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Hydrophobic interaction chromatography of proteins: Thermodynamic analysis of conformational changes

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ABSTRACT

For BSA and β-lactoglobulin adsorption to hydrophobic interaction chromatography (HIC) stationary phases leads to conformational changes. In order to study the enthalpy (ΔH_{ads}), entropy (ΔS_{ads}), free energy (ΔG_{ads}) and heat capacity ($\Delta c_{p,ads}$) changes associated with adsorption we evaluated chromatographic data by the non-linear van't Hoff model. Additionally, we performed isothermal titration calorimetry (ITC) experiments. van't Hoff analysis revealed that a temperature raise from 278 to 308 K increasingly favoured adsorption seen by a decrease of ΔG_{ads} from −12.9 to −20.5 kJ/mol for BSA and from −6.6 to −13.2 kJ/mol for β-lactoglobulin. $\Delta c_{p,ads}$ values were positive at 1.2 m $(NH_4)_2SO_4$ and negative at 0.7 m $(NH_4)_2SO_4$. Positive $\Delta c_{p,ads}$ values imply hydration of apolar groups and protein unfolding. These results further corroborate conformational changes upon adsorption and their dependence on mobile phase $(NH_4)_2SO_4$ concentration. ITC measurements showed that ΔH_{ads} is dependent on surface coverage already at very low loadings. Discrepancies between ΔH_{ads} determined by van't Hoff analysis and ITC were observed. We explain this with protein conformational changes upon adsorption which are not accounted for by van't Hoff analysis.

1. Introduction

Thermodynamic analysis of protein surface interactions is essential for understanding the binding mechanism and the design of new separation processes. However, thermodynamic data for protein adsorption are scarcely available. Hydrophobic interaction chromatography (HIC) is a method used frequently in bioseparations [1,2]. Chromatography based on hydrophobic interactions was first reported by Shepard and Tiselius, then termed salting-out chromatography [3]. Hofstee further developed the method and Hjertén finally termed it hydrophobic interaction chromatography [4,5]. Since then various parameters influencing protein surface interactions in HIC have been studied. A wide range of experimental conditions and their effect on the binding characteristics have been investigated already. This includes the impact of pH, stationary phase ligand and matrix, type and concentration of salt in the mobile phase as well as type of protein [6–10]. It is known that hydrophobic interactions have a strong temperature dependency [11]. This was corroborated for temperature effects in HIC [12–15]. Methods of choice to study the impact of temperature and salt on the thermodynamic quantities associated with adsorption of proteins in HIC are classical van't Hoff analysis and isothermal titration calorimetry (ITC) in combination with batch adsorption and pulse response experiments.

The theoretical framework for studying retention thermodynamics using van't Hoff analysis was presented by Vailaya and Horváth. They studied the adsorption of dansyl derivates of amino acids [13]. Rowe et al. studied the adsorption of acetyl amino acid methyl esters on hydrophobic surfaces [16]. Several groups studied protein adsorption to hydrophobic surfaces using van't Hoff analysis [6,10,17–20]. ITC is a valuable tool to study the heat flow resulting from the interaction of a protein with a ligand [21]. In recent years ITC has also been successfully used to study protein surface interactions [22,23]. ITC is the only method to directly measure the heat flow associated with adsorption of a protein to a chromatographic stationary phase. Chen applied ITC to protein adsorption in HIC [24]. Discrepancies between thermodynamic quantities determined by van't Hoff analysis and ITC have been reported [18,25]. ITC as well as van't Hoff analysis are used frequently in the context of protein adsorption. However, more work has to be done to further elaborate the theoretical framework in order to fully understand the underlying principles [26].

Usually, the design of novel separation processes is based on trial and error where the large number of process parameters

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is evaluated empirically resulting in high process development costs. Understanding the influence of these process parameters on the level of thermodynamics will ultimately speed up process development times [27]. A broader knowledge base will contribute to understand phenomena such as unspecific adsorption and incomplete elution. A comprehensive understanding using thermodynamic analysis of biomolecular interactions is not only important in the context of protein adsorption in chromatography. Thermodynamic parameters associated with protein adsorption can also provide valuable information for the biomedical community where materials are engineered to exhibit a high protein adsorption resistance [28,29]. In this work we provide a thermodynamic analysis of protein adsorption in HIC and discuss how these thermodynamic quantities can be determined. Furthermore, we compare van't Hoff analysis and ITC with regard to thermodynamic analysis of protein adsorption. The impact of temperature, $(NH_4)_2SO_4$ concentration and surface coverage on thermodynamics of adsorption are discussed in the context of protein conformational changes.

2. Experimental

2.1. Instrumentation

For batch adsorption experiments the Stuart SB3 tube rotator from Barloworld Scientific (Staffordshire, UK) was used. Pulse response experiments were carried out with the ÄKTAprime-system from GE Healthcare (Uppsala, Sweden). Batch adsorption as well as pulse response experiments were performed at a defined temperature in the 2023 Minicold-lab from LKB Bromma. For ITC experiments the Thermal Activity Monitor (TAM) III nanocalorimeter from Thermometric (Jarfälla, Sweden) was used.

2.2. Stationary phase and columns

Butyl Sepharose 4 Fast Flow (FF) was purchased from GE Healthcare (Uppsala, Sweden). HR 10/10 columns were acquired from GE Healthcare.

2.3. Buffers and proteins

$(NH_4)_2SO_4$ at different molalities (0.7 and 1.2 m) was added to a 20 mM sodium phosphate buffer. A pH of 7.3 was used. All buffers were filtered through 0.22 μm filters manufactured by Millipore (Bedford, USA). The ingredients were acquired from Merck (Vienna, Austria). BSA and β-lactoglobulin were purchased from Sigma–Aldrich (Vienna, Austria).

2.4. Batch adsorption experiments

Batch adsorption experiments were used to determine equilibrium adsorption isotherms. Protein and stationary phase were incubated together under constant movement. Protein concentration in the supernatant was measured with a UV/vis spectrophotometer.

2.5. Pulse response experiments

Pulse response experiments were performed with HR 10/10 columns at an approximate bed height of 100 mm and at a linear flow rate of 100 cm/h. Proteins were dissolved at a concentration of 10 mg/ml. Protein pulses were applied with a 100 μl sample loop. Isocratic runs were designed as follows: equilibration with 3 column volumes (CVs), injection of the protein pulse, and elution with 5 CVs.

2.6. ITC measurements

TAM III was equilibrated for 24 h at each measuring temperature to achieve a stable baseline. Before each experiment TAM III was calibrated. 1 ml glass ampoules and a gold propeller stirrer at 120 rpm were used. For protein injection a 250 μl Hamilton syringe with a stainless steel canula was used. For ITC measurements the stationary phase slurry (1:4) was prepared in the ampoule and the syringe was filled with protein solution (6.0 mg/ml). Beforehand, slurry and protein solution were carefully degassed. Consecutive injections of 10 μl were used. After each experiment a diligent cleaning procedure was performed with detergent (Decon90), ethanol and HPLC-grade water. Baseline stability problems did not allow measurements below 288 K.

3. Theory

The theoretical framework for the determination of thermodynamic quantities in HIC using van't Hoff analysis was presented by Vailaya and Horváth in Ref. [13]. Retention in chromatography is given in terms of the retention factor k':

$$k' = \frac{V_r - V_0}{V_0} = \frac{t_r - t_0}{t_0} \quad (1)$$

with V_0 (ml) accounting for the retention volume of a non-retained solute, t_0 (s) for the retention time of a non-retained solute and V_r (ml) and t_r (s) as retention volume and time of a retained solute, respectively. k' is also given by

$$k' = K\phi \quad (2)$$

K is the equilibrium constant and the phase ratio ϕ is given by

$$\phi = \frac{1-\varepsilon}{\varepsilon} \quad (3)$$

ε is the porosity of the packed column. Consequently, K can either be determined by pulse response experiments using Eq. (2) or from the adsorption isotherm. Adsorption isotherm experimental data used for determination of K is generally fitted using the Langmuir equation:

$$q = q_{\max}\frac{K_a C}{1 + K_a C} \quad (4)$$

where K_a expresses the protein binding affinity, C denotes the protein concentration in the mobile phase under equilibrium conditions and q the protein concentration adsorbed per unit stationary phase. We extrapolate q/C to infinite dilution for calculation of K:

$$K = \lim_{C\to 0}\left(\frac{q}{C}\right) \quad (5)$$

K has been used for the calculation of the Gibbs energy change associated with the adsorption of a protein to a stationary phase, ΔG_{ads}, in numerous works [10,17–20]:

$$\Delta G_{ads} = -RT\ln(K) \quad (6)$$

R is the universal gas constant and T is the temperature. The enthalpy change associated with the adsorption of a protein to a stationary phase, ΔH_{ads}, can be determined directly using ITC or in an indirect way using van't Hoff analysis. In ITC, the heat Q, resulting from protein adsorption to a stationary phase, is calculated by integrating the power P associated with the interaction of protein and adsorbent over time t:

$$Q_{ads} = \int_{t_1}^{t_2} P\ \Delta t \quad (7)$$

Q_{ads} is related to ΔH_{ads} by

$$\Delta H_{ads} = \frac{Q_{ads}}{Vq} \quad (8)$$

V is the volume of stationary phase used. For calculation of the entropy change associated with the adsorption of a protein to a stationary phase, ΔS_{ads}, the fundamental property relation for the Gibbs energy is used:

$$\Delta G_{ads} = \Delta H_{ads} - T\Delta S_{ads} \quad (9)$$

For ΔH_{ads}, ΔS_{ads} and the heat capacity change attributed to the adsorption of a protein to a stationary phase, $\Delta c_{p,ads}$, being temperature dependent Horváth et al. introduced the "quadratic equation" in the context of van't Hoff analysis [13]:

$$\ln(k') = a + \frac{b}{T} + \frac{c}{T^2} + \ln(\phi) \quad (10)$$

a, b and c are the parameters of the "quadratic equation". These parameters are used for the calculation of ΔH_{ads}, ΔS_{ads} and $\Delta c_{p,ads}$:

$$\Delta H_{ads} = -R\left(b + \frac{2c}{T}\right) \quad (11)$$

$$\Delta S_{ads} = R\left(a - \frac{c}{T^2}\right) \quad (12)$$

$$\Delta c_{p,ads} = \frac{2Rc}{T^2} \quad (13)$$

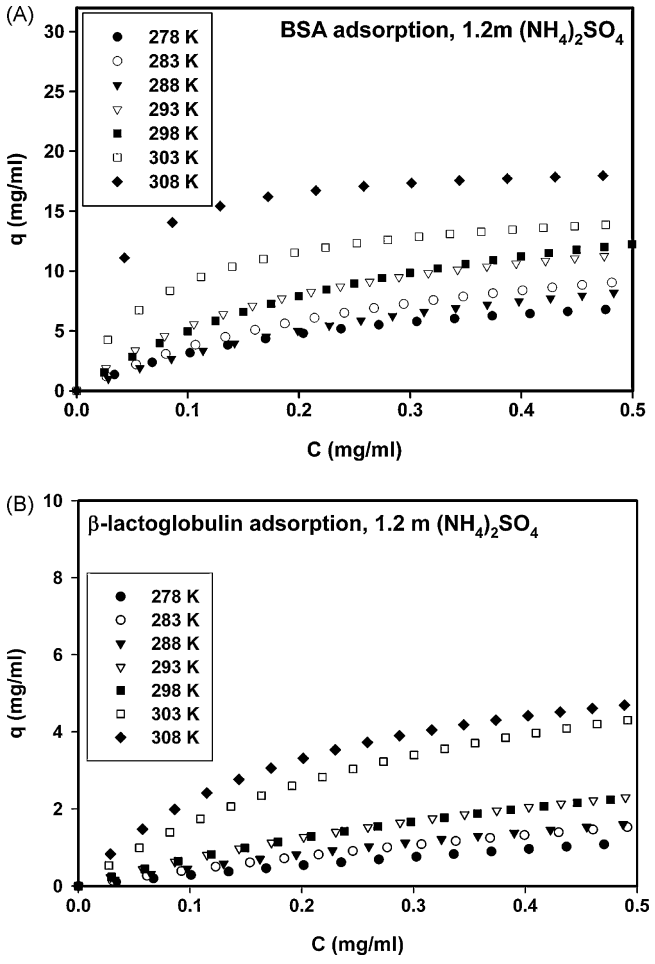

Fig. 1. Langmuir isotherms for the adsorption of BSA (A) and β-lactoglobulin (B) to Butyl Sepharose 4 FF at an $(NH_4)_2SO_4$ concentration of 1.2 m. Experiments were performed at seven different temperatures (278, 283, 288, 293, 298, 303, 308 K). Experimental data was fitted with Eq. (10). Due to clarity only fits are shown.

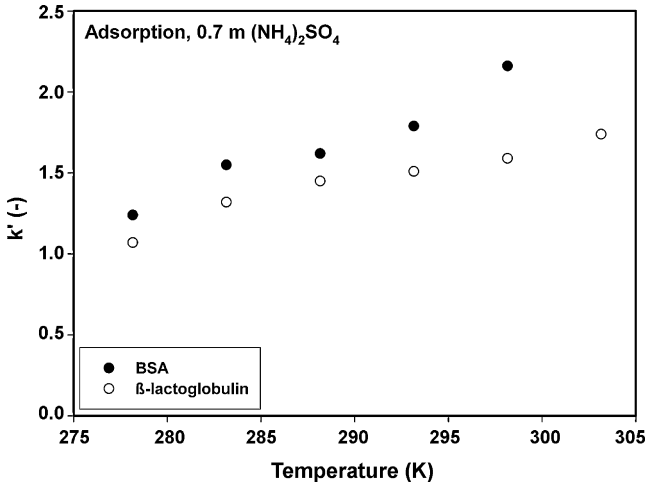

Fig. 2. Pulse response experiments for interaction of BSA (●) and β-lactoglobulin (○) with Butyl Sepharose 4 FF at an $(NH_4)_2SO_4$ concentration of 0.7 m.

4. Results and discussion

Fig. 1 shows Langmuir isotherms for the adsorption of BSA (A) and β-lactoglobulin (B) to Butyl Sepharose 4 FF at an $(NH_4)_2SO_4$ concentration of 1.2 m. For these batch adsorption studies the tem-

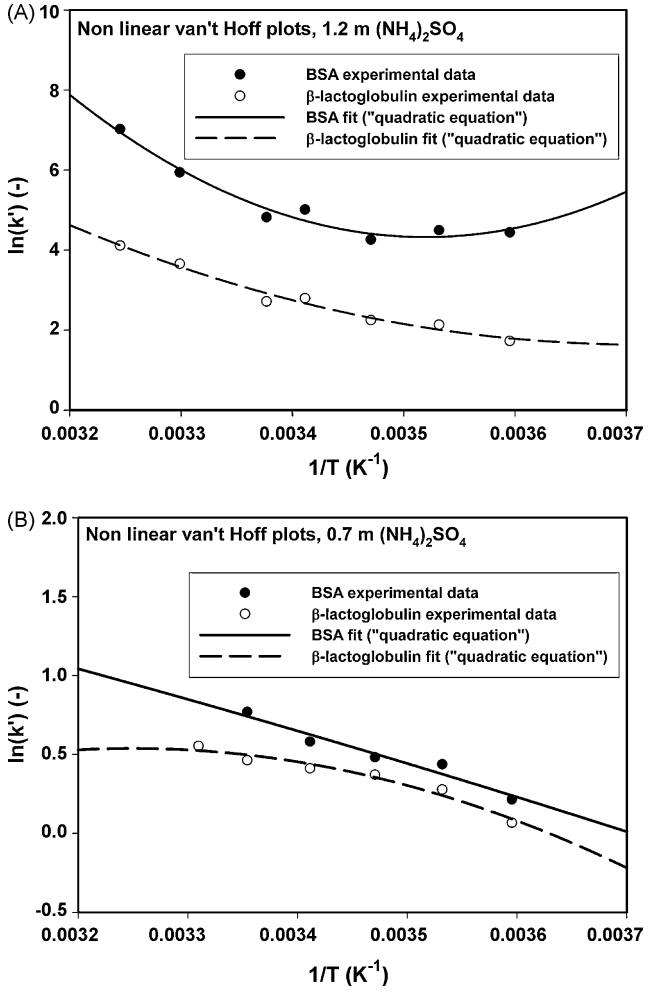

Fig. 3. Non-linear van't Hoff plots for adsorption of BSA (●) and β-lactoglobulin (○) at an $(NH_4)_2SO_4$ concentration of 1.2 m (A) and 0.7 m (B). Experimental data of BSA and β-lactoglobulin was fitted with the "quadratic equation".

perature was varied from 278 to 308 K. It is obvious for both proteins that capacity as well as binding affinity increased with increasing temperature. The higher the temperature was the steeper the adsorption isotherms became. For BSA the strongest temperature effect was observed when increasing temperature from 303 to 308 K. BSA adsorption exhibited the greatest leap between 298 and 303 K. The overall binding capacity was significantly higher for BSA.

k' was determined by pulse response experiments. The more a protein is retained by the stationary phase the higher are the values for k'. The results for adsorption of BSA and β-lactoglobulin to Butyl Sepharose 4 FF at an $(NH_4)_2SO_4$ concentration of 0.7 m are shown in Fig. 2. Increasing temperature led to increasing protein retention times resulting in increasing values for k'.

In van't Hoff plots the $\ln(k')$ is plotted versus the inverse temperature. According to Eq. (2) k' can either be determined by batch adsorption experiments or by pulse response experiments. For strong protein surface interactions at an $(NH_4)_2SO_4$ concentration of 1.2 m batch adsorption experiments were used (Fig. 1), whereas weaker interactions at an $(NH_4)_2SO_4$ concentration of 0.7 m were investigated via pulse response experiments (Fig. 2). Resulting van't Hoff plots are shown in Fig. 3 for adsorption of BSA and β-lactoglobulin at an $(NH_4)_2SO_4$ concentration of 1.2 (A) and 0.7 m (B), respectively. Experimental data in Fig. 3 was fitted with Eq. (10). Fitting parameters derived thereof were used for calculation of thermodynamic quantities associated with the adsorption of BSA and β-lactoglobulin to Butyl Sepharose 4 FF (ΔH_{ads}, ΔS_{ads} and $\Delta c_{p,ads}$). ΔG_{ads} was calculated using Eq. (8). The square of correlation (R^2) was used to assess the goodness of the fits. R^2 was always above 0.95 indicating very good correlation.

In Fig. 4 the energetic signature associated with the adsorption of BSA and β-lactoglobulin to Butyl Sepharose 4 FF is presented. The thermodynamic quantities ΔH_{ads}, ΔS_{ads} and ΔG_{ads} are shown for 7 different temperatures from 278 to 308 K in 5 K temperature increments. Fig. 4 presents thermodynamic quantities for adsorption of BSA (A) and β-lactoglobulin (B) at an $(NH_4)_2SO_4$ concentration of 1.2 m. Additionally, thermodynamic quantities attributed to adsorption at an $(NH_4)_2SO_4$ concentration of 0.7 m are shown for BSA (C) and β-lactoglobulin (D). In all cases ΔG_{ads} is becoming more negative with increasing temperature. For adsorption of BSA at $(NH_4)_2SO_4$ concentrations of 1.2 and 0.7 m ΔG_{ads} is decreasing from −12.9 to −20.5 kJ/mol and from −3.0 to −5.1 kJ/mol, respectively. Adsorption of β-lactoglobulin at $(NH_4)_2SO_4$ concentrations of 1.2 and 0.7 m is accompanied by a decrease of ΔG_{ads} from −6.6 to −13.2 kJ/mol and from −2.6 to −4.1 kJ/mol, respectively. The interaction of proteins with hydrophobic surfaces are driven by an increase in entropy caused by the release and rearrangement of water molecules [8]. Fig. 4 shows that the protein surface interactions are generally driven by the change in entropy. Only for adsorption of BSA at an $(NH_4)_2SO_4$ concentration of 1.2 m and very low temperatures a negative ΔH_{ads} was observed.

$\Delta c_{p,ads}$ values at $(NH_4)_2SO_4$ concentrations of 1.2 and 0.7 m are shown in Fig. 5. At an $(NH_4)_2SO_4$ concentration of 1.2 m the val-

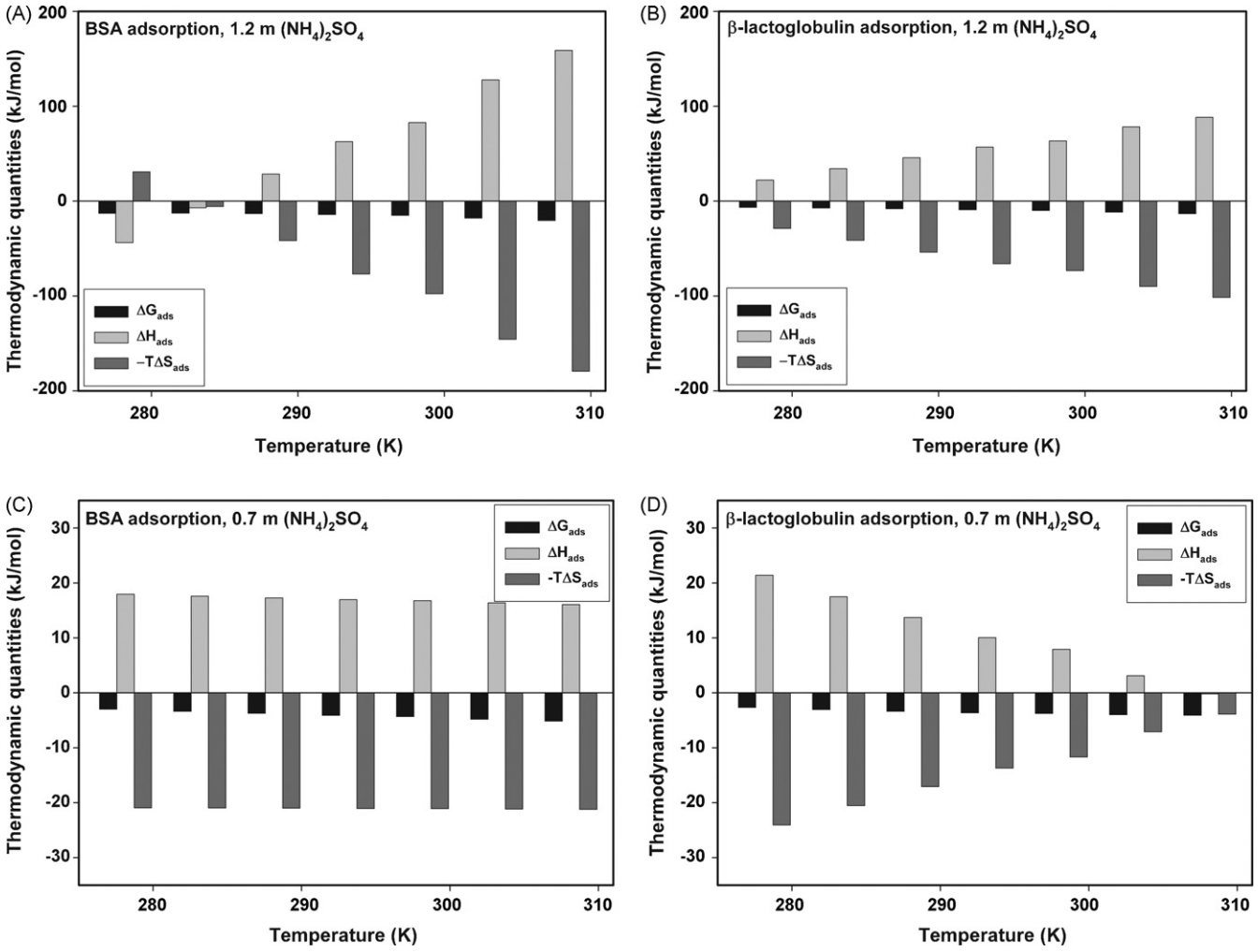

Fig. 4. Energetic signature attributed to the adsorption of BSA and β-lactoglobulin to Butyl Sepharose 4 FF at seven different temperatures (278, 283, 288, 293, 298, 303 and 308 K): BSA (A) and β-lactoglobulin (B) at an $(NH_4)_2SO_4$ concentration of 1.2 m as well as BSA (C) and β-lactoglobulin (D) at an $(NH_4)_2SO_4$ concentration of 0.7.

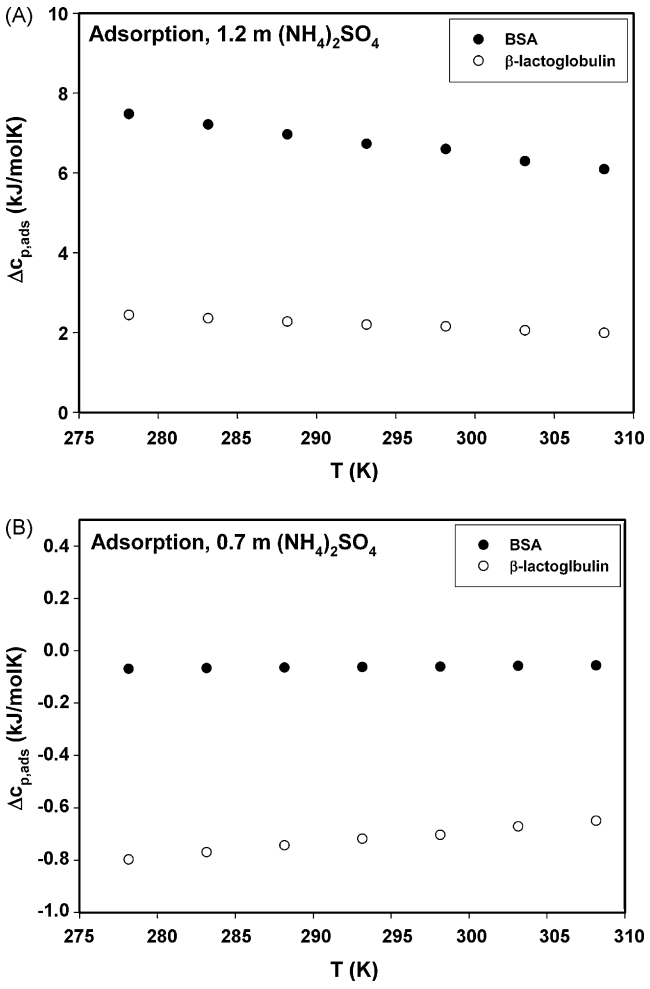

Fig. 5. $\Delta c_{p,ads}$ for interaction of BSA (●) and β-lactoglobulin (○) with Butyl Sepharose 4 FF at an $(NH_4)_2SO_4$ concentration of 1.2 m (A) and 0.7 m (B).

ues for $\Delta c_{p,ads}$ are throughout positive (Fig. 5A). For both, BSA and β-lactoglobulin, $\Delta c_{p,ads}$ is decreasing with increasing temperature. This trend is more pronounced for BSA, where $\Delta c_{p,ads}$ decreases from 7.5 to 6.1 J/(mol K). In the case of β-lactoglobulin, we observed a decrease from 2.4 to 2.0 J/(mol K). However, at an $(NH_4)_2SO_4$ concentration of 0.7 m the values for $\Delta c_{p,ads}$ are in the negative range (Fig. 5B). Conversely to the higher ammonium sulfate concentration, $\Delta c_{p,ads}$ is increasing with increasing T. This is more pronounced for β-lactoglobulin where $\Delta c_{p,ads}$ increased from −0.8 to −0.6 J/(mol K). We observed a marginal increase from −0.1 to −0.05 J/(mol K) for BSA. Recently, we showed that BSA and β-lactoglobulin undergo conformational changes upon adsorption to HIC stationary phases [30]. Δc_p in general provides rich insight in protein solvation and conformation [31,32]. Three factors determine the heat capacity of a protein: primary structure, non-covalent interactions arising from secondary and tertiary structure formation and protein hydration. The main contribution arises from the primary structure. However, protein conformation and hydration account for 15–40%, depending on the protein. Upon protein binding in HIC water is released from the protein hydration layer to the bulk [8]. Additionally, conformational changes come into play for the adsorption of BSA and β-lactoglobulin under the conditions investigated. This means that the changes in $\Delta c_{p,ads}$ are due to changes in the hydration layer and of the conformation. Conformational changes in the adsorbed state alter the non-covalent interactions in the protein on the level of both, secondary and tertiary structure as well as the protein hydration [30]. By looking

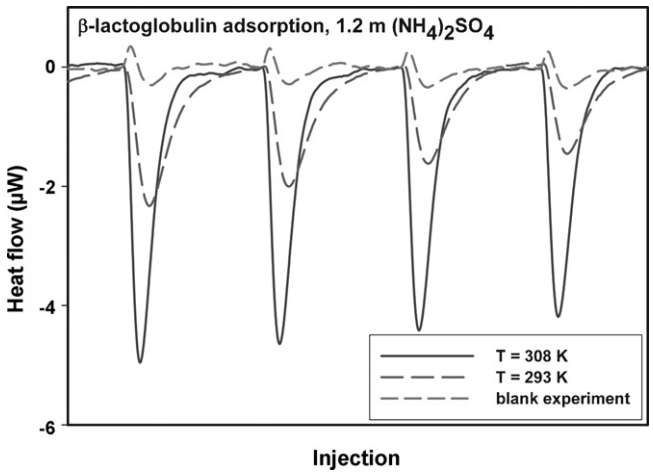

Fig. 6. Typical plot of raw data for the adsorption of β-lactoglobulin at 293 and 308 K; additionally, a blank experiment at 298 K is shown.

at the sign of Δc_p, one can distinguish between solvation of apolar (+) and polar (−) groups [32]. Both, BSA and β-lactoglobulin exhibit positive $\Delta c_{p,ads}$ at an $(NH_4)_2SO_4$ concentration of 1.2 m and a negative $\Delta c_{p,ads}$ at an $(NH_4)_2SO_4$ concentration of 0.7 m. Here, we assume that the conformational changes upon adsorption lead to exposure of apolar groups that are buried on the inside of these globular proteins in their native state. Consequently, the obtained values for $\Delta c_{p,ads}$ are in good agreement with previous work where we showed that a high $(NH_4)_2SO_4$ concentration of 1.2 m leads to more pronounced conformational changes [30]. Furthermore, unfolding of globular proteins is associated with a positive Δc_p [32]. The fact that the values for $\Delta c_{p,ads}$ in Fig. 5A are higher than in Fig. 5B supports these results. In this context, many factors influence protein conformation and solvation. At this point, we know that changes in the hydration layer and conformational changes upon adsorption are the main contributions to $\Delta c_{p,ads}$. However, the exact extent to which these factors contribute remains subject to further studies.

Since ITC experiments were performed under constant pressure it follows from the 1st law of thermodynamics that the heat measured by ITC is equal to ΔH_{ads}. When a protein is injected to a stationary phase $(\Delta H_{ads})^{prot}$ is measured. Importantly, not only the adsorption of the protein to the chromatographic stationary phase results in a heat flow. One also must consider the heat of dilution of the protein $(\Delta H_{dil})^{prot}$, the heat of dilution of the stationary phase $(\Delta H_{dil})^{sp}$ and the heat flow resulting from ion adsorption $(\Delta H_{ads})^{ion}$. Blank experiments must be performed and ΔH_{ads} is then calculated according to Eq. (14):

$$\Delta H_{ads} = (\Delta H_{ads})^{prot} - (\Delta H_{dil})^{prot} - (\Delta H_{dil})^{sp} - (\Delta H_{ads})^{ion} \quad (14)$$

Fig. 6 shows a typical plot of raw data for the adsorption of β-lactoglobulin at different temperatures. Also a blank experiment is shown. According to the measurement principle applied by TAM III, a negative heat flow corresponds to a positive enthalpy change. Fig. 6 illustrates that ΔH_{ads} increases with increasing temperature. A clear trend is obvious showing that temperature changes result in significant changes of ΔH_{ads}. As expected the blank experiment yielded a non-specific heat flow that had to be considered. A blank experiment at 293 K is exemplarily shown in Fig. 6.

Fig. 7 shows ITC data measured for adsorption to Butyl Sepharose 4 FF at 2 exemplary temperatures (293 and 308 K): ΔH_{ads} of BSA (A) and β-lactoglobulin (B) at an $(NH_4)_2SO_4$ concentration of 1.2 m as well as ΔH_{ads} of BSA (C) and β-lactoglobulin (D) at an $(NH_4)_2SO_4$ concentration of 0.7 m; in all cases the higher temperatures resulted in higher ΔH_{ads} values. Increasing $(NH_4)_2SO_4$

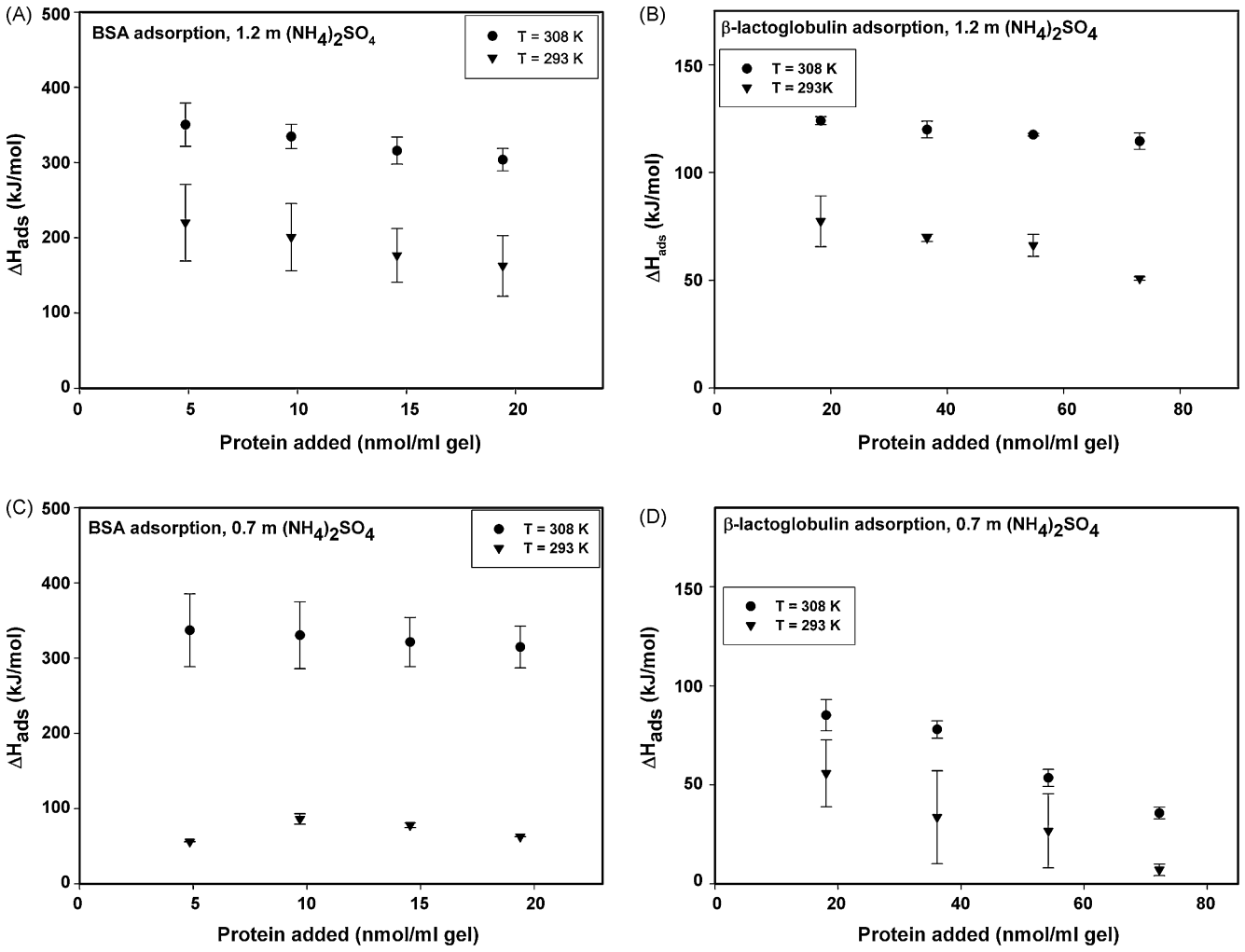

Fig. 7. ΔH_{ads} for BSA (A) and β-lactoglobulin (B) at an $(NH_4)_2SO_4$ concentration of 1.2 m as well as for the adsorption of BSA (C) and β-lactoglobulin (D) at an $(NH_4)_2SO_4$ concentration of 0.7 m. Stationary phase was Butyl Sepharose 4 FF. Experiments were performed in triplicates as indicated by the error bars.

concentration also resulted in increased ΔH_{ads} values. It is known that protein conformational changes respectively protein denaturation require energy [33]. This energy consumption adds to the positive ΔH values associated with the lone adsorption. We hypothesize that the differences in ΔH_{ads} for adsorption at different $(NH_4)_2SO_4$ concentrations as well as temperatures are partly caused by increasing amount of conformational changes. In an earlier work we have already shown that conformational changes upon adsorption increase with $(NH_4)_2SO_4$ concentrations and temperature [30].

Furthermore, ITC enabled us to specifically investigate the impact of surface coverage on ΔH_{ads}. It is well known that surface coverage already plays a crucial role at low loadings [34]. We observed trends of decreasing ΔH_{ads} with increasing surface coverage at a specific $(NH_4)_2SO_4$ concentration and temperature (Fig. 7). The enthalpy changes associated with conformational changes add to ΔH_{ads} determined by ITC. Consequently, lower extent of protein conformational changes will result in lower ΔH_{ads} values since less energy is consumed. We explain the decrease in ΔH_{ads} with lowered extent of conformational changes. Increasing surface coverage led to less unfolding of BSA and β-lactoglobulin. Fogle et al. shows for α-lactalbumin that the protein structure is stabilized at high loadings [19].

ITC is the only way to directly determine ΔH_{ads}. Researchers have reported discrepancies between enthalpy changes determined by van't Hoff analysis and those measured directly via ITC [18,25]. We also observed discrepancies for the adsorption of BSA and β-lactoglobulin to Butyl Sepharose at $(NH_4)_2SO_4$ concentrations of 1.2 and 0.7 m (Table 1). The higher the temperatures the higher were the discrepancies between ΔH_{ads} determined by van't

Table 1
Comparison of ΔH_{ads} derived from van't Hoff analysis and ITC for BSA and β-lactoglobulin. For ITC experiments the mean value of the 1st injection is used.

Molality (mol/kg)	Temperature (K)	BSA ΔH_{ads} (kJ/mol)		β-Lactoglobulin ΔH_{ads} (kJ/mol)	
		van't Hoff	ITC	vant' Hoff	ITC
0.7	293	17.3	56.1	10.1	55.8
0.7	308	16.1	337.1	−0.2	90.8
1.2	293	62.2	215.1	56.9	77.9
1.2	308	158.6	345.3	88.3	123.8

Hoff analysis and ITC. This again can be explained by the fact that higher temperatures tend to destabilize the protein structure resulting in more pronounced conformational changes. This fact is reflected in the higher discrepancies at elevated temperatures. However, the tendencies for ΔH_{ads} determined by both methods as far as $(NH_4)_2SO_4$ concentration and temperature effects are concerned were comparable. We know that protein conformational changes upon adsorption play a crucial role for BSA and β-lactoglobulin under the conditions investigated [30]. According to van't Hoff analysis ΔH_{ads} is calculated on basis of the reversible equilibrium process and, as a consequence of that, does not account for protein conformational changes. In contrast, ITC measures all enthalpy changes associated with protein adsorption, including those arising from conformational changes. These method inherent differences are responsible for the observed discrepancies.

5. Conclusions

In this study we performed a thermodynamic analysis of the adsorption of BSA and β-lactoglobulin to Butyl Sepharose 4 FF. The full set of thermodynamic quantities (ΔG_{ads}, ΔH_{ads}, ΔS_{ads}, $\Delta c_{p,ads}$) associated with the adsorption of BSA and β-lactoglobulin were determined indirectly by van't Hoff analysis. ΔH_{ads} was directly measured by ITC. We were able to thermodynamically track the impact of mobile phase $(NH_4)_2SO_4$ concentration and temperature on protein adsorption in HIC. The higher $(NH_4)_2SO_4$ concentration and temperature were the more favorable protein surface interactions became. ITC measurements pointed out that ΔH_{ads} is dependent on surface coverage. This was obvious already at low loadings. BSA and β-lactoglobulin are stabilized on the surface with increasing loading. We showed that ITC is the superior method to van't Hoff analysis when analyzing ΔH_{ads} involving protein conformational changes. The determination of ΔS_{ads} is a complex topic and remains a goal of major importance [22]. Thermodynamic quantities are rich in insight and offer a multitude of information about the processes associated with protein surface interactions. The link to protein conformation and conformational changes is essential for modeling and prediction of breakthrough behavior. We have already shown the importance of incorporating spreading effects into existing models [35]. Unspecific adsorption and incomplete elution are persistent problems in process chromatography. A typical process chromatography step has 50–100 operating parameters which all may influence protein stability and adsorption behavior [36]. By correlating thermodynamic and structural information with operating parameters such as temperature and concentration of salt our results will help to fine-tune existing models in order to correctly predict chromatographic behavior.

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