi-Trap (Amersham Pharmacia Biotech). HEWL was obtained from Sigma (St. Louis, MO, USA) and used without further purification (lot 65H7025, cat. No. L-6876). Deuterium oxide was obtained from Isotec (Miamisburg, OH, USA). HPLC grade acetonitrile (ACN) was from Sigma-Aldrich (St. Louis, MO, USA). All experiments were conducted at ambient temperature. Retention times were measured using an Äkta Explorer chromatography system (Amersham Pharmacia Biotech).

Capacity factors were determined for each column by loading a 20-μl sample of HEWL (33.4 mg/ml) on the column in 10 m*M* sodium acetate (pH 4.2) and eluting isocratically in ACN–10 m*M* sodium acetate (60:40, v/v) to mimic NMR sample preparation. A sodium chloride injection was used to determine the elution volume of a non-retained species.

2.2. Isotope-exchange labeling of adsorbed protein

The method used to prepare protein samples labeled by hydrogen–deuterium exchange during adsorption (surface-labeled samples) and corresponding control samples for NMR spectroscopy analysis is outlined in Fig. 1. Preparation of the surface-labeled sample is described in detail below; preparation of the control sample was identical except that $^{2}H_2O$ was not employed until the elution step as noted in Fig. 1B. The initial protein sample (1.0 ml of ~20 mg/ml HEWL in load buffer (10 m*M* phosphate buffer, pH 7) was adsorbed to the RPLC column with load buffer as the mobile phase. Flow was then stopped and the protein was incubated on the surface for 2 h. Following incubation, flow was resumed with labeling buffer (load buffer prepared at pH 6.6* with $^{2}H_2O$; * indicates pH uncorrected for isotope effects [31]). Two column volumes of labeling buffer were passed through the column in 4 min. During labeling, the protein remained adsorbed to the surface, but solvent-exposed amide hydrogens exchanged rapidly for deuterons. Based on literature studies of unstructured peptides [27] the characteristic exchange time for exchange for fully solvent exposed residues under these conditions is ≈6.0 s. Deuterated solvents were used from this point onward to ensure that none of the exchange-labeled deuterated sites could be repopulated by hydrogen atoms. After the 4 min. labeling period, the mobile phase was changed to quench buffer (pH* 3.8, 10 m*M* sodium acetate in $^{2}H_2O$). At this pH, the hydrogen–deuterium exchange rate constant is re-

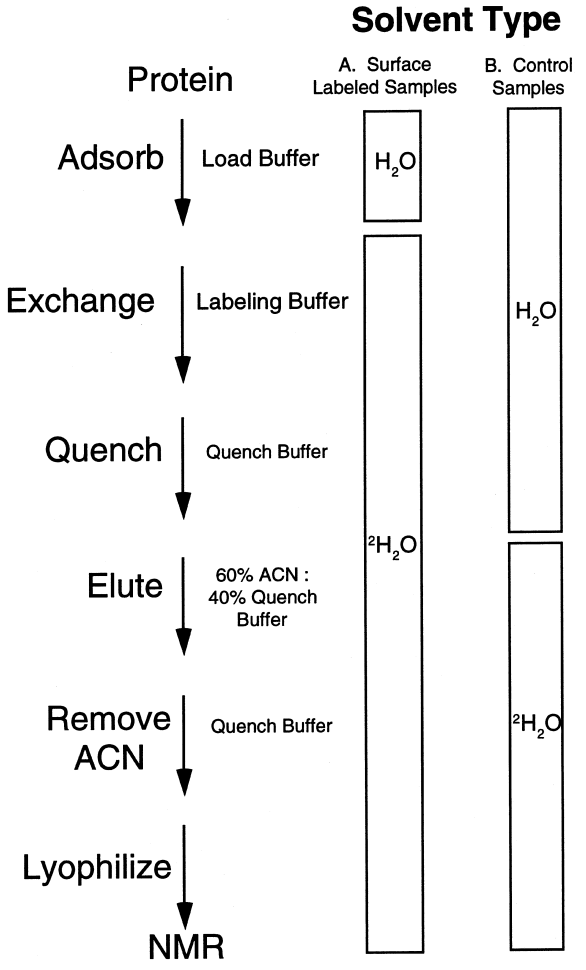

Fig. 1. (A) Surface-labeled sample was subjected to hydrogen–deuterium exchange while adsorbed to the resin. (B) Control samples were not exposed to ^2H_2O until after quenching to block exchange.

duced by a factor of ≈630. Two column volumes of quench buffer were passed through the column over 4 min. Following the quench step, the protein was eluted from the RPLC column isocratically using ACN–quench buffer, pH* 4.88 (60:40, v/v). Protein elution was detected using a 280 nm UV flow cell. As soon as protein began to elute, flow was redirected through to an SEC column pre-equilibrated with quench buffer to remove ACN. A conductivity flow cell (Cole Parmer) was used to verify that the protein eluted from the SEC column did not contain any ACN. It was necessary to remove ACN as soon as possible since HEWL is not completely native in ACN, but refolds to the native state once ACN is removed (see Section 3). Though quenching the exchange reaction with pH helps protect the label, the best way to protect the label is to refold the protein to the native state. In the native state, the exchange rate constant of residues inside the protein can be as much as 10^8 less than that of residues on the surface which are directly exposed to the solvent [25]. The protein fraction collected from the SEC column was frozen at −70°C within 10 min of elution, lyophilized for 48 h, and stored at −70°C to quench further exchange. In preparation for NMR, the sample was reconstituted with ^2H_2O. The pH* was adjusted to 3.8 with ^2HCl and NaO^2H. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS; 3 m*M* final concentration in sample) was added as a chemical shift reference.

2.3. NMR spectroscopy

All NMR spectra were obtained on a Varian UnityPlus 500 spectrometer operating at 497.541 MHz. For one-dimensional (1D) spectra, 8192 complex points were obtained with a spectral width of 6250 Hz, and 64 scans were obtained with a repetition time of 5 s. The 90° pulse width was calibrated for each sample. Two dimensional spectra were obtained using the HOHAHA (Homonuclear Hartman-Hahn) pulse sequence [32]. As with 1D spectra, the 90° pulse width was calibrated for each sample. The mixing time was 60 ms, 2048 complex points were obtained for 512 T_1 increments, the spectral width was 6250 Hz in both dimensions, and 32 scans (repetition time 1 s between scans) were averaged for each 1D slice to increase signal to noise. Total acquisition time for 2D spectra was ≈12 h. All spectra were obtained at 25°C.

NMR spectra were processed using FELIX 95.0 software (Molecular Simulations). The first data point in each spectrum was linearly predicted using estimates from the next 100 data points. All spectra were apodized using sinebell squared multiplication. The residual water peak was removed using time domain convolution. Peak assignments were made using peak coordinates from Redfield and Dobson [33]. The height of each peak was determined using the macro xpk_hgt_vol.mac obtained from Palmer

[34]. To allow comparison of peaks in different spectra, all peak heights in a given spectrum were normalized using two non-exchanging peaks ($C_{3,5}$–$C_{2,6}$ on Y23 and $C_{3,5}$–$C_{2,6}$ on Y53). Fractional occupancy (FO) values were then computed using normalized peak heights for each reporter group as described in Eq. (1)

$$\text{FO} = \frac{\text{Normalized peak height in experimental spectrum}}{\text{Normalized peak height in native spectrum}} \quad (1)$$

Although hydrogen exchange data is normally presented in terms of protection factors, the data here is presented in terms of fractional occupancies since data was only obtained for one exchange time, whereas protection factors are generally calculated from several exchange times.

To confirm reproducibility of the hydrogen exchange technique, two of the control and one of the surface-labeled experiments were repeated. The original and repeat data sets for the C_4 control sample are shown in Fig. 2 as a representative example. Depending on the residue, spectrum-to-spectrum variation led to a relative error in peak height of less than 5% (for residues with the largest peaks in the native state) to 35% (for residues with smallest peaks). On average, the change in peak height from original to repeat was less than ten percent of the largest peak. It is also clear from Fig. 2 that the pattern of peak heights is maintained across experiments.

2.4. Circular dichroism (CD)

All near-UV CD spectra were obtained using a Jasco J-720 spectrapolarimeter and a quartz cell with 1-mm path length. Each spectrum was the averaged result of five scans. Samples were obtained by

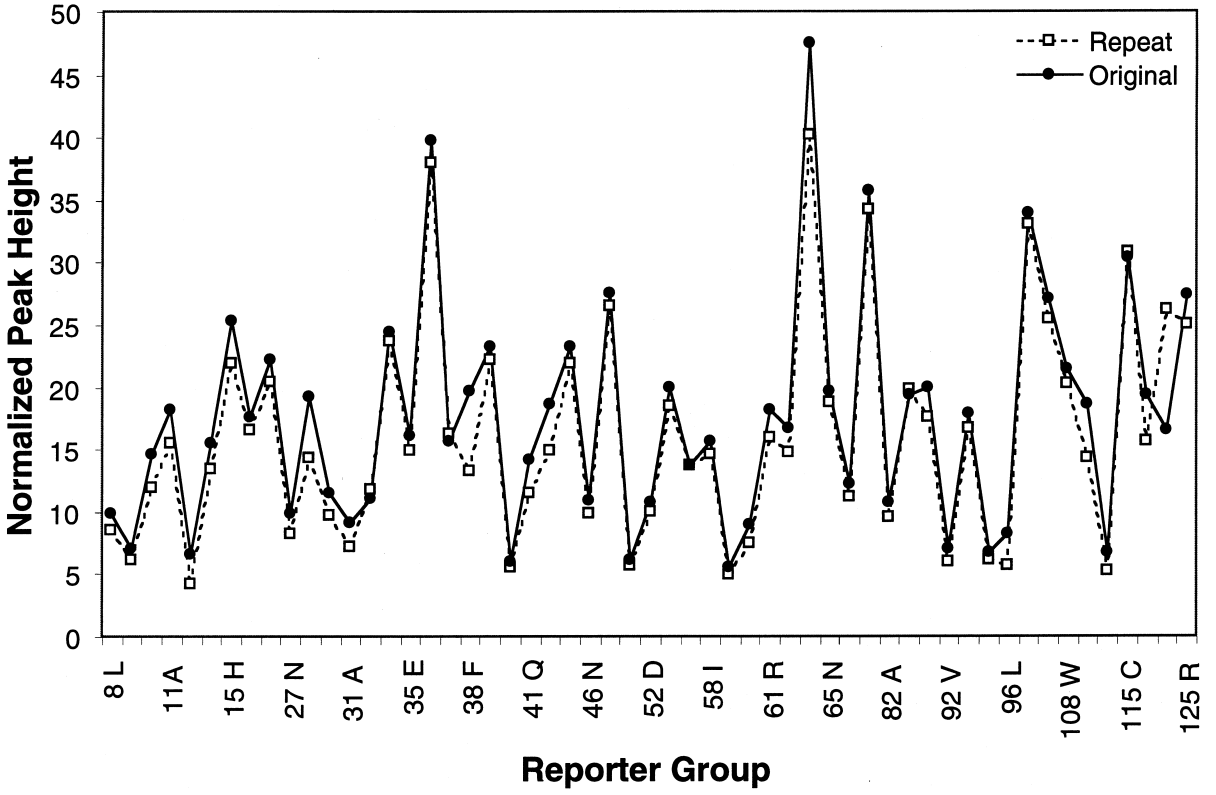

Fig. 2. Normalized peak heights are comparable for original and repeat C_4 control samples (shown as a representative pair).

diluting each NMR sample to ~1.8 mg/ml with 10 m*M* sodium acetate, pH 4.2. A reference scan of the buffer was subtracted from each protein spectrum.

3. Results and discussion

Samples were prepared according to the protocols described in Figs. 1A and B (see Section 2). The protein was used in native, protonated form, and exchange was allowed to occur from adsorption until elution. Protonated amides produce a peak on the ^{1}H NMR spectrum, whereas deuterated amides do not produce a peak. Hence, a reduction in peak height in the surface-labeled sample indicates that the corresponding residue was more exposed to the deuterated solvent, and thus was more able to exchange a proton for a deuteron. To account for exchange in post-adsorption steps in Fig. 1A, a control was performed (see Fig. 1B) in which the protein was not exposed to $^{2}H_{2}O$ until the elution step. The degree of additional proton exchange in the surface-labeled sample relative to the control is indicative of increased solvent exposure resulting from conformational changes due to surface adsorption, hydrogen bonding [35] will affect the ability of an amide to exchange.

It is important to ascertain that the protein has refolded to the native state before the NMR experiment to allow the use of published peak assignments. Furthermore, returning the protein to the native state after adsorption helps protect residues from additional exchange. To confirm that HEWL returned to the native state after the protocol in Fig. 1, CD spectra of the control samples and the native protein were obtained and are shown in Fig. 3. The near-UV region of the CD spectrum is sensitive to tertiary structure of the protein [36]. The control spectra are

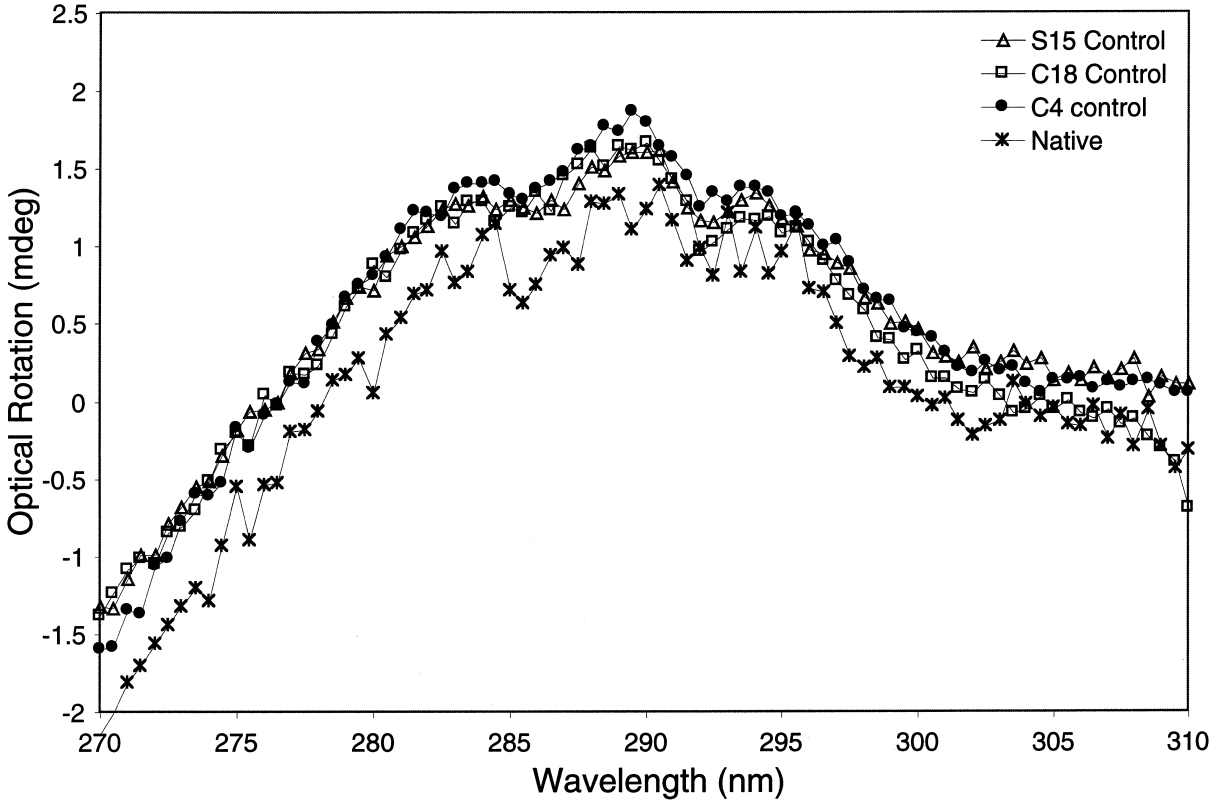

Fig. 3. CD spectra for all control samples and that of native HEWL illustrate that the protein refolds back to the native conformation after processing.

not significantly different from the native spectra, showing that the protein refolds to the native state after adsorption, elution, and further sample preparation. As further evidence that the protein refolded back to the native state, the aliphatic region of 1D NMR spectra for all deuterium surface-labeled samples and the native protein were collected and found to be identical (see Fig. 4), with the exception of the large peak at 2.0 ppm in the surface-labeled samples. This peak is from sodium acetate in the surface-labeled sample.

In addition to confirming that the protein has refolded to the native state after adsorption, it is also important to determine how other elements of sample preparation affect the protein structure. In particular, ACN, which is used to elute the protein from each of the RPLC surfaces, may cause some denaturation. Control samples were prepared in exactly the same

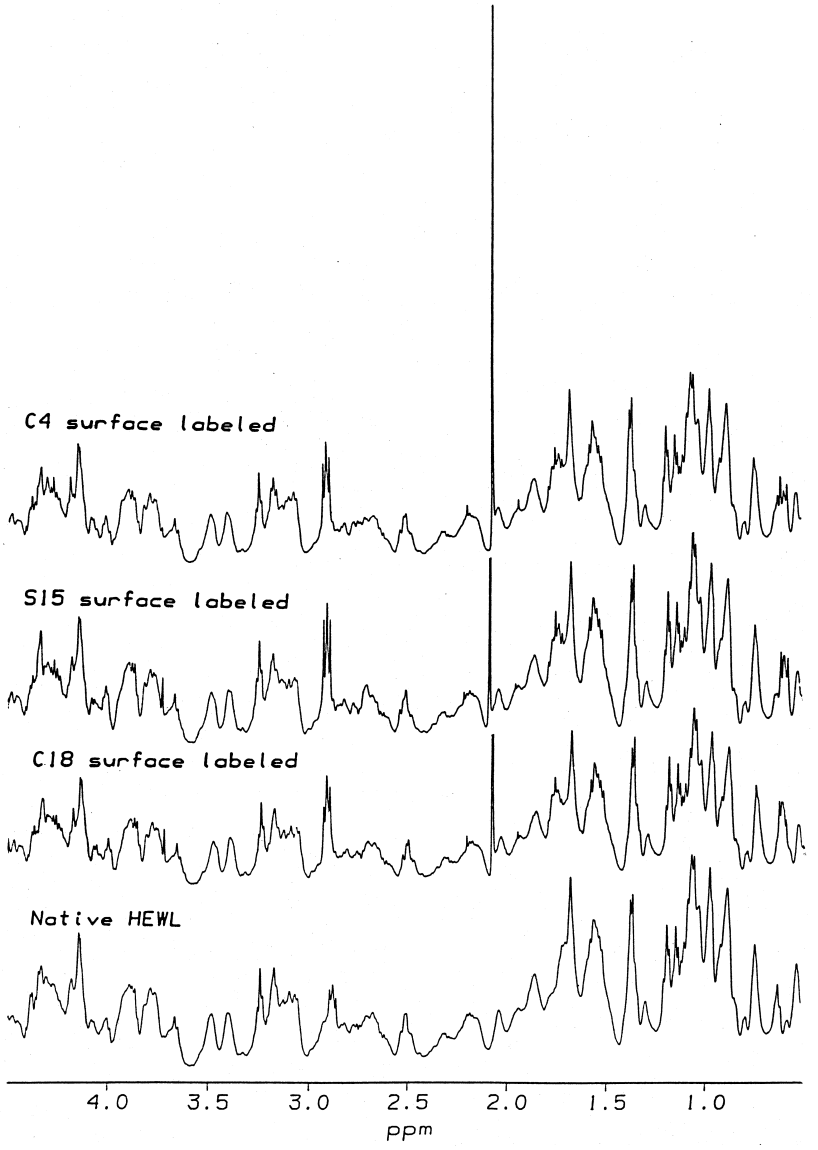

Fig. 4. Aliphatic regions of the 1D spectra for the native protein and all surface-labeled samples demonstrate that the protein refolds back to the native conformation after processing.

way as the surface-labeled samples, except that the control sample was not exposed to deuterated solvent until elution (see Fig. 1B). Quenched conditions (pH* 3.8) were used during adsorption so that hydrogen exchange labeling would reflect the surface-exposed conformation as much as possible. Quenched conditions substantially reduce but do not completely arrest the hydrogen exchange reaction. Thus, if the protein is or becomes unfolded subsequent to the quench step in Fig. 1, some exchange will occur. Otherwise, the fractional occupancies for all residues in the control samples should be unity. Fig. 5 shows the normalized peak heights of control samples for each of the three surfaces examined. To minimize the effects of native state exchange, only the residues exhibiting the slowest exchange in the native state were analyzed. Some exchange is evident, however, such losses were generally not more than 50% (fractional occupancy of 0.5), and in most cases are substantially less. The values for the C_4 and Source 15 surfaces were essentially identical for all reporter groups. Normalized peak heights for the C_{18} surface follow the same trends as for the C_4 and Source 15 surfaces, but are decreased overall in degree by approximately 7–10 normalized peak height units. This may indicate greater unfolding on the C_{18} surface which leads to more isotope exchange during elution. In any case, the residue-to-residue variation in normalized peak height is the same for all three control samples. Thus, it appears that the impact of elements of the protocol other than surface exposure on the pattern of exchange in the controls is primarily surface independent.

Like the control samples, the fractional occupancies for the 56 reporter residues were calculated according to Eq. (1) for samples subjected to surface-labeling. Table 1 shows the average degree of exchange for all reporter groups calculated for the

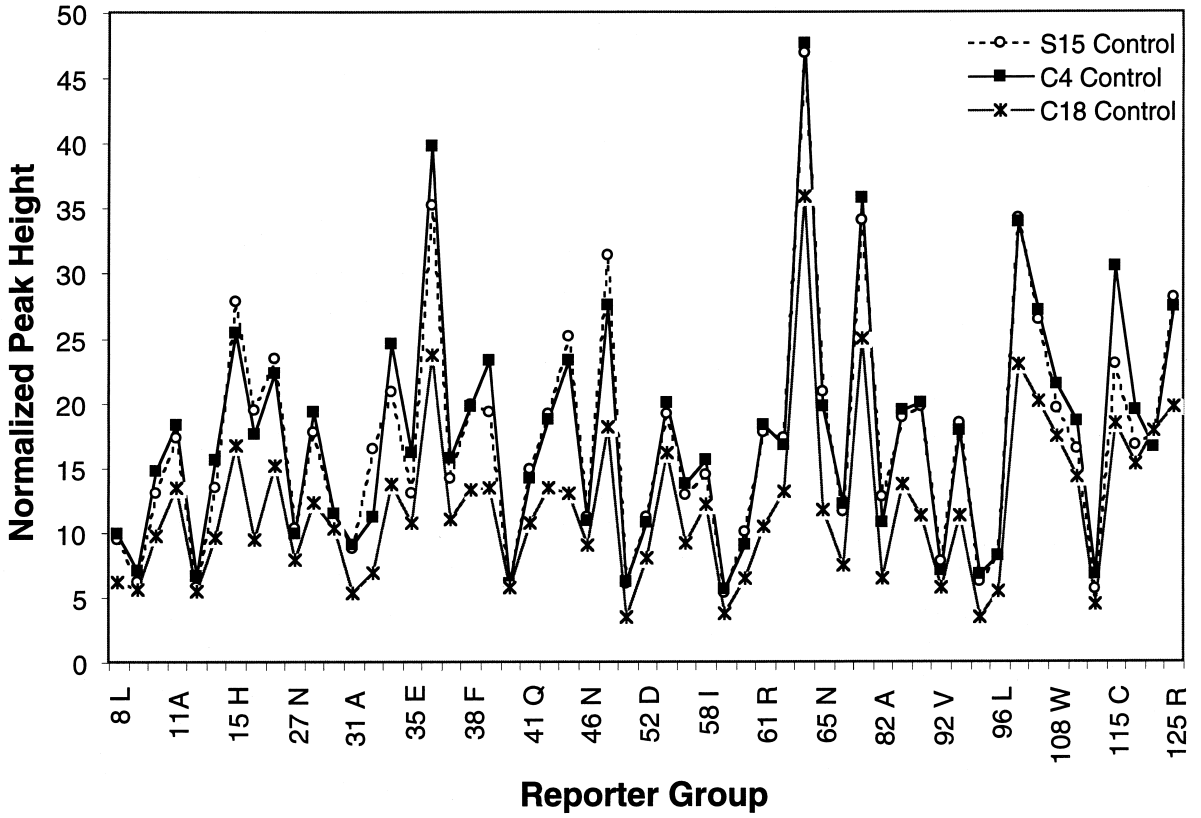

Fig. 5. Normalized peak heights for the control samples show similar trends for all three surfaces.

Table 1
Average fractional occupancies; averages are of all reporter groups in a single sample

Surface	Average fractional occupancy	
	Control protein	Surface-labeled protein
C_4	0.79	0.24
C_{18}	0.53	0.06
Source 15	0.78	0.05

surface-labeled and control samples. For each of the three surfaces, several residues were solvent exposed during adsorption, as is indicated by a reduction in fractional occupancy of the surface-labeled sample relative to the corresponding controls. It is noteworthy that for all surfaces, some residues were protected from exchange, indicating that some structure was preserved in all cases.

Reporter residue fractional occupancies of surface-labeled samples are compared to those of the control samples for C_4, C_{18}, and Source 15 surfaces in Fig. 6. If native structure was preserved, one would expect the fractional occupancy to be unity for all reporter groups. However, even for the controls this is clearly not the case, indicating that both the control and surface-labeled samples experienced additional hydrogen–deuterium exchange relative to the native state. Residues with zero fractional occupancy values are caused by high degrees of solvent exposure, suggesting that the structure around these residues was substantially unfolded. Residues with non-zero fractional occupancies were protected to some degree from solvent exposure, suggesting that there was some structure around these residues. However, it is not possible to ascertain if this structure was like that of the native state.

To determine the effect of surface adsorption on protein structure, one should focus on the difference between the fractional occupancy values for the control and surface-labeled samples. In almost all cases examined, the residues which show some protection from exchange (non-zero fractional occupancy) in the surface labeled sample have fractional occupancy values lower than those for corresponding residues in the control sample. This provides evidence that surface adsorption affects all regions of the protein to at least some extent. However, in all cases there are some protected residues in the surface-labeled sample, indicating that there is some structure preserved in the surface-adsorbed state.

Fig. 6A and B shows the effect of adsorption to a C_4 surface. Regions of HEWL protected from exchange on the C_4 surface correspond well to distinct areas of secondary structure, as is evident from the clustering of protection in primarily alpha helices. Residues with non-zero fractional occupancy values are highlighted on the X-ray structure of HEWL in Fig. 6B to further illustrate this effect. HEWL is often considered to have two major regions of secondary structure, an α-helical domain and a β-sheet/loop domain, shown on the left and right, respectively, in Fig. 6B. The regions of greatest protection for the C_4 surface-labeled sample are near helices A, B and D, all in the α-helical domain. The coil region close to the C-terminus of the protein, which wraps around the core of the α-domain, (residues V99-R125) also shows substantial protection. One would expect to see the greatest protection in regions of α-helix or β-sheet since, as long as the region remains folded, the involvement of amides in hydrogen bonded secondary structure will help protect them from hydrogen exchange. Since these residues in the C-terminal coil region remained protected in the surface-labeled sample, it may be concluded that this region of the protein remained well folded while the molecule was adsorbed to the surface. In contrast, helix C, which is close to the β-sheet/loop domain both spatially and in the primary sequence, is not protected from exchange. Furthermore, residues in the region of sheet from F38 to the start of helix C at A82 which form the core of the β-sheet/loop domain are mainly unprotected. Fractional occupancy values for the few protected residues in the β-domain are substantially less than those in the protected helices. Of the residues which do show some protection, all except S50 are in loop regions outside the β-sheet structure. Thus, overall, the C_4 surface-labeled sample shows substantial protection in the α-helical domain and very little in the β-sheet/loop domain.

Fig. 6C and D shows the effect of adsorption to the C_{18} surface on HEWL conformation. In Fig. 6C, fractional occupancy values for the surface-labeled and control samples are shown for all reporter groups. There are substantially fewer residues with

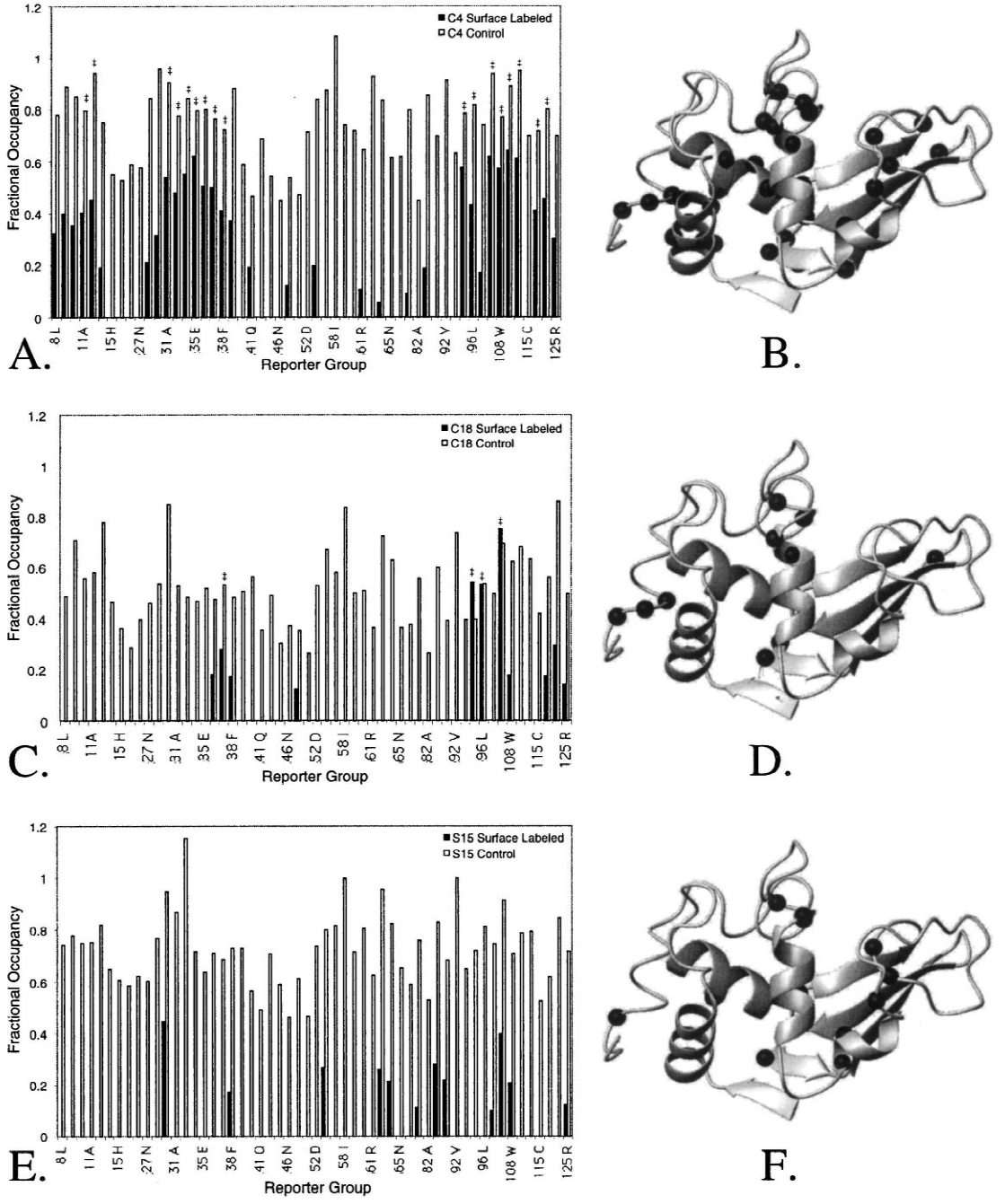

Fig. 6. Fractional occupancy values as a function of reporter group (referenced by residue sequence number) illustrating surface-induced unfolding in HEWL adsorbed to (A) C_4, (C) C_{18}, and (E) Source 15 RPLC surfaces. X-ray structures with superimposed dark spheres indicating location of amides protected from exchange while adsorbed to (B) C_4, (D) C_{18}, and (F) Source 15 resins. For clarity, only every fourth reported group is labeled in A, C and E. ‡ indicates that surface-labeled fractional occupancy value differs from control fractional occupancy value by at least two standard deviations. Nonzero fractional occupancies were only shown for residues with heights greater than three times the spectrum noise level. The displayed protein structures were prepared with the program MOLMOL [39].

non-zero fractional occupancy values for the C_{18} surface-labeled sample than for the C_4 surface-labeled sample (shown in Fig. 6A), suggesting that the C_{18} surface is much more denaturing than the C_4 surface. This is consistent with expectations since the longer alkyl chains on the C_{18} surface may provide a more hydrophobic, and thus more denaturing, environment for protein adsorption. Further, the carbon loading for the C_{18} surface used in this study is 15.1–16.6% (w/w) vs. 1.8–2.4% (w/w) for the C_4 surface (Amersham Pharmacia Biotech). Thus, the C_{18} surface appears to have more alkyl chains per unit mass than the C_4 surface, which may also contribute to the denaturing ability of the C_{18} surface. However, it is not possible to draw any further detailed conclusions about the relationship between surface environment and protein unfolding as the exact chain density and orientation on the surface is unknown. Although the C_{18} surface-labeled sample shows significantly less protection than the C_4 surface-labeled sample, there are some similarities in the structure retained on the two surfaces. The residues which remain unexchanged on the C_{18} surface are a subset of the residues protected on the C_4 surface, with the greatest similarities near the protein's C-terminus. In contrast to protein adsorbed to the C_4 surface, protein adsorbed to the C_{18} surface shows no protection in any of the residues in the loop regions surrounding the β-sheet structure.

As was noted previously, the C_{18} control sample showed the same pattern of fractional occupancies as the other surfaces, but the values were reduced by approximately 0.2 (see Fig. 5). Since the C_{18} surface was very strongly denaturing, the C_{18} control sample may have been substantially unfolded when it was eluted from the surface. Under the elution conditions used, ACN–quench buffer, pH* 4.88 (60:40), hydrogen–deuterium exchange was not completely quenched. If the C_{18} control sample was more unfolded and/or refolded more slowly than controls from the other surfaces, it may have exchanged more than the other control samples during elution. This suggests that the refolding time during elution is slower than the intrinsic exchange time ($\approx$6 min), since there is exchange in the all control samples beyond that in the native, as is seen from the fractional occupancy values which are less than one.

Fig. 6E and F shows the effect of adsorption to a Source 15 surface on HEWL conformation. The number of non-zero fractional occupancies in the Source 15 surface-labeled sample is much closer to that for the C_{18} surface-labeled sample than for the C_4 surface-labeled sample. As can be seen from Fig. 6E, many of the residues protected upon exposure to the Source 15 surface are a subset of the residues protected on the C_4 surface, but several are different from those protected on the C_{18} surface. In addition, two of the residues protected on the Source 15 surface (W63 and L84) were not protected on the C_4 surface. Overall, the number of non-zero fractional occupancies in the Source 15 surface-labeled sample is much closer to that for the C_{18} surface-labeled sample than for the C_4 surface-labeled sample. The Source 15 surface is composed of polystyrene–divinylbenzene with no attached ligands and is considered quite hydrophobic. The high degree of exchange observed suggests the protein is substantially unfolded on Source 15. This is consistent with the hypothesis that increased surface hydrophobicity results in increased protein unfolding. The Source 15 result is especially interesting in that it clearly shows different patterns of protection than with the C_4 or C_{18} surfaces. This suggests that HEWL may interact and unfold differently with the different materials. Such behavior would substantially complicate the ability to describe protein–surface interactions. However, further experiments, including the analysis of adsorbed protein conformation on additional surface types as well as detailed characterization of surface hydrophobicity and other properties, are needed to elucidate the nature of these differences and further test this hypothesis.

Nagadome et al. [24] examined the adsorption of HEWL to hydroxyapatite using similar methods to those used in this study. They observed that the bulk of amide reporter groups were unaffected by adsorption to the hydrophilic calcium phosphate surface of hydroxyapatite surface, suggesting little denaturation occurred. However, residues, Ala-9, Ala-11, Lys-13, and Leu-83, exhibited slower H–^{2}H exchange in the surface bound molecule. The authors concluded that Ala-9, Ala-11, and Lys-13 form part of a possible binding site for HEWL on hydroxyapatite which is protected from exchange upon adsorption. Their results suggest that hydrogen ex-

change NMR techniques may be useful for identifying specific interactions between proteins and chromatographic surfaces in a way similar to hydrogen-exchange NMR studies of protein–protein interactions [37]. In contrast, in the present study, all four of these residues along with many others showed increased exchange after exposure to each of the three surfaces examined, indicating that increased solvent exposure, and therefore unfolding, occurred. These differences in degree and pattern of exchange are certainly not surprising given the very different nature of the surfaces involved. However, the observations of Nagadome et al. demonstrate that adsorption to a surface can increase the protection from hydrogen exchange. Although increases in protection upon surface exposure compared to controls were not observed in this study, it could be that some of the protection from exchange we observed may be due to reduced solvent access caused by strong adsorption to the hydrophobic surface. Such an effect may be responsible for the scattered pattern of protection observed in the loops at the edges of the sheet regions in protein adsorbed to the more hydrophobic surfaces, especially for the Source 15 surface.

It is also interesting to try to relate the preserved regions of structure to other observations of partially denatured states of lysozyme which have been only recently elucidated using similar hydrogen-exchange NMR techniques. Buck et al. [21] used hydrogen exchange to examine the pattern of HEWL unfolding in 50% trifluoroethanol (TFE). They found greatest protection in the helical regions, with regions of protected residues very similar to those observed on the C_4 surface (helices A, B, D, and the C-terminal segment). All three surfaces showed some additional 'scattered' sites of protection, generally at a lower level of fractional occupancy, which do not seem to correspond with patterns in the TFE state of lysozyme. The significance of these isolated regions of protection is not yet clear.

The NMR results above indicate that there is some unfolding of HEWL on RPLC surfaces. It might be expected that degree of unfolding would correlate with retention time. Capacity factor (k') values were computed for each surface using Eq. (2)

$$k' = (V_{\text{retained}} - V_o)/V_o \quad (2)$$

where V_{retained} is the volume of 60% ACN needed to elute the protein and V_o is the volume of mobile phase required to move a non-retained species, NaCl, through the column. k' values were obtained isocratically for HEWL on the three surfaces with the same elution buffer used to prepare the NMR samples are shown in Table 2. Somewhat surprisingly, the C_4 and C_{18} surfaces had similar capacity factors yet showed very different degrees of unfolding for adsorbed protein. Furthermore, the C_{18} and Source 15 surfaces showed roughly the same, greater degree of unfolding by NMR, albeit with different patterns of protection, and yet had very different k' values. Further experiments (data not shown) were performed which included incubating the protein while adsorbed to the surface for 2 h before isocratic elution, as well as gradient elution with and without two hour incubation in the adsorbed state. In all cases, the trends in the retention data were the same as is shown in Table 2. Although there is some variation of degree of unfolding with surface type, it does not seem to be directly correlated retention. Factors which can complicate this relationship include the amount of hydrophobic contact area, separation between the protein and surface, and the role of the cosolvent in these interactions. Thus, changes in the surface type or carbon loading may tip a delicate balance, resulting in complex response.

Pearson and Regnier [38] made observations supporting those of this work. They found that protein retention was only weakly dependent on alkyl chain length (C_2 to C_{22}). However, they reported that selectivity (retention volume of A/retention volume of B) was influenced by alkyl chain length, with the C_4 surface having the greatest selectivity overall for the proteins they examined. Our results show that the C_4 surface-adsorbed protein has the greatest degree of residual structure. If

Table 2
Capacity factors (k') for the two silica surfaces (C_4 and C_{18}) are larger than that for the polystyrene–divinylbenzene surface (Source 15); measurements obtained via isocratic elution of HEWL in the buffer used for preparation of NMR samples

Surface	k'	Standard deviation
C_4	0.376	0.022
C_{18}	0.415	0.016
Source 15	0.263	0.032

the degree and nature of residual conformation is an important determinant of retention, this may explain why C_4 surfaces exhibit better selectivity properties.

Although this study provides the first residue-level picture of protein unfolding on chromatographic surfaces, there are some important limitations of these measurements. Using the methods presented here, it is not generally possible to obtain detailed information about the distribution of adsorbed conformers since the NMR spectrum is only an average of all conformational states present. For example, at any given residue, NMR cannot distinguish whether half the protein molecules are completely solvent exposed at that residue, or whether all the molecules are partially solvent exposed. However, from the nonnative exchange patterns observed here, it can be concluded that the nonnative species remain partially protected from hydrogen exchange, presumably retaining some elements of native structure. While there may be a predominant adsorbed conformation for each RPLC surface, an ensemble of adsorbed conformations with different degrees of unfolding is certainly possible.

Second, using the methods described here, it is not possible to detect any changes in hydrogen exchange rates for rapidly exchanging residues on or near the surface of the native protein. Since $\approx$12 h is required to obtain an NMR spectrum under our conditions, essentially all amide hydrogens on the surface of the protein will exchange during the NMR measurement and any labeling information will be lost.

Finally, slow denaturation kinetics on the RPLC surface [8] may affect the measurements. In the surface exposure experiments reported here, the protein was incubated on the surface for 2 h before deuterium labeling to allow such surface rearrangements to occur before labeling commenced. Furthermore, the labeling step was performed at high pH to allow for rapid exchange, in an effort to obtain a 'snapshot' of the surface adsorbed protein conformation. However, there are likely interesting conformational kinetics which are averaged during the labeling period, or which occur before the labeling was performed in these experiments. Further hydrogen-exchange labeling studies to determine the effect of duration of adsorption on protein conformation will be required to address these issues.

4. Conclusions

The present data clearly show that HEWL unfolds when interacting with RPLC surfaces. A residue level picture of unfolding is presented which shows that the protein unfolds to varying extents and possibly different ways on each surface, but that some residual structure is retained in the surface adsorbed protein in all cases examined. The retained structure is concentrated primarily in the helical domain, and to a lesser extent in the β-sheet/loop domain. No simple correlation between retention and surface hydrophobicity or degree of surface unfolding was found for the surfaces examined. Further studies will be required to distinguish the precise and complex contributions of conformation to retention and selectivity.

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