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Flow microcalorimetric measurements for bovine serum albumin on reversed-phase and anion-exchange supports under overloaded conditions

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Abstract

Heat of adsorption data using flow microcalorimetry is reported for the adsorption of bovine serum albumin (BSA) on C_{18} and C_4 chromatographic supports. Exothermic heats were obtained in all cases. Data for the effect of salt indicate that conformational changes in adsorbed protein appear to be greatest in the absence of salt. Also, the specific surface area of the support was found to influence behavior more strongly than the length of the carbon ligand. Heats of adsorption of BSA on an ion-exchange support were also measured. Endothermic heats were obtained in all cases. The data indicate that the observed heat effects may be strongly influenced by the release of water from the surface.

Keywords: Calorimetry; Thermodynamic parameters; Heat of adsorption; Adsorption; Albumin; Proteins

1. Introduction

Ion-exchange chromatography (IEC) and hydrophobic interaction chromatography (HIC) are two very popular methodologies used in the purification of proteins. Reversed-phase liquid chromatography (RPLC), although less popular than IEC or HIC can also be used for protein purification provided the desired protein does not readily denature in the presence of a strong hydrophobic stationary phase. Although these techniques are commonly used, the mechanisms that establish equilibrium characteristics such as capacity and selectivity are not adequately quantified. A number of models [1–6] have been proposed, with limited success. This is mainly because of non-idealities originating from intermolecular interactions are not satisfactorily described, particularly under overloaded conditions.

An approach that is often used is to characterize behavior with isotherm measurements at a limited set of conditions. The experimental isotherms are fitted to empirical models, such as the Langmuir or bi-Langmuir, and used. While this approach is convenient and relatively direct, it is nonetheless risky. Since the isotherm parameters are not linked fundamentally to underlying mechanisms, extrapolation or interpolation can lead to erroneous predictions. For example, it has been found in IEC that it is difficult to predict the effects of salt on capacity under overloaded conditions [7,8]. Raje and Pinto [7] have argued that these difficulties arise from the lack of adequate information in the adsorption isotherm. In an effort to bridge this gap some researchers [9–12] have turned to calorimetry.

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The equilibrium capacity of a surface is dictated by the Gibbs free energy change for adsorption (ΔG_{ads}). Since the ΔG_{ads} is dependent upon the overall enthalpy change, (ΔH_{ads}), associated with adsorption and since this is a measurable quantity, it is reasonable to expect that calorimetry can provide valuable insight on the underlying mechanisms of adsorption.

Calorimetry has been used to study the adsorption of proteins. It has been shown that under chromatographic conditions protein adsorption can be endothermic. Bowen and Hughes [9] have demonstrated that the selection of stationary phase and process conditions can influence whether protein adsorption is endothermic or exothermic. Esquibel-King et al. [11] have reported bimodal heats of adsorption for bovine serum albumin (BSA) on hydrophobic supports. Exothermic heats of adsorption indicate the dominance of attractive forces between the surface and the adsorbing protein, and/or attractive forces between adsorbed molecules on the surface. Endothermic heats of adsorption imply the overall adsorption process is entropically driven. In regards to the endothermic nature of protein adsorption, Raje [13] has postulated that protein configuration, protein reorientation and/or water release from the surface can lead to endothermic heats of adsorption. Perkins et al. [6] and Esquibel-King et al. [11] have used the preferential interaction analysis approach to show that significant amounts of water are released from the surface of several hydrophobic chromatographic supports. Lin et al. [12] have clearly shown the influence of stationary phase dehydration on the magnitude of the ΔH_{ads}. These data suggest that the desolvation of the stationary phase may significantly mask the heat released from the primary interaction of protein molecules with the adsorbent.

In this paper we report the results of a study of protein adsorption on chromatographic supports using flow microcalorimetry. Although attempts to study protein adsorption energetics have been made using Van 't Hoff plots [14–16], this approach is limited to the linear portion of the isotherm. Since most production-scale chromatographic protein purifications take place in the non-linear region of the isotherm, it is imperative to measure heats of adsorption under conditions existing in the column. Flow microcalorimetry (FMC) lends itself well for such studies. It is operated in a manner that is virtually identical to a laboratory-scale chromatographic unit. The objectives of the FMC studies were twofold: to study of the energetics of overloaded protein chromatography in RPLC and IEC, and to highlight any similarities and differences.

2. Experimental

2.1. Materials and apparatus

BSA was purchased from Sigma (St. Louis, MO, USA) and used without further purification. Two siliceous-based, RPLC stationary phases were used. C_4 ligands were bonded to surface of one support and C_{18} ligands were bonded to the surface of the second support. The C_4 support (15 μm, 300 Å pores) was purchased from Phenomenex (San Diego, CA, USA). The C_{18} support (17 μm, 300 Å pores) was purchased from Chrom-Tech (Minneapolis, MN, USA). Both reversed-phase supports were cross-linked and endcapped. The IEC support is also siliceous-based with an average diameter of 50 μm and an average pore size of 1000 Å. The surface contained cross-linked polyethylenimine (PEI) ligands. The IEC support was purchased from Millipore (Bedford, MA, USA).

The mobile phases used in the RPLC experiments were water containing 0.1% trifluoroacetic acid (TFA), 20 m*M* Tris containing 1.0 *M* sodium chloride (NaCl), and 20 m*M* Tris containing 4.0 *M* NaCl. The carrier fluid for the IEC experiments was a 20 m*M* Tris buffer (pH 7.2). Sodium chloride was used as modulator for all IEC experiments. The TFA, NaCl and trizma base (Tris) were purchased from Fisher Scientific (Hanover Park, IL, USA).

2.2. Flow microcalorimetry

The FMC system (Gilson Instruments, Westerville, OH, USA) is operated similar to a liquid chromatograph. The column or cell volume is 0.171 ml. The flow-rate through the cell is controlled by precision syringe micropumps. Interfaced with the cell are two highly sensitive thermisters. These instruments are capable of detecting small temperature changes within the cell that are associated with the adsorption

of an analyte onto the surface of a particular adsorbent. A block heater is used to monitor and control the cell temperature. As in a chromatograph, the FMC system is equipped with a configurable injection loop to accommodate different injection volumes. The effluent was collected and analyzed with a Milton Roy spectrophotometer.

The FMC system is initially filled with a specified volume of adsorbent. The next step (although not always necessary) is the evacuation of the cell. The evacuation process to a vacuum pressure of 30 in.Hg usually requires 24 h (1 in.Hg=338.638 Pa). The purpose of this step is to remove all air from the resin surface. Once the cell has been successfully evacuated, the contents are ''wetted'' with the carrier fluid. Following wetting the syringe pumps are turned on and the adsorbent is equilibrated with the carrier solution. Once the system has reached thermal equilibrium, the sample (dissolved in the carrier fluid) is loaded into the injection loop, and introduced into the cell by switching a multiport valve. The adsorption of the sample onto adsorbent surface causes a change in cell temperature which is converted to a heat signal by the FMC system through an experimentally determined calibration factor. (The calibration factor was obtained using the 50 μm PEI particles). Once the mass in the effluent is quantified with the spectrophotometer, a simple mass balance is performed to determine the quantity of sample adsorbed. From these data the specific heat of adsorption is calculated.

2.3. Van 't Hoff analysis

Van 't Hoff data were collected on an HP1100 chromatograph (Hewlett-Packard, Atlanta, GA, USA) unit with a 5.0×0.21 cm I.D. column at 10, 15, 20, 25, 30 and 35°C. The column was equilibrated with 0.1% aqueous TFA with different concentrations of acetonitrile at a flow-rate of 0.1 ml/min. Elution times were obtained by injecting 5 μl of 2.0 mg/ml BSA. The response was monitored with a UV detector at 280 nm.

2.4. Surface Area Analysis

A Micromeritics Gemini 2360 (Norcross, GA, USA) BET apparatus was used to measure the surface area of the C_{18} support. The surface area of the C_4 support was measured with a Micromeritics ASAP 2010 BET machine. Both samples were degassed with helium for 2 h. After degassing each sample was exposed to nitrogen for the analysis.

3. Results and discussion

The ΔH_{ads} of BSA on the C_{18} reversed-phase support is shown in Fig. 1. As seen in this figure the ΔH_{ads} is exothermic in all cases. Note however that when the carrier fluid contains salt an overall reduction in the heat of adsorption occurs. This may be due to differences in protein conformation in the presence of salt. Also very noticeable is the decrease in the ΔH_{ads} with an increase in surface concentration of protein. This effect is indicative of increasingly stronger repulsive interactions between protein molecules with the same charge. Neither of these effects is present when BSA adsorbs to the surface of the reversed-phased C_4 support (Fig. 2). This may be due in part to surface area differences between the C_4 and C_{18} supports. From BET measurements the total surface area of the C_4 support was calculated to be 133.5 m^2/g and 67 m^2/g for the C_{18} support. Thus, competition for adsorption sites on the C_{18} support is much greater because of the limited surface area. Mobile phase composition did not affect ΔH_{ads} on the C_4 as it did on the C_{18} support. This may be because the overall hydrophobicity of the C_4 resin is greater simply because the C_4

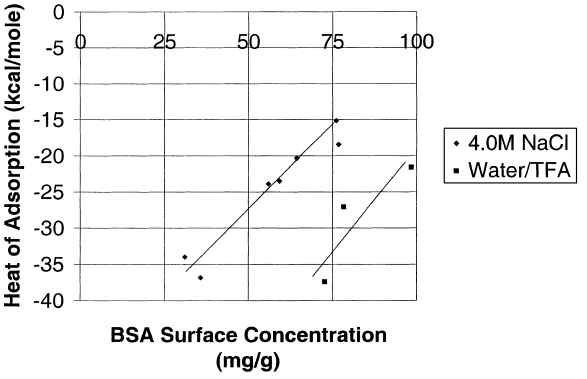

Fig. 1. Heat of adsorption vs. BSA surface concentration (C_{18} support).

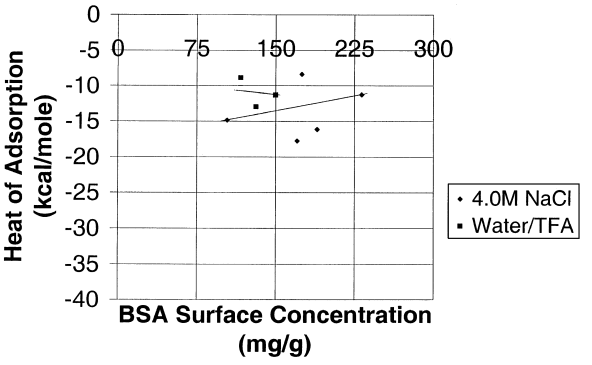

Fig. 2. Heat of adsorption vs. BSA surface concentration (C_4 support).

possesses more surface area per gram. As the hydrophobicity of the stationary phase increases, the ability to distinguish differences in protein conformation diminishes.

Fig. 3 compares the ΔH_{ads} for BSA on the C_4 and C_{18} supports in the presence of salt. Note that when we compare the average ΔH_{ads} of the C_4 against the ΔH_{ads} on the C_{18} at the highest coverage very little difference is observed in the heat of adsorption. In both cases BSA is essentially adsorbing onto carbon atoms. It is reasonable to expect that the ΔH_{ads} onto carbons should not change regardless of chain length because BSA is much larger than any of the ligands on either support. Since the surface is saturated in both cases the repulsive interactions between protein molecules should be equivalent thus producing similar heats of adsorption. We could not compare the

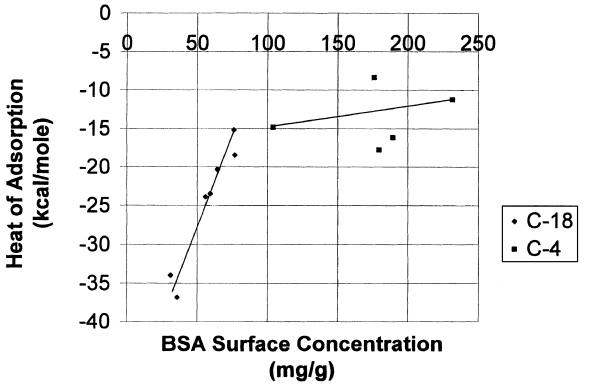

Fig. 3. Heat of adsorption vs. BSA surface concentration. (A comparison between C_4 and C_{18} in the presence of 4.0 *M* NaCl).

average ΔH_{ads} of the C_4 against the average ΔH_{ads} on C_{18} at the lowest surface concentration (where repulsive interactions are assumed to be minimal) because it was difficult to distinguish heat signals on the C_4 at low surface coverage. In the calorimetry experiments reported by Lin et al. [12] a noticeable difference in the heat of adsorption (migration in the endothermic direction) was observed when the carbon chain length of the sorbent surface was increased. Increasing the carbon chain length and ligand density effectively increases the hydrophobicity of the surface. It was postulated that these differences in ΔH_{ads} values were due to surface dehydration. Surface dehydration increased as the ligand chain length and density was increased thus moving the ΔH_{ads} in an endothermic direction. Unfortunately, we could not test for the same effect in our experiments

Typical heat of adsorption signals for the data in Figs. 1 and 2 are shown in Fig. 4. The difference in the two types of signals (Fig. 4a and b) can be attributed to the mobile phase conditions. In Fig. 4a the signal is composed two peaks. The first peak in time is due to the adsorption of BSA onto the surface. The second peak is suspected to be the heat released after a conformational change of the protein molecule on the surface. This conformational change is mostly occurring after the first peak has reached it maximum value. In this stage BSA is probably opening its structure to provide better contact between its hydrophobic interior and the hydrophobic surface. This phenomenon may be responsible for producing the highly non-linear Van ‘t Hoff plots shown in Fig. 5. Esquibel-King et al. [11] and Gill et al. [10] have already shown that the Van ‘t Hoff approach must be used cautiously for obtaining ΔH_{ads} for proteins. It is seen in Fig. 5 that in the case of RPLC conformational changes of the protein on the surface of the support can produce significant breaks in the slope of the plot, and thereby complicate the evaluation of the standard state $\Delta H_{ads.}$

In the presence of salt only one peak is observed in the FMC signal (Fig. 4b). When salt is present the protein wants to minimize its interaction with the salt [17] as much as possible, thus reducing the degree of the conformational changes on the surface. This minimizes the interaction between the hydrophobic protein interior and the salt, but reduces the overall

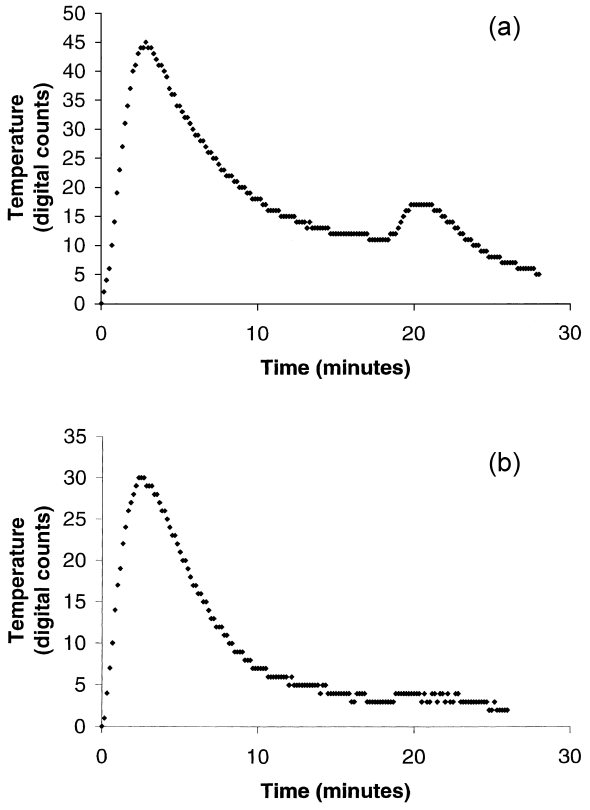

Fig. 4. Typical heat signals for BSA adsorption onto a reversed-phase support in the presence of (a) 0.1% aqueous TFA and (b) 4.0 *M* NaCl.

heat released in the form of a second peak. Some conformational changes do take place in the presence of salt and this has been clearly shown by Tibbs and Fernandez [18]. However in our study the heat evolved was too small to produce a distinguishable second exothermic peak.

The heat of adsorption of BSA onto an ion-exchange support (PEI), as shown in Figs. 6 and 7, was observed to be endothermic in all cases studied. Fig. 6 illustrates the effect of pH on the heat of adsorption. At pH 8 the charge capacity of the surface is lower than at pH 7.2. Fig. 7 illustrates the effect of salt on the heat of adsorption. The data collected at 0 m*M* NaCl is of particular interest because under this condition the attraction between BSA and the surface should be the strongest. The PEI surface contains both hydrocarbons and positively charged amine moieties on the surface. We have

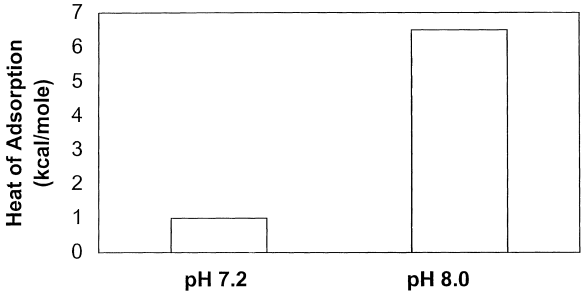

Fig. 6. Heat of adsorption of BSA onto an anion-exchange support at pH 7 and pH 8 (0.0 m*M* NaCl). The surface coverage was approximately 85 mg/g in each case.

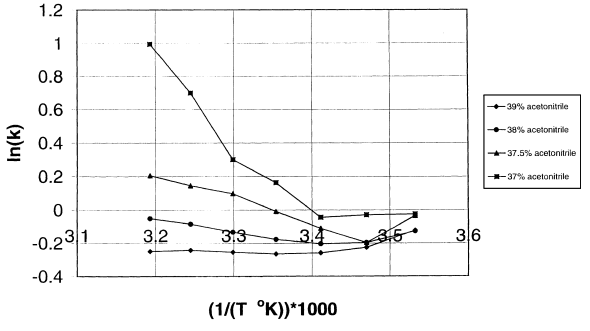

Fig. 5. Van 't Hoff plot for the retention of BSA on a reversed-phase C_4 support.

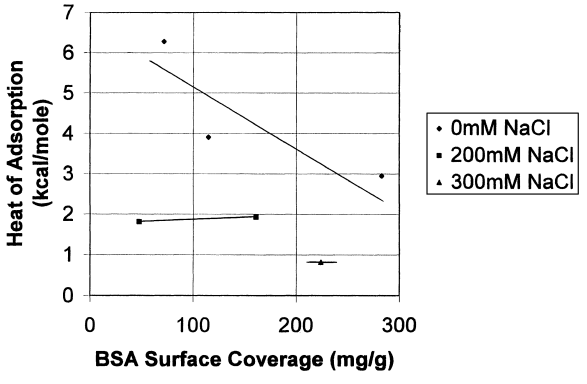

Fig. 7. Heat of adsorption vs. BSA surface concentration (anion-exchange support).

already seen in our studies that the heat of adsorption onto carbon atoms produces exothermic heats of adsorption. Additionally, it is reasonable to expect that when a negatively charged protein such as BSA comes in contact with positively charged amine groups heat should be evolved. However the opposite effect is observed. A possible explanation may be surface dehydration. The water release and the subsequent dilution of mobile phase ions (an endothermic process) may actually mask the heat released when a negatively charged protein such as BSA is adsorbed to a positively charged surface. As shown in Fig. 7 the endothermic heat of adsorption decreases as the mobile phase salt concentration increases. This suggests screening of the ionogenic groups by the modulator ions. This effect reduces the ion-exchange interaction between BSA and the surface. Another possible contribution to the reduction of the endothermic ΔH_{ads} could be the minimization of the repulsive interactions between adsorbed protein molecules. As seen in our C_{18} studies, proteins with the same charge will experience repulsive interactions with each other on the surface. The negative surface charge on the protein is neutralized by the presence of positively charged sodium ions. In effect the sodium ions are screening the repulsive interaction between protein molecules on the surface. This same effect has been reported by Arafat [19] in studies on the adsorption of phenol onto activated carbon in the presence of salt. Bowen and Hughes [9] have also reported this effect for the adsorption of BSA although they did not specifically attribute this trend to dilution or the minimization of the repulsive interactions.

4. Conclusions

It has been demonstrated that the ΔH_{ads} data obtained using FMC provides valuable insight on the adsorption of proteins. These data indicate that protein adsorption involves more than just interaction between the protein and the adsorbent surface. Displacement of water, reconfiguration or reorientation of the protein on the surface and ionic shielding are also in general important. It has been shown in our study and other investigations [9,11] that the mobile phase composition will significantly influence the energetics and the overall adsorption process. The adsorption of BSA onto an IEC support produced endothermic heats of adsorption that decreased in magnitude with the addition of salt. The reduction in the endothermic heat may be due to weaker repulsive interactions between surface protein molecules, or screening of the ion-exchange fixed sites by the modulator ions. The heats of adsorption for BSA on a reversed-phase surface are generally strongly exothermic because of the strong affinity between the hydrophobic surface and hydrophobic components of BSA. Our studies also revealed that mobile phase composition can influence the degree of protein conformational changes on the surface. Additionally conformational changes of the protein may complicate the interpretation of protein Van ‘t Hoff data generated under reversed-phase conditions.

Based on these results it is surmised that to develop a model that predicts the adsorption behavior under both the linear and overloaded chromatographic conditions, it is important to understand factors such as water release, protein reorientation and protein conformational changes, in addition to the primary adsorption interaction.

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