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Changes in solvent exposure reveal the kinetics and equilibria of adsorbed protein unfolding in hydrophobic interaction chromatography

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Abstract

Hydrogen exchange has been a useful technique for studying the conformational state of proteins, both in bulk solution and at interfaces, for several decades. Here, we propose a physically-based model of simultaneous protein adsorption, unfolding and hydrogen exchange in HIC. An accompanying experimental protocol, utilizing mass spectrometry to quantify deuterium labeling, enables the determination of both the equilibrium partitioning between conformational states and pseudo-first order rate constants for folding and unfolding of adsorbed protein. Unlike chromatographic techniques, which rely on the interpretation of bulk phase behavior, this methodology utilizes the measurement of a molecular property (solvent exposure) and provides insight into the nature of the unfolded conformation in the adsorbed phase. Three model proteins of varying conformational stability, α -chymotrypsinogen A, β -lactoglobulin B, and *holo* α -lactalbumin, are studied on Sepharose™ HIC resins possessing assorted ligand chemistries and densities. α -Chymotrypsinogen, the most conformationally stable protein in the set, exhibits no change in solvent exposure at all of the conditions studied, even when isocratic pulse-response chromatography suggests nearly irreversible adsorption. Apparent unfolding energies of adsorbed β -lactoglobulin B and *holo* α -lactalbumin range from -4 to 3 kJ/mol and are dependent on resin properties and salt concentration. Characteristic pseudo-first order rate constants for surface-induced unfolding are 0.2 to 0.9 min⁻¹. While poor protein recovery in HIC is often associated with irreversible unfolding, this study documents that non-eluting behavior can occur when surface unfolding is reversible or does not occur at all. Further, this hydrogen exchange technique can be used to assess the conformation of adsorbed protein under conditions where the protein is non-eluting and chromatographic methods are not applicable.

Keywords

Hydrophobic interaction chromatography; protein folding; protein adsorption; thermodynamics; kinetics

1. Introduction

While affinity and ion-exchange are more commonly utilized chromatographic modes in commercial biotherapeutic production [1], hydrophobic interaction chromatography is an orthogonal mode that is effective in removing difficult to separate species such as

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aggregated and misfolded forms of the target protein or target-related variants [2–6]. A complication that can arise in the use of hydrophobic adsorbents for bioseparations is the tendency of these stationary phases to induce denaturation of adsorbed proteins. Conformational change during HIC can affect process performance by reducing yields or changing selectivity [7–10]. Experimental evidence for protein unfolding on HIC resins has been published in the literature for more than two decades [11–13], yet this phenomenon remains relatively poorly understood with regard to the thermodynamics, kinetics and molecular mechanisms.

One approach to studying protein unfolding in HIC has been to incorporate conformational change into mathematical models of chromatographic behavior. Initial work in this area was performed by Karger and co-workers, utilizing gradient elution chromatography to study the effects of temperature, stationary phase and mobile phase conditions on conformation change [12–14]. To and Lenhoff [15] recently applied a reversible unfolding model to describe asymmetric peaks in pulse-response isocratic elution HIC. In our recent articles [16–18], a four-state model of protein conformation and adsorption in HIC has been adapted from models of combined reaction and chromatographic separation [19–21] and used to investigate surface-induced unfolding and the effects of stability-modifying solution conditions and mutations on chromatographic behavior. An irreversible unfolding model has been proposed by Jungbauer and co-workers [22,23], and has been used to determine unfolding rate constants in HIC. These chromatographic techniques rely on the interpretation of phase partitioning behavior to describe a conformational change that occurs on the hydrophobic surface, without direct observation of unfolding.

Several additional techniques have been employed to gain insight into the molecular details and mechanism of HIC-induced unfolding. Spectroscopic methods including circular dichroism [18], fluorescence [24,25] and infrared spectroscopy [23] have detected changes in the structure of adsorbed proteins, but may be restricted to solution phase measurements or chromatographic materials that are either translucent or nanoscale particles. Further, it is difficult to interpret such data when multiple conformations of adsorbed protein may be present and the results are only semi-quantitative. Isothermal titration calorimetry (ITC) has been used to detect changes in the heat capacity of proteins adsorbed and unfolded on hydrophobic surfaces [26,27]. ITC has to this point only been shown to be an indicator of conformation change, without interpretation regarding the nature of the unfolded molecule or the unfolding process.

Hydrogen exchange has proven to be a useful tool for the study of protein conformation both in solution [28–30], and adsorbed at solid interfaces [31–35]. Coupling hydrogen exchange with mass spectrometry (HX-MS) has allowed the efficient determination of conformation change of adsorbed protein, and insights into the effects of stationary and mobile phase properties on protein conformation during HIC have been gained [18,36–38]. Application of this technique to the quantitative description of adsorbed protein unfolding has been limited. Tibbs-Jones et al. [38] quantified the free energy of unfolding of a stable protein in the adsorbed phase, while Fogle et al. [36] characterized unfolding kinetics via an empirical rate model.

In this study, we propose a kinetic model and accompanying experimental protocol for batch adsorption and hydrogen exchange labeling of proteins in HIC. Previous work demonstrated such an approach for the limiting case of a marginally unstable system at elevated temperature [16], where adsorption equilibrium was reestablished rapidly compared to the characteristic time scale for unfolding. Here the method is applied generally to investigate the conformational equilibria and kinetics of HIC systems exhibiting chromatographic behavior suggestive of unfolding. Variables investigated include protein solution-phase

conformational stability, stationary phase properties and salt concentration. The results provide quantitative detail of protein instability during HIC, while also offering insight into the molecular structure beyond what can be determined chromatographically.

2. Theoretical Aspects

2.1 A stoichiometric water-displacement model of adsorbed protein unfolding

In a recent publication, we utilized the idea of stoichiometric water displacement from hydrophobic interfaces initially proposed by Geng et al. [39] to derive thermodynamic relations describing the effects of salt concentration, resin properties, and loading on protein retention in HIC [40]. The adsorption of proteins in HIC is described by the following mechanism:

(Refer to Nomenclature for definition of symbols.) A similar mechanism is proposed to describe protein unfolding during HIC. Consider that unfolding of an adsorbed protein results in increased contacts with hydrophobic ligands, thus increasing the number of displaced water molecules,

Here, there is a net release of interfacial water molecules per unfolding event that depends on the increased number of protein-ligand contacts ($\omega \xi$) and the increase in solvent exposure of previously-buried residues (χ). By similarity with the adsorption model, this model suggests that conditions favoring protein adsorption in HIC will also promote unfolding.

Expressions for the rates of folding and unfolding of adsorbed protein are

$$r_{fold,S} = k_{fold,S} [U_s] [W]^{(\omega \xi - \chi)} \quad (1)$$

$$r_{unf,S} = k_{unf,S} [N_s] [L]^\omega \quad (2)$$

Further, at a constant salt concentration and in the limit of low protein loading where the concentration of free ligands is nearly constant, pseudo-first order rate expressions can be defined,

$$r_{fold,S} = k'_{fold,S} [U_s] \quad (3)$$

$$r_{unf,S} = k'_{unf,S} [N_s] \quad (4)$$

The equilibrium for adsorbed protein unfolding can be described by the partition coefficient, defined as

$$A_{unf,S} = \frac{[U_S]}{[N_S]} \quad (5)$$

2.2 Unfolding and hydrogen-deuterium exchange kinetics

The applicable principles of protein labeling with deuterium for this study have been described previously [16] and are reviewed briefly here. Amide hydrogen atoms in the peptide backbone may exchange with deuterons in solution at a rate that depends on solvent accessibility and hydrogen bonding [41], which are in turn dependent on the three-dimensional structure of the molecule [42]. Protein unfolding increases the solvent exposure of buried residues, resulting in increased hydrogen exchange in a deuterium-rich solvent.

At ambient temperature and neutral pH, the intrinsic rate of hydrogen-deuterium exchange for amide hydrogens is on the order of 10^3 min^{-1} [43], several orders of magnitude greater than the unfolding rates of adsorbed protein observed in this work (see Results). In this kinetic regime (termed EX1), the mass distribution of a protein will be bimodal, with a low mass population consisting of molecules that have not unfolded in the presence of deuterium and a high mass population of protein that has received increased labeling due to unfolding. The acquisition of increased labeling can be assumed to be instantaneous upon solvent exposure of buried residues under these conditions.

Figure 1 presents a schematic depiction of a batch system in which adsorption, conformation change and deuterium labeling occur simultaneously. Adsorption and conformation change are reversible processes, while hydrogen exchange labeling is considered to be irreversible in a deuterium-rich solvent. Unfolded protein in the adsorbed phase is treated as irreversibly bound. Material balances for the low-mass species N and N_S yield the system of differential equations

$$\frac{d[N]}{dt} V_m = -(k_{unf} + k_{ads,N}) V_m [N] + k_{des,N} V_S [N_S] \quad (6)$$

$$\frac{d[N_S]}{dt} V_S = k_{ads,N} V_m [N] - (k_{des,N} + k_{unf,S}) V_S [N_S] \quad (7)$$

If the protein is highly stable in solution, such that $k_{unf} \approx 0$, then Equation 6 can be simplified to

$$\frac{d[N]}{dt} V_m = -k_{ads,N} V_m [N] + k_{des,N} V_S [N_S] \quad (8)$$

Equations 7 and 8 can be solved analytically, resulting in the governing equations

$$[N] = c_1 e^{\lambda_1 t} + c_2 e^{\lambda_2 t} \quad (9)$$

$$[N_s] = c_1 \frac{V_m(k_{ads,N} + \lambda_1)}{V_s k_{des,N}} e^{\lambda_1 t} + c_2 \frac{V_m(k_{ads,N} + \lambda_2)}{V_s k_{des,N}} e^{\lambda_2 t} \quad (10)$$

where the constants and eigenvalues are

$$c_1 = \frac{V_s k_{des,N} [N_s]_0 - V_m [N]_0 (k_{ads,N} + \lambda_2)}{V_m (\lambda_1 - \lambda_2)} \quad (11)$$

$$c_2 = [N]_0 - c_1 \quad (12)$$

$$\lambda_1 = \frac{-(k_{ads,N} + k_{des,N} + k_{unf,S}) + \sqrt{(k_{ads,N} + k_{des,N} + k_{unf,S})^2 - 4k_{ads,N}k_{unf,S}}}{2} \quad (13)$$

$$\lambda_2 = \frac{-(k_{ads,N} + k_{des,N} + k_{unf,S}) - \sqrt{(k_{ads,N} + k_{des,N} + k_{unf,S})^2 - 4k_{ads,N}k_{unf,S}}}{2} \quad (14)$$

2.3 Analysis of labeled protein structure

The deuterium labeling procedure utilized in this study results in a time-dependent increase in the mass of protein molecules. While the hydrogen-exchange rate of each residue is a unique function of many variables, we classify all residues as fast, medium, or slow exchangers. Following Tobler and Fernandez [44], we describe the fractional labeling of protein with the empirical model

$$\frac{D}{D+H} = 1 - f_{fast} e^{-k_{fast} t} - f_{med} e^{-k_{med} t} - f_{slow} e^{-k_{slow} t} \quad (15)$$

Further, at the conditions of the experiments in this work, the intrinsic exchange rate is sufficiently rapid that we consider the fast-exchanging residues to be instantly labeled upon the addition of labeling buffer. We define the slow-exchanging residues to be those residues that do not acquire labeling in the time frame of the experiments. Therefore, Equation 15 can be simplified to

$$\frac{D}{D+H} = 1 - f_{med} e^{-k_{med} t} - f_{slow} \quad (16)$$

Equation 16 is fit to data for the change in mass of protein with labeling time (minimization of sum of squared errors) to determine the parameters f_{fast} , f_{med} , f_{slow} and k_{med} .

3. Materials and Methods

3.1 Materials

Bovine α -lactalbumin, bovine β -lactoglobulin B, and bovine α -chymotrypsinogen A were obtained from Sigma (St. Louis, MO, USA) and used without further purification. All other chemicals were reagent grade or better. The HIC media used in this study were Phenyl SepharoseTM 6 Fast Flow (low substitution), Phenyl SepharoseTM 6 Fast Flow (high substitution) and Butyl SepharoseTM 4 Fast Flow bulk resins purchased from GE Healthcare (Piscataway, NJ, USA).

3.2 Isocratic elution chromatography

β -Lactoglobulin B and α -chymotrypsinogen A were eluted isocratically on Phenyl SepharoseTM 6 Fast Flow (high substitution) and Butyl SepharoseTM 4 Fast Flow media. The media were packed according to manufacturer guidelines into HR5/2 columns (GE Healthcare) to bed volumes of 0.4 (phenyl) and 0.5 ml (butyl). In each chromatographic run, 50 μ L of a 2.5 mg/mL protein solution was loaded and eluted at a constant flow rate of 0.2 mL/min. The mobile phase consisted of 100 mM MOPS, pH 7.0 buffer and ammonium sulfate at concentrations up to 1.4 M was used as the modifier. The outlet of the column was monitored by UV absorbance at 280 nm throughout the entire chromatographic run. Chromatography was performed on an AKTA Explorer 10 chromatography system provided by GE Healthcare (Piscataway, NJ, USA).

3.3 Batch adsorption and hydrogen-deuterium isotope exchange

Hydrogen-deuterium isotope exchange was performed to detect conformational change of proteins adsorbed to HIC resins. Batch adsorption and deuterium labeling were performed according to the previously documented protocol [16], with changes as described below.

In each experiment, 100 μ L of 4–5 mg/mL protein solution was equilibrated with 200 μ L of resin slurry, 50% v/v settled bed, for 30 minutes at ambient temperature (22–25 °C). Protein solutions and resin slurries were prepared at pH 7.0, 50 mM MOPS and ammonium sulfate concentrations of 0.8–1.4 M. The proportions of protein, settled resin bed and buffer in the batch adsorption systems ensured that resin loading did not exceed 4 mg protein per mL of settled bed, and protein concentration in the supernatant did not exceed 1.7 mg/mL. Ultraviolet (UV) absorbance measurements of the supernatant at 280 nm indicated that the concentration of protein in the supernatant was not measurably changed after the 30 minute equilibration period.

After establishing equilibrium, 1800 μ L of deuterated salt buffer was added to the mixture. Labeling was conducted at ambient temperature for labeling times of 10–600 s, and labeling was quenched by removing a 500 μ L aliquot of the slurry and adding to a chilled microcentrifuge tube containing 50 μ L of quench buffer (100 mM PO_4^{3-} , pH 2.5). The resin and supernatant were separated by centrifugation for 1 minute at 5900 g with a 0.22 μ m Amicon centrifugal filter (Millipore, Billerica, MA, USA). The UV absorbance of the supernatant was measured at 280 nm, and used with an independently determined extinction coefficient to determine the amount of protein in solution. The mass distribution of protein in the supernatant was determined by mass spectrometry.

Protein adsorbed to the resin was eluted with 200 μ L of deuterated elution buffer (pD 3.0, 8M Gdn-HCl, 25 mM PO_4^{3-}) at 0 °C. This buffer composition and temperature were selected to minimize the amide hydrogen exchange rate and prevent loss of deuterium labels, while maximizing protein recovery. Mass spectra of unlabeled protein under the elution condition showed negligible acquisition of labels at the elution condition (data not

shown). The microcentrifuge vial containing the elution mixture was agitated and placed on ice for 2 minutes, and filtration was performed as described above to separate supernatant and resin. The supernatant containing eluted protein was analyzed by mass spectrometry.

Control experiments were performed by labeling each protein per the labeling protocol described above at 0.8 M ammonium sulfate in the absence of HIC resin. These controls were also conducted at labeling times of 10–600 s.

3.4 Mass spectrometry

Mass spectrometry was performed to analyze protein mass distributions after deuterium labeling following the method described previously [16]. Briefly, protein solutions were injected onto a 200 μ L or 500 μ L stainless steel sample loop, which was mounted on a 2-way, 6-port injection valve (Rheodyne, Rohnert Park, CA, USA). Protein molecules were concentrated and de-salted on a 12.5 mm $\times$ 2.1 mm I.D. Zorbax C4 guard column (Agilent Technologies, Wilmington, DE, USA) for 2 min at 0.5 mL/min. Following de-salting, protein was eluted from the RPC column with a 5 minute gradient from 5 to 80% Solvent B. Solvent A was distilled water with 0.05% TFA, and solvent B was acetonitrile with 0.04% TFA. HPLC solvents were de-gassed on-line and delivered by a SpectraSystem P4000 gradient pump (Thermo Electron Corporation, San Jose, CA, USA). For deuterium-labeled protein, the sample loop, injection valve, RPC column, and solvent inlet line were submerged in an ice-water bath to minimize back-exchange.

Protein eluted from the RPC column was pumped directly to the electrospray ionization (ESI) source of an LCQ Duo mass spectrometer (Thermo Electron Corporation, San Jose, CA, USA). Flow was split from 0.5 mL/min to approximately 30 μ L/min immediately prior to the ESI source. The ESI source capillary temperature was 200°C and the spray voltage was 4.2 kV. Mass spectral data was collected in single-ion monitoring (SIM) mode in the following mass-to-charge (m/z) ranges: 1770–1790 for α -lactalbumin, 1825–1850 for β -lactoglobulin B, and 1972–1997 for α -chymotrypsinogen A. These ranges correspond to the detection of ions with respective mass-to-charge ratios of +8, +10 and +13.

4. Results

4.1 Isocratic elution chromatography of unstable HIC systems

In this study, we sought to examine the behavior of HIC systems in which the protein is conformationally unstable when adsorbed to the stationary phase. It has been proposed that one indicator of unfolding during HIC is incomplete elution during isocratic chromatography [12,13,45]. Potential proteins for the current work were identified based on isocratic pulse-response chromatographic behavior.

Incomplete elution during isocratic HIC was observed for two globular proteins, β -lactoglobulin B and α -chymotrypsinogen A, on the phenyl (high substitution) and butyl resins. Sample chromatograms for adsorption to butyl resin are shown in Figure 2. β -Lactoglobulin B undergoes a sharp transition in retention behavior with a small change in mobile phase modifier concentration when adsorbed to the butyl resin; the protein is relatively weakly retained at 0.8 M ammonium sulfate, but appears to be irreversibly bound at 1.0 M salt and must be removed from the column by a wash with salt-free buffer. On the same resin, the retention strength of α -chymotrypsinogen A increases more gradually with salt concentration and the protein becomes irreversibly bound at 1.4 M ammonium sulfate. On the high substitution phenyl resin, both β -lactoglobulin B and α -chymotrypsinogen A are non-eluting over the range of ammonium sulfate concentrations investigated (data not shown).

The unfolding behavior of α -lactalbumin adsorbed to hydrophobic surfaces has been examined previously [24,25,32,36] and extensively in the current series of articles [16–18]. *Holo* α -lactalbumin was studied when adsorbed to low substitution Phenyl Sepharose™ resin at elevated temperature (55 °C), and it was shown that while conformation change occurs during HIC, the unfolding is limited and has little effect on the observed partitioning [16]. In contrast, however, recently published HIC isocratic elution chromatography data suggests that on the more hydrophobic resins and conditions considered in this study, conformation change may be more significant and result in extended tailing of isocratic elution chromatograms [8,40]. Although elution of *holo* α -lactalbumin is nearly complete in the chromatography experiments, we included this protein in studies utilizing deuterium labeling to determine if the extent and rate of unfolding could be quantified by the present technique.

4.2 Hydrogen exchange during HIC adsorption

Holo α -lactalbumin, β -lactoglobulin B and α -chymotrypsinogen A were batch adsorbed to butyl and phenyl (high substitution) Sepharose™ Fast Flow HIC resins to allow comparison with the adsorption conditions of isocratic elution chromatography. Experiments were performed using ammonium sulfate concentrations of 0.8–1.4 M in the mobile phase. Under these conditions, the protein was labeled with deuterium to detect and quantify the amount of protein that had undergone conformational change during the labeling period. In EX1 hydrogen-deuterium exchange, the distribution of protein labeling states at a particular labeling time depends on both the equilibrium and kinetics of conformation change. Labeling experiments were performed at multiple labeling times to allow application of the derived governing equations (Equations 9 and 10).

Sample mass spectra of protein desorbed from the resin after labeling are shown in Figure 3. The spectra display two distinct behaviors with labeling time. Some mass distributions are bimodal, though overlap between the distributions can exist (Figure 3b). When two peaks are observed, the low molecular weight peak decreases in amplitude with labeling time, while the high molecular weight peak increases, consistent with EX1 behavior. The peaks also increase in mass with labeling time, indicating that there are residues that are partially protected from solvent exposure and acquire deuterium at a reduced rate relative to fully solvent-exposed residues.

The peak behavior in these spectra are representative for each protein species. α -chymotrypsinogen A (Figure 3a) displayed only a single peak for each resin investigated over the range of salt concentrations, even at chromatographic conditions where the protein did not elute in isocratic elution chromatography. Both β -lactoglobulin B (Figure 3b) and α -lactalbumin (Figure 3c) displayed bimodal mass distributions at the various experimental conditions.

The masses for each protein after desorption from the HIC resins are compared with solution-phase controls in Figures 4, 5 and 6. All control experiments exhibited single-peak spectra with substantial solvent protection, confirming native structure in solution. The spectra detected for α -chymotrypsinogen A when adsorbed to HIC resins are consistent with the behavior of the solution phase controls, indicating that the conformation of the protein is not changed upon adsorption. For both β -lactoglobulin B and α -lactalbumin, the low mass peak in the bimodal distributions corresponds closely with the mass of protein labeled in solution. The parameters of Equation 16 for each protein are reported in Table 1. For all proteins, there was no statistical difference in the fraction of residues that are highly protected (f_{slow}) between the solution-phase controls and the low-mass peaks.

Figures 5 and 6 show the fractional labeling of unfolded β -lactoglobulin B and α -lactalbumin adsorbed to the butyl and high substitution phenyl resins at all of the salt concentrations studied. For both proteins, the solvent exposure of the unfolded protein does not change with increasing ammonium sulfate in the mobile phase. However, in the case of β -lactoglobulin B, the unfolded conformations differ between the two resin types. On the butyl resin, nearly all of the residues are labeled within 10 minutes, while when adsorbed to the high-substitution phenyl resin, a larger fraction of the residues remains protected. Table 1 indicates that there is a significant difference in the solvent exposure of the unfolded conformation on these resins. Conversely, α -lactalbumin is highly solvent-exposed on both resins.

The bimodal mass distributions displayed by β -lactoglobulin B and α -lactalbumin (Figures 3b and 3c) indicate that conformation change occurs for these proteins during HIC. Bimodal mass distributions are observed not only for protein desorbed from the resin after labeling, but also protein in the supernatant, as shown in Figure 7. Since control experiments indicate that unfolding does not occur in the solution phase on this time scale, this result demonstrates that protein that unfolds upon adsorption desorbs to the supernatant, either directly or by a surface-refolding pathway.

To investigate the effect of resin ligand density on HIC-induced unfolding, deuterium labeling experiments were performed using β -lactoglobulin B and *holo* α -lactalbumin adsorbed to low-substitution phenyl resin, which has a manufacturer-reported nominal ligand density (20 $\mu\text{mol/ml}$) that is half of the reported ligand density of the high-substitution phenyl resin (40 $\mu\text{mol/ml}$). Representative data in Figure 8 illustrate that β -lactoglobulin B desorbed from the resin after labeling gave no indication of conformation change at salt concentrations up to 1.4 M, while α -lactalbumin shows an increased mass peak beginning to form at long labeling times and high salt concentrations (1.2 and 1.4 M). These data suggest that unfolding of these proteins adsorbed to the low-substitution phenyl resin is limited and occurs on a characteristic time scale that is substantially longer than that observed with the high-substitution phenyl and butyl resins. For these reasons, kinetic modeling of data collected with the low-substitution phenyl resin was not performed.

In these deuterium labeling experiments, the adsorption systems are initially at equilibrium, but this equilibrium is disrupted by the addition of labeling buffer. Specifically, the protein concentration in the supernatant is reduced by dilution, resulting in a driving force for net protein desorption. In previous application of this experimental protocol, the concentration of protein in the supernatant was found to have reached a constant value at times much shorter than the characteristic time for unfolding on the resin surface [16]. However, supernatant protein concentration profiles in Figure 9 show that in the current experiments, equilibrium is not always re-established within the labeling times investigated.

The governing equations for simultaneous adsorption/desorption, conformation change and hydrogen exchange (Equations 9 and 10) require the concentrations of unlabeled protein in each phase at multiple labeling times. The concentration of protein in the mobile phase is determined by UV spectroscopy, and the concentration in the stationary phase computed by overall material balance. The fraction of protein in each phase that has not unfolded during the labeling period is computed from the mass spectra. Each spectrum is modeled as the sum of two Gaussian population distributions, and the relative amounts of protein in each population are determined by the areas.

The governing equations, Equations 9–14, have pseudo-first order rate constants and the initial conditions as the adjustable parameters. As a result, both the kinetics and thermodynamics of adsorbed protein unfolding during HIC can be quantified with this

experimental protocol. Data were fit to the Equations 9 and 10 by adjusting the rate constants $k'_{unf,S}$, $k_{ads,N}$ and $k_{des,N}$, as well as the surface-unfolding partition coefficient ($A_{unf,S}$). At equilibrium the rates of adsorbed protein folding and unfolding are equal, and Equations 3–5 yield the following expression to calculate the pseudo-first order adsorbed protein folding rate constant:

$$k'_{fold,S} = \frac{k'_{unf,S}}{A_{unf,S}} \quad (17)$$

Model parameters were determined by minimizing the sum of squared errors with the Microsoft Excel Solver add-in, and standard errors were computed with the SolverAid macro [46]. For all systems except β -lactoglobulin B adsorbed to Butyl SepharoseTM resin, strong partitioning of protein into the adsorbed phase at 1.4 M ammonium sulfate resulted in concentrations of protein in the supernatant that were too low for labeling analysis, and modeling was not performed at this condition.

Figure 10 plots representative data for a set of adsorption and labeling experiments at multiple labeling times, along with the model fit of Equations 9 and 10. Unlabeled protein is lost from the surface with labeling time, as expected. Interestingly, the amount of unlabeled protein in the supernatant initially increases, before eventually decaying. This is a result of the competing effects of unlabeled protein desorption and labeling of adsorbed protein. For the system in Figure 10, some of the adsorbed protein is in the native conformation at equilibrium. Upon addition of labeling buffer, protein that desorbs from the resin results in an increase in unlabeled protein in the supernatant. However, as the experiment proceeds, both adsorption and desorption continue to occur dynamically. Adsorbed protein also continues to unfold and refold on the resin surface, resulting in the overall amount of unlabeled protein decreasing with time. Eventually, the concentration of unlabeled protein in the supernatant decreases as more of the molecules in the system have undergone unfolding in the presence of the deuterium labeling buffer. As seen in Figure 10, the proposed model is able to describe this phenomenon with fitted values of the model parameters.

Figure 11 shows the quantity $-RT \ln A_{unf,surf}$ plotted against ammonium sulfate concentration for both α -lactalbumin and β -lactoglobulin B on the butyl and high substitution phenyl resins. More negative values of this plotted quantity are indicative of stronger surface-induced unfolding. The results show that unfolding becomes more energetically favorable with increased salt concentration in most systems. A sharp transition is observed for β -lactoglobulin B adsorbed to Butyl SepharoseTM between 0.8 and 1.0 M salt, consistent with the independently observed chromatographic behavior (Figure 2). For the same resin and salt concentration, unfolding of β -lactoglobulin B is generally more thermodynamically favored compared to α -lactalbumin. Similarly, the butyl resin is observed to be more denaturing than the phenyl resin for both proteins. When compared to folding stabilities from the literature reported in Table 2 (a quantity identical to $-RT \ln A$ as defined here), however, it can be seen that the change in folding stability is approximately -34 to -38 kJ/mol for α -lactalbumin, while the stability of β -lactoglobulin B is decreased by approximately 27 to 32 kJ/mol. Thus, it appears that while β -lactoglobulin B is less stable than α -lactalbumin at comparable adsorption conditions, the destabilizing effect of adsorption to these HIC resins is greater for α -lactalbumin.

Pseudo-first order rate constants for folding and unfolding on the chromatographic surface are plotted in Figure 12. No overall trend with salt concentration is observed, and the effect of the resin properties differs for each protein. Unfolding of α -lactalbumin on the surface appears to generally be more rapid than that of β -lactoglobulin B for the same resin and salt

concentration. However, refolding is also faster for α -lactalbumin, indicating that this protein explores conformational space more rapidly than β -lactoglobulin B when adsorbed to these hydrophobic surfaces. Forge et al. [47] reported that the solution-phase folding rate constant for α -lactalbumin in 10 mM CaCl_2 (pH 7.2, 20 °C) is 20.2 s^{-1} , several orders of magnitude greater than the folding rates determined here. Likewise, application of Equation 15 to the problem of solution-phase folding/unfolding and the folding stability reported in Table 2 suggests an unfolding rate constant for α -lactalbumin on the order of 10^{-4} min^{-1} , substantially slower than unfolding rates of adsorbed protein. Although data are not available in the literature for the folding rate of β -lactoglobulin in solution at the conditions of interest here, the relative folding rates of these two proteins can be estimated based on the correlation with relative contact order shown by Plaxco and co-workers [48]. Utilizing the online protein contact order calculator from the University of Alberta (<http://busby2.cs.ualberta.ca/>) and PDB files listed in Table 2, the relative contact orders of β -lactoglobulin B and α -lactalbumin are 0.13 and 0.10, respectively. This result indicates that, in solution, β -lactoglobulin is expected to have a slower folding rate than α -lactalbumin, consistent with the relative folding rates of the adsorbed species.

Although the intention of this work is to study the conformational behavior of proteins during HIC, the non-equilibrium nature of this protocol requires the description of dynamic protein adsorption and desorption during the labeling experiment. The pseudo-first order rate constants were found to range in order of magnitude from $0.1 - 1 \text{ min}^{-1}$, and the model fits of the data were relatively insensitive to the values of these parameters, with standard errors of 50–100% of the parameter value for most systems. Studies of protein mass transfer in hydrophobic chromatography resins have been reported in the literature [15,49], and explicit mass transfer studies and detailed modeling may prove to enhance the method proposed here in the future.

5. Discussion

This work has developed an experimental protocol and appropriate model of protein adsorption and unfolding in HIC, so that both the kinetics and thermodynamics of protein conformational change during HIC may be quantified. Previous studies have utilized modeling of chromatographic data for this purpose, so the present results can be compared with those studies.

Jungbauer and co-workers have previously identified β -lactoglobulin as unstable on butyl-based HIC resins using a chromatographic technique [22,23,45]. In that work, isocratic elution chromatograms are generated, and it is observed that some of the protein is not eluted until a wash step is initiated. This is interpreted as an indication of protein unfolding so that integration of peaks in the isocratic elution and wash should yield the fraction of protein that was unfolded during the experiment. These experiments are performed at multiple flow rates to obtain data at varied residence times, and the data are used with an irreversible unfolding model to characterize the kinetics of surface-induced unfolding.

One of the resins used in the current work, Butyl SepharoseTM 4 Fast Flow, was also investigated by Ueberbacher and co-workers [23], and ammonium sulfate was used as the mobile phase modifier, allowing direct comparison for the unfolding behavior of β -lactoglobulin B. At 25 °C and 0.5 – 0.7 M ammonium sulfate, the data of Ueberbacher and co-workers indicates unfolding rate constants of $k \approx 0.05 - 0.15 \text{ min}^{-1}$ [23]. This rate is close to the pseudo-first order rate constants determined here, $k_{unf,surf} = 0.2 - 0.3 \text{ min}^{-1}$, despite the use of an irreversible unfolding model. The somewhat higher value of the rate constant in this work may be a result of accounting for reversible conformation change on the surface, even though at ammonium sulfate concentrations $\geq 1.0 \text{ M}$, the folding rate

constants were determined to be much lower than the unfolding rate ($k_{fold,surf} = 0.05 - 0.1 \text{ min}^{-1}$). It must also be noted that lot-to-lot differences in the ligand density of commercial HIC resins yields differences in observed chromatographic behavior [40] and may also result in variability in the observed unfolding behavior.

The data of Ueberbacher et al. displays a significant transition in the unfolding behavior with ammonium sulfate concentration from 0.6 – 1.0 M [23]. With an irreversible unfolding model, such a transition must be attributed to an increase in the kinetics of surface-induced unfolding with salt concentration. However, the results obtained in the current study suggest that there is a significant increase in the amount of unfolded protein on the resin at equilibrium when the salt concentration is increased from 0.8 M to 1.0 M, without an accompanying increase in the unfolding rate. The rate of refolding is therefore calculated to decrease, indicating that energetically favorable unfolding and slow refolding are responsible for the observed chromatographic response. This demonstrates that in order to discern the cause of an unfolding-induced loss in chromatographic recovery, both thermodynamics and kinetics must be considered. Again, differences in the ligand density of the butyl resins used in these studies may contribute to the observed difference in salt concentrations over which a shift in behavior occurs.

It must be noted that, in solution at ambient temperature and neutral pH, bovine β -lactoglobulin exists predominantly as a homodimer [50] and dissociation of the dimer may play a role in the HIC behavior. It is unlikely that the dimeric structure stays intact when the protein denatures, and the mass spectra corresponding to high solvent exposure observed in the deuterium labeling experiments most certainly coincide with protein that has dissociated and unfolded. The lower mass spectra are consistent with solution phase control experiments, which were performed at 0.8 M ammonium sulfate. Literature studies have shown that increasing ionic strength favors dimer formation by β -lactoglobulin [51–53], suggesting that the control experiments are representative of the dimeric form. If the “native” peak in the adsorbed protein mass spectra actually corresponded to protein that had dissociated to monomer, but remained folded, we might expect that there would be some increase in labeling due to exposure of residues at the protein-protein interface. This is not observed, which supports the existence of dimer in the adsorbed phase.

The results for α -lactalbumin deuterium labeling during HIC confirm that conformation change occurs at ambient temperature on more hydrophobic resins. The measured partition coefficients for surface unfolding, though, indicate that unfolding is less energetically favored relative to β -lactoglobulin B, and values of the quantity $-RT \ln A_{unf,surf}$ that are positive correspond with unfolding that is thermodynamically unfavorable. The partition coefficient $A_{unf,surf}$ as defined by Equation 5, is often considered to be equivalent to the equilibrium constant by assuming species ideality, and $-RT \ln A_{unf,surf}$ under such an assumption is equivalent to the Gibbs free energy change of adsorbed protein unfolding. However, as has been recently discussed by Møllerup [54], the assumption of ideality is not rigorous and we do not make such an assertion here.

To and Lenhoff have described the elution behavior of proteins exhibiting tailing behavior in isocratic HIC by incorporating reversible surface unfolding into the modeling equations for adsorption and mass transfer [15]. Although results are not presented for the α -lactalbumin systems studied here, it is shown that the tailing behavior can be described by adsorbed protein unfolding equilibrium constants having values less than one, equivalent to the quantity $-RT \ln A_{unf,surf}$ having value greater than zero. These results are in qualitative agreement with our α -lactalbumin data, and support the idea that unfolding of proteins adsorbed to HIC resins that is less energetically favorable results in misshapen isocratic elution chromatograms, while more favorable unfolding leads to loss of recovery.

Comparison of folding and unfolding rates for α -lactalbumin in solution and when adsorbed to the HIC resins indicates that unfolding rate is increased upon adsorption, while the folding rate is decreased. Although the experiments themselves do not reveal a mechanism for the effect of the surface on these rates, we hypothesize that the presence of the hydrophobic ligands changes the effective activation energy for each transition. When unfolding, the protein will sample transitional conformations between fully folded and fully unfolded states, and these transitional conformations may be stabilized on the HIC surface relative to solution, reducing the energetic barrier for unfolding. For folding of adsorbed protein, it is expected that many of the protein-ligand hydrophobic interactions that are present for protein in the unfolded state will be lost upon refolding. These interactions are energetically favorable, and therefore impose an increased energetic barrier to folding compared to that of protein in solution.

The experiments conducted in this study have focused on the conformational behavior of proteins adsorbed to HIC resins only at low loading, where we assume loading effects to be negligible. Fogle et al. [36] have used a similar protocol to study the conformation of *holo* α -lactalbumin adsorbed to a phenyl SepharoseTM resin at increased loadings. An empirical first order rate model was applied, and the apparent rate constant for surface-induced unfolding was found to decrease with increased loading. Although a qualitative analysis by the current model is not possible, since solution phase concentrations and mass spectra of protein in the supernatant were not obtained, we find that the data of Fogle and co-workers agree qualitatively with Equation 2, in that we expect increased loading to decrease the unfolding rate of adsorbed protein based on a decreased concentration of free ligands.

The theory presented in this paper states that an adsorbed protein increases the number of ligands contacted upon unfolding, and the rate of unfolding is therefore dependent on the concentration of free ligands. From a physical standpoint, this may be interpreted as inhibition of unfolding due to competition of closely packed adsorbed protein molecules for free ligands, and steric interactions between proteins might also be expected to play a role. However, Fogle et al. calculated the maximum surface coverage of the resin in their experiments as only 58% of accessible area [36], and proposed that the inhibition of adsorbed protein unfolding rates with increased loading is not due to steric interactions, but to the probability that an individual protein molecule will be adsorbed at a site with a locally high concentration of hydrophobic ligand. This mechanism is consistent with the theory proposed in this work.

The solvent exposure of adsorbed α -chymotrypsinogen A was unaltered compared to solution control experiments, for all resins and salt concentrations investigated, even at very highly retaining conditions where the protein binding appears to be irreversible. This is an important result indicating that, in some cases, irreversible adsorption may not coincide with conformation change. Further, we might expect that an increase in solvent protection (decrease in amount of labeling) would result from strong association with the resin surface, but this is also not observed for α -chymotrypsinogen A. The possibility that conformation change and solvent exclusion due to protein-resin contact are occurring simultaneously, resulting in a net neutral effect on the global hydrogen exchange behavior, cannot be ruled out by the present work.

The conformational behavior of α -chymotrypsinogen A on Butyl SepharoseTM resin was previously investigated by Tibbs-Jones and Fernandez [38] using HX-MS. The solvent exposure of the adsorbed protein was observed to increase upon adsorption, although to a lesser extent than in the fully-labeled control experiment. The extent of labeling was independent of the salt concentration at which labeling was performed. This work contrasts with the present study, in which no change in solvent exposure with salt was observed for

the same protein-resin system. However, the protocol of Tibbs-Jones and Fernandez utilized protein loading at 2 M ammonium sulfate prior to labeling, even for experiments where the labeling was performed at a lower salt concentration. Differences in the labeling behavior may be attributed to a kinetic limitation in refolding from a partially unfolded state that was favored at the 2 M salt condition. Further, sensitivity to lot-to-lot differences in ligand density of the resin may once again restrict the extent to which the results can be compared quantitatively.

A significant advantage of the current work is that a universal protocol and modeling can be applied independent of the retention behavior of the chromatographic system. In this work we have identified systems in which the protein is stable despite very strong retention (α -chymotrypsinogen A) and quantified the kinetics and thermodynamics of adsorption-induced unfolding of another system (β -lactoglobulin B) that exhibits non-eluting behavior. Isocratic chromatographic methods to study this phenomenon, in contrast, require at least partial elution of the protein. Further, the HX-MS protocol enables the identification of conformation change via a change in a property at the molecular level (overall solvent exposure) while avoiding disadvantages associated with spectroscopic methods, such as interference from the stationary phase.

It is desirable to relate the biophysical behavior of proteins to molecular properties or descriptors in order to identify trends, and selected properties of the three proteins selected for investigation by hydrogen-exchange labeling are summarized in Table 2. The conformational stabilities of each protein in the absence of salt, but at similar pH to that used in the present work have been reported in the literature [55–57]. A four-state model of protein adsorption and unfolding [17,18] proposed that the conformational stability of proteins in the adsorbed and solution phases are coupled in a thermodynamic cycle, suggesting that the unfolding behavior of these proteins on the resin surface may follow the same trend exhibited in solution. We observe that the stabilities of our three proteins in this study correspond with their unfolding behavior in HIC. α -chymotrypsinogen A is the most stable, and does not unfold during HIC, even under conditions where the isocratic chromatogram is non-eluting. α -Lactalbumin possesses an intermediate stability in the set and unfolds when adsorbed, while β -lactoglobulin B is the least stable and exhibits the most unfolding. The stabilities of these latter two proteins are greatly reduced on the chromatographic surface relative to the solution phase. Another model protein, hen egg white lysozyme, has been shown to be conformationally stable on HIC resins in independent studies [23,38]. Though it is a structural homologue of α -lactalbumin [58], the reported structural stability of lysozyme (43.1 kJ/mol) [56] is substantially greater than that of *holo* α -lactalbumin. Together, these observations suggest a relationship between the conformational stability of proteins in solution and adsorbed to HIC resins.

The molecular scheme proposed here connects the unfolding of adsorbed protein in HIC to water molecule displacement from the hydrophobic ligands. This suggests that the relative denaturing effect of an HIC resin on an adsorbed protein would be proportional to the relative increase in surface area of the protein upon unfolding. Proteins that have increased area when unfolded would be expected to have greater numbers of water molecules displaced from the hydrophobic interface ($\omega_{\text{H}}^{\text{H}}$). Myers et al. found that the change in surface area on unfolding correlates very strongly with the number of residues in a protein [59], and this correlation was used to estimate the change in surface area on unfolding of the three proteins considered in this study (reported in Table 2). Table 2 also includes changes in solvent accessible surface area upon unfolding calculated using the software Naccess 2.1.1, and it can be seen that these results differ quantitatively from the correlation of Myers et al., but the trend in the relative changes in area of the three proteins is consistent. In each case, the surface areas do not trend with the observed stability of the adsorbed proteins. Further,

the change in stability of α -lactalbumin is greater than that of β -lactoglobulin B when these proteins are adsorbed, though the calculations indicate that the increase in surface area upon unfolding is less for α -lactalbumin. Changes in hydrophobic surface area upon unfolding for each protein were also computed by Naccess 2.1.1 (Table 2), and again there is no apparent trend with either the adsorbed protein stability or the change in stability conferred by adsorption to the hydrophobic surfaces.

We suggest that unfolding of an adsorbed protein will also result in solvent exposure of some buried hydrophobic residues, creating additional hydrophobic contact with the solvent. The balance between increased contact with the resin and increased solvent exposure of the hydrophobic core will ultimately determine the stability of the adsorbed protein, and the change in surface area of a protein upon unfolding may not be a reliable sole indicator of these competing forces.

The adiabatic compressibility has been suggested as a property that may correlate with conformation change on adsorption in HIC [8,60], and this too is consistent with the results of this study, though the compressibilities of α -lactalbumin and β -lactoglobulin reported in the literature [61] are very similar. It is noteworthy that the compressibility of α -lactalbumin was determined in the absence of calcium, and binding of calcium ions is known to affect the conformational stability of the molecule in solution [56] and during HIC [18,62]. Thus, the compressibility of the calcium-bound form of the molecule used in our study may be lower than the literature reported value. Regardless, the data here are for only three proteins, and data for a much larger set is required before any relationships between HIC-induced unfolding behavior and molecular properties can be established.

6. Conclusions

A physically-based model of adsorbed protein conformation change during HIC has been employed with a corresponding experimental protocol, utilizing hydrogen-deuterium exchange to quantify the equilibrium partitioning and kinetics of adsorbed protein folding and unfolding. Three distinct combinations of isocratic elution and conformation behavior were observed. α -Chymotrypsinogen A, at conditions where the protein is non-eluting, apparently retains native structure. Under similarly non-eluting conditions, β -lactoglobulin B unfolds with slow refolding kinetics. Less unfolding, but with rapid conformational dynamics, is observed for α -lactalbumin, which elutes with extended tailing. These results are in good qualitative agreement with chromatographic data in the literature. Thus, while different chromatographic methods and modeling are required to study protein unfolding based on elution behavior, the HX-MS method proposed here can be successfully applied to systems with widely different behaviors. This allows for direct comparison of systems of different proteins, resins and salt concentrations. Further, by measuring a molecular property, we are able to distinguish the conformational changes undergone by the adsorbed protein, and showed that for β -lactoglobulin B, the conformation of unfolded protein depends on the nature of the resin. With study of more systems, generalized relationships among protein and resin properties and HIC-induced unfolding may be found.

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7. Nomenclature

$A_{unf,S}$ Partition coefficient for adsorbed protein unfolding

c_1, c_2	Constants in the solution of the governing differential equations
D	Number of amide groups that are deuterated
f_{fast}	Fraction of amide groups that exchange with rapid kinetics
f_{med}	Fraction of amide groups that exchange with medium kinetics
f_{slow}	Fraction of amide groups that exchange with slow kinetics
H	Number of amide groups that are not deuterated
$k_{ads,N}$	Rate constant for native protein adsorption
$k_{des,N}$	Rate constant for native protein desorption
k_{fast}	First-order rate constant for fast hydrogen-deuterium exchange
k_{fold}	Rate constant for protein folding in solution
$k_{fold,S}$	Rate constant for adsorbed protein folding
k_{med}	First-order rate constant for medium hydrogen-deuterium exchange
k_{slow}	First-order rate constant for slow hydrogen-deuterium exchange
k_{unf}	Rate constant for protein unfolding in solution
$k_{unf,S}$	Rate constant for adsorbed protein unfolding
$k'_{fold,S}$	Pseudo-first order rate constant for adsorbed protein folding
$k'_{unf,S}$	Pseudo-first order rate constant for adsorbed protein unfolding
L	Free ligand on the resin surface
N	Native protein in solution
N_s	Adsorbed, native protein
N^*	Native protein in solution that has acquired complete deuterium labeling
N_s^*	Native protein adsorbed to the resin that has acquired complete deuterium labeling
$[N_s]_0$	Concentration of adsorbed, native protein at the initial condition
$[N]_0$	Concentration of native protein in solution at the initial condition
$r_{fold,S}$	Folding rate of adsorbed protein
$r_{unf,S}$	Unfolding rate of adsorbed protein
t	Labeling time
U	Unfolded protein in solution
U_s	Adsorbed, unfolded protein
U^*	Unfolded protein in solution that has acquired complete deuterium labeling
U_s^*	Unfolded protein adsorbed to the resin that has acquired complete deuterium labeling
V_m	Mobile phase volume
V_s	Stationary phase volume (dry resin)
W	Water molecule displaced from ligand contact

χ	Stoichiometric number of water molecules contacting previously buried hydrophobic surface of adsorbed protein upon unfolding
λ_1, λ_2	Eigenvalues in the solution of the governing differential equations
ν	Stoichiometric number of ligands contacted by an adsorbed, native protein
ω	Stoichiometric number of additional resin ligands contacted upon adsorbed protein unfolding
ξ	Stoichiometric number of water molecules displaced per contacted ligand

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Figure 1.

Schematic depiction of simultaneous protein adsorption/desorption, folding/unfolding and hydrogen-deuterium exchange during HIC. The letters N and U denote native and unfolded species, respectively, while the subscript S denotes adsorbed species and symbols with an asterisk indicate protein with increased labeling as a result of unfolding.

Figure 2. Isocratic elution chromatograms for (a) β -lactoglobulin B and (b) α -chymotrypsinogen A adsorbed to Butyl SepharoseTM 4 Fast Flow at various concentrations of ammonium sulfate.

Figure 3.

Mass spectra of protein desorbed from Phenyl Sepharose™ 6 Fast Flow (high substitution) resin after labeling with deuterium for varied labeling times: (a) α -chymotrypsinogen A, 1.0 M ammonium sulfate; (b) β -lactoglobulin B, 0.8 M ammonium sulfate; (c) *holo* α -lactalbumin, 0.8 M ammonium sulfate. Mass centroids for unlabeled (solid) and fully-labeled (dashed) controls are shown by vertical lines.

Figure 4.

Fractional labeling vs. labeling time for α -chymotrypsinogen A eluted from (a) Butyl Sepharose™ 4 Fast Flow and (b) Phenyl Sepharose™ 6 Fast Flow (high substitution) resins at ammonium sulfate concentrations of 0.8 M (squares), 1.0 M (diamonds), 1.2 M (circles) and 1.4 M (triangles). Solid lines are the model fits to solution phase control data (data points not shown).

Figure 5.

Fractional labeling vs. labeling time for β -lactoglobulin B eluted from (a) Butyl SepharoseTM 4 Fast Flow and (b) Phenyl SepharoseTM 6 Fast Flow (high substitution) resins at ammonium sulfate concentrations of 0.8 M (squares), 1.0 M (diamonds), 1.2 M (circles) and 1.4 M (triangles). Data are shown for low-mass (open symbols) and high-mass (filled symbols) mass spectra. Solid lines are the model fits to solution phase control data (data points not shown) and dashed lines are the model fits to the high-mass data.

Figure 6. Fractional labeling vs. labeling time for *holo* α -lactalbumin eluted from (a) Butyl Sepharose™ 4 Fast Flow and (b) Phenyl Sepharose™ 6 Fast Flow (high substitution) resins at ammonium sulfate concentrations of 0.8 M (squares), 1.0 M (diamonds), 1.2 M (circles) and 1.4 M (triangles). Data are shown for low-mass (open symbols) and high-mass (filled symbols) mass spectra. Solid lines are the model fits to solution phase control data (data points not shown) and dashed lines are the model fits to the high-mass data.

Figure 7.

Mass spectra of supernatant protein in the presence of Phenyl Sepharose™ 6 Fast Flow (high substitution) after labeling with deuterium for varied labeling times: (a) β -lactoglobulin B, 0.8 M ammonium sulfate; (b) *holo* α -lactalbumin, 0.8 M ammonium sulfate. Mass centroids for unlabeled (solid) and fully-labeled (dashed) controls are shown by vertical lines.

Figure 8. Mass spectra of protein desorbed from Phenyl SepharoseTM 6 Fast Flow (low substitution) after labeling with deuterium for varied labeling times: (a) β -lactoglobulin B, 1.4 M ammonium sulfate; (b) *holo* α -lactalbumin, 1.2 M ammonium sulfate. Mass centroids for unlabeled (solid) and fully-labeled (dashed) controls are shown by vertical lines.

Figure 9. Concentration of α -lactalbumin (filled symbols) and β -lactoglobulin B (open symbols) in the supernatant for the experimental systems Butyl SepharoseTM 4 Fast Flow-1.2 M ammonium sulfate (squares) and Phenyl SepharoseTM 6 Fast Flow (high substitution)-0.8 M ammonium sulfate (triangles) plotted against labeling time.

Figure 10.

Amount of low molecular weight (less labeled) *holo* α -lactalbumin in the adsorbed (filled symbols) and mobile (open symbols) phases vs. labeling time for adsorption to Phenyl Sepharose™ 6 Fast Flow (high substitution) resin in 1.0 M ammonium sulfate. Solid lines are the model of Equations 9 and 10 fitted to the data.

Figure 11.

The quantity $-RT \ln A_{unf,S}$ for each experimental system at the studied mobile-phase ammonium sulfate concentrations. Error bars represent the standard error of the model fit.

Figure 12.

Pseudo-first order rate constants for (a) adsorbed protein unfolding and (b) adsorbed protein folding for each experimental system at the studied mobile phase ammonium sulfate concentrations. Error bars for (a) represent the standard error from the model fit, and error bars for (b) from propagation of errors.

Table I

Characterization of deuterium labeling behavior in solution and adsorbed to HIC resins

Protein	Media	f_{slow}	f_{med}	f_{fast}	$k_{med} (min^{-1})$
α -chymotrypsinogen A	Solution	0.28 ± 0.02	0.23 ± 0.01	0.49 ± 0.01	0.53 ± 0.05
	Butyl	0.31 ± 0.02	0.24 ± 0.01	0.45 ± 0.01	0.73 ± 0.08
	Phenyl (high sub)	0.29 ± 0.02	0.21 ± 0.01	0.50 ± 0.01	0.64 ± 0.08
β -lactoglobulin B (Native)	Solution	0.39 ± 0.05	0.25 ± 0.03	0.36 ± 0.02	0.46 ± 0.12
	Butyl	0.34 ± 0.05	0.26 ± 0.03	0.40 ± 0.02	0.31 ± 0.12
	Phenyl (high sub)	0.40 ± 0.09	0.17 ± 0.07	0.43 ± 0.02	0.68 ± 0.63
β -lactoglobulin B (Unfolded)	Butyl	0.09 ± 0.04	0.28 ± 0.02	0.63 ± 0.02	0.91 ± 0.17
	Phenyl (high sub)	0.19 ± 0.04	0.18 ± 0.02	0.63 ± 0.02	0.82 ± 0.23
	Solution	0.41 ± 0.06	0.15 ± 0.03	0.44 ± 0.03	1.45 ± 0.65
α -lactalbumin (Native)	Butyl	0.41 ± 0.04	0.17 ± 0.02	0.42 ± 0.02	0.84 ± 0.22
	Phenyl (high sub)	0.38 ± 0.04	0.19 ± 0.02	0.43 ± 0.02	0.81 ± 0.22
	Butyl	0.08 ± 0.08	0.19 ± 0.04	0.73 ± 0.04	2.75 ± 0.97
α -lactalbumin (Unfolded)	Phenyl (high sub)	0.07 ± 0.04	0.17 ± 0.02	0.76 ± 0.02	1.87 ± 0.44

Table II

Select protein properties.

Protein	PDB Reference	Molecular Weight (kDa)	Number of Residues	Folding Stability at 25 °C, pH 7 (kJ/mol)	ΔASA of Unfolding (Overall) (10 ³ Å ²)	ΔASA of Unfolding (Hydrophobic) (10 ³ Å ²)	Adiabatic Compressibility at 25 °C (10 ⁻¹² cm ² /dyne) ^d
α-chymotrypsinogen A	1CHG	25.7	245	52 ^b	18.3 ^e 26.0 ^f	15.7 ^f	4.05
α-lactalbumin	1F6S	14.3	123	37 ^c	6.9 ^e 12.8 ^f	7.5 ^f	8.27
β-lactoglobulin B	1BEB	18.3	162	28 ^d	12.4 ^e 17.3 ^f	10.4 ^f	8.45

^aGekko and Noguchi [57]

^bCupo et al. [51]

^cIn 12 mM CaCl₂, Ikeguchi et al. [52]

^dKella and Kinsella [53]

^eComputed from the correlation of Myers et al. [55]

^fComputed with Naccess 2.1.1