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Unfolding of a model protein on ion exchange and mixed mode chromatography surfaces

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ABSTRACT

Recent studies with proteins indicate that conformational changes and aggregation can occur during ion exchange chromatography (IEC). Such behavior is not usually expected, but could lead to decreased yield and product degradation from both IEC and multi mode chromatography (MMC) that has ligands of both hydrophobic and charged functionalities. In this study, we used hydrogen exchange mass spectrometry to investigate unfolding of the model protein BSA on IEC and MMC surfaces under different solution conditions at 25 °C. Increased solvent exposure, indicating greater unfolding relative to that in solution, was found for protein adsorbed on cationic IEC and MMC surfaces in the pH range of 3.0 to 4.5, where BSA has decreased stability in solution. There was no effect of anionic surfaces at pH values in the range from 6.0 to 9.0. Differences of solvent exposure of whole molecules when adsorbed and in solution suggest that adsorbed BSA unfolds at lower pH values and may show aggregation, depending upon pH and the surface type. Measurements on digested peptides showed that classifications of stability can be made for various regions; these are generally retained as pH is changed. When salt was added to MMC systems, where electrostatic interactions would be minimized, less solvent exposure was seen, implying that it is the cationic moieties, rather than the hydrophobic ligands, which cause greater surface unfolding at low salt concentrations. These results suggest that proteins of lower stability may exhibit unfolding and aggregation during IEC and MMC separations, as they can with hydrophobic interaction chromatography.

1. Background and introduction

Ion exchange chromatography (IEC) is a valuable tool for protein purification in the pharmaceutical industry. IEC can be used as a capture step for therapeutic proteins, as an orthogonal polishing step to hydrophobic interaction chromatography (HIC), or even in assisting the refolding of unfolded protein molecules.

IEC has the added advantage that, in general, protein unfolding and aggregation are not observed, but there is recent evidence that IEC surfaces, in addition to HIC surfaces, can also cause unfolding and aggregation of proteins. Unusual elution profiles with multiple peaks and lower than expected yields had been previously observed, but role of surface unfolding was uncertain [1–3]. Even in cases without unusual elution profiles, unexpected increases in high molecular weight species have been observed after IEC steps, suggesting surface induced unfolding and aggregation [3,4]. In one study, the unfolding and aggregation of an IgG1 on a strong cation exchanger was demonstrated using size exclusion chromatography (SEC) and hydrogen exchange mass spectrometry (HXMS) [4]. In that study, residence time, temperature, pH, and ionic strength all influenced protein unfolding and aggregation on the IEC surface. Protein conformation in IEC has been shown to affect chromatographic separations for proteins unfolded with urea or dithiothreitol [5,6]. These effects may have positive or negative effects on selectivity in separations. For example, if the unfolding is reversible, it could improve resolution as applied previously in hydrophobic interaction chromatography (HIC) [7–10].

There seem to be no detailed studies on how different operational variables affect unfolding in IEC. Studies with protein unfolding in HIC have demonstrated that both mobile and stationary phase properties are important to consider [11,12], so it is likely that these are also important in IEC unfolding. Of particular interest is how the mobile phase variables known to affect binding, such as pH and ionic strength, also affect unfolding. For stationary phases, it is uncertain if a protein that unfolds on a cation exchanger will also unfold on an anion exchanger or vice versa. Further, it is unclear if proteins unfold more or less on strong IEC media (charged over a

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wide pH range) or versus weak media (charged only over a narrow pH range).

Understanding unfolding on IEC surfaces would also be important for mixed mode chromatography (MMC). MMC stationary phases have both IEC and HIC characteristics to enhance removal of certain impurities by providing operational advantages over single mode steps such as IEC or HIC. The existence of multiple interactions makes difficult thorough understanding of the adsorption mechanisms [13], particularly how each type of interaction contributes to protein destabilization.

In this study, the binding and unfolding behavior of a model multi domain protein, bovine serum albumin (BSA), was examined on a series of IEC and MMC surfaces. It was hypothesized that by changing the degree of electrostatic interaction between the protein and IEC surface (through pH) the unfolding of BSA would vary. It was also hypothesized that changing the type of electrostatic interaction (by switching from cationic to anionic surfaces) would also affect BSA unfolding. Finally, it was hypothesized that by varying pH and ammonium sulfate concentration for BSA adsorbed on MMC surfaces, the individual effects of electrostatic and hydrophobic interactions on unfolding might be delineated.

Although the degree that BSA would unfold on these surfaces was uncertain, the low stability of BSA, and knowledge of its unfolding on HIC [12], made it a suitable candidate. To start, the effect of pH and ammonium sulfate concentration were examined with UV measurements and hydrogen exchange mass spectrometry (HXMS) to identify condition(s) giving unfolding. Additional HXMS experiments were then done with proteolytic digestion to observe how changing pH and type of IEC surface affects the different regions of BSA. Finally, the unfolding of BSA on Capto MMC with varying pH was done to try distinguishing electrostatic from hydrophobic interactions.

2. Materials and methods

2.1. Materials and equipment

BSA, citric acid, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Aldrich® (St. Louis, MO, USA). BSA was of BioReagent grade with a purity of ≥96% and vendor reported isolectric point of 5.3 and molecular weight of ~66 kDa. Monosodium citrate and disodium citrate were purchased from ACROS Organics (New Jersey, USA). Guanidine hydrochloride (GdnHCl) was purchased from MP Biomedicals (Solon, OH, USA). Disodium phosphate was purchased from Thermo Fisher Scientific Inc. (Fair Lawn, NJ, USA). Tris(2 carboxyethyl)phosphine hydrochloride (TCEP) was purchased from Thermo Fisher Scientific Inc. (Rockford, IL, USA).

The IEX resins, SP Sepharose® Fast Flow (FF) and Q Sepharose® Fast Flow (FF), and MMC resins, Capto™ MMC and Capto™ Adhere, used in this study were purchased from GE Healthcare (Uppsala, Sweden). Ultrafree® MC centrifugal filter units were purchased from Thermo Fisher Scientific (Houston, TX, USA) for the separation of supernatant liquid from resin particles.

The sample pump, LabAlliance™ Series I, was purchased from Thermo Fisher Scientific (Houston, TX, USA). The C_4 (TR1/25109/01, 1 mm inner diameter by 8 mm length) and C_8 (TR1/25109/02, 1 mm inner diameter by 8 mm length) desalting columns were purchased from Michrom Bioresources (Auburn, CA). Porcine pepsin was purchased from Sigma-Aldrich (Saint Louis, MO, USA) and immobilized on POROS-20AL packing media in a small column (2.1 mm inner diameter by 60 mm length) from Applied Biosystems (Grand Island, NY, USA). The XBridge C_{18} column (186003563, 2.1 mm inner diameter by 50 mm length, 3.5 μm pore size) was purchased from Waters (Milford, MA). The Surveyor MS HPLC pump and LTQ linear electrospray ionization quadrupole ion trap mass spectrometer were purchased from Thermo Finnigan (San Jose, CA, USA).

2.2. Sample preparation and measurements

Protein structure was studied as a function of pH and added salt in solution only and with different chromatographic media added. Hydrogen/deuterium exchange was done for most conditions with whole protein molecules and for peptides obtained by protein digestion. These include both equilibrium and rate studies.

2.2.1. pH studies

For no surface control (solution) experiments, 5 μL of 20 mg/mL protein solution was mixed with 45 μL of deuterated buffer at room temperature. The protein solutions and labeling buffers were prepared at pH 3.0, 3.5, 4.0, and 4.5 in 50 mM citrate for SP Sepharose FF and pH 6.5, 7.0, 7.5, and 8.0 in 50 mM phosphate for Q Sepharose. Labeling times varied for each experiment, as outlined below. After labeling, 5 μL of quench buffer (150 mM potassium phosphate, pH 1.5), kept in an ice bath, was added, bringing the final solution pH to 2.6, near the pH minimum of the hydrogen deuterium exchange reaction. Samples were kept at room temperature for 40 s before 145 μL of desorption buffer was added to the solution. The desorption buffer was at pH 2.6, with 100 mM citric acid, 8 M GdnHCl, 100 mM TCEP, and 27 mM EDTA in H_2O. After addition of desorption buffer, the sample was placed in ice for 2 min before being kept at room temperature for 40 s. Then, 600 μL of 95% H_2O, 5% acetonitrile, 0.1% formic acid, and 0.01% trifluoroacetic acid were added to dilute the protein and GdnHCl concentration. Solution phase samples were placed at room temperature for 40 s to replicate the time between sample quenching and introduction into the MS for the adsorbed phase experiments with two additional centrifugation steps (40 s each).

For adsorbed phase experiments, 35 μL of 20 mg/mL protein solution was added to 65 μL of resin slurry (50:50 dry resin:working buffer) in an Ultrafree® MC centrifugal filter unit within a 1.5 mL microcentrifuge tube. The samples were equilibrated overnight to ensure adsorption equilibrium. Prior to labeling, the sample was centrifuged at 7.4 rcf for 30 s to separate supernatant liquid. The concentration of the supernatant was measured via UV at 280 nm to obtain protein binding under the different conditions by material balance. To initiate labeling, 100 μL of deuterated buffer was added to the filter unit at room temperature. Labeling times were the same as those for the solution experiments, as described below. After labeling, 10 μL of quench buffer was added to the filter unit and the microcentrifuge tube was immediately centrifuged at 7.4 rcf for 30 s. The filter unit was transferred to a new microcentrifuge tube in ice and 145 μL of desorption buffer was added. The sample was placed in ice for 2 min and then centrifuged at 7.4 rcf for 30 s. Finally, 600 μL of sample pump solution was added to dilute the protein and GdnHCl concentration.

Labeling times varied with surface and pH. For whole protein experiments on Q Sepharose and Capto Adhere, labeling was 10 min for all pHs. For whole protein experiments on SP Sepharose FF and Capto MMC a different labeling time for each pH was used: 100, 50, 10, and 5 min for pH 3.0, 3.5, 4.0, and 4.5, respectively. This tiered labeling time accounts for the logarithmic decay of the hydrogen deuterium exchange rate with pH [14]. This use of tiered labeling was intended to ensure a measurable level of deuterium uptake at the lower pHs. The tiered labeling times were also used for the no surface (solution) control experiments. The tiered labeling strategy was not used for experiments at pH 5.0 to 8.0 as the exchange rate is sufficiently high enough that allows measurable levels of deuterium uptake. It should be noted that this can create a false positive that unfolding occurs as pH increases. The important focus here is determining if the labeling increase is different between