Microcalorimetry Study of the Interactions between Poly(*N*-isopropylacrylamide) Microgels and Amino Acids

Ohad Kimhi† and Havazelet Bianco-Peled*,‡

The Inter-Departmental Program for Biotechnology and Department of Chemical Engineering, Technion—Israel Institute of Technology, Haifa 32000, Israel

Received March 14, 2002. In Final Form: August 15, 2002

Here, we present the first direct measurement of the binding enthalpy between two model amino acids, valine and aspartic acid, and poly(*N*-isopropylacrylamide) (PNIPA) microgels. Equilibrium binding isotherms of the two acids showed enhanced binding with a temperature elevation from 25 to 37 °C. The binding of aspartic acid was somewhat higher than valine at 25 °C, whereas at 37 °C, the binding of valine was higher because of its more hydrophobic nature. Isothermal titration calorimetry measurements showed that the binding of valine was endothermic, which implies an entropy-driven binding mechanism. Contrary, the binding of aspartic acid was exothermic, suggesting a binding mechanism based on interactions such as hydrogen bonding. Differential scanning calorimetry measurements detected a 0.4 °C lowering of the gel phase transition temperature in dilute solutions of either valine or aspartic acid relative to buffer. Our results show that a direct measurement of the binding enthalpy gives further insight into the balance of binding mechanisms between biochemicals and PNIPA gel.

Introduction

Stimuli-responsive polymers, often termed as "smart materials", have recently become a major source of interest for scientists in many fields of research. The physical nature of these materials may be manipulated by applying external stimulation such as changes in temperature, pH, or salt concentration.[1] Poly(*N*-isopropylacrylamide) (PNIPA) is one of the most investigated smart polymers. This polymer is temperature sensitive; that is, it undergoes a hydrophilic−hydrophobic transition at a "lower critical solution temperature" (LCST) of about 32 °C. Likewise, PNIPA hydrogels collapse, deswell, and become more hydrophobic above the LCST.[2]

Several authors have suggested the use of PNIPA in different bioseparation techniques. Early papers described an extraction process,[3,4] in which a collapsed PNIPA gel is placed in an aqueous solution of large biomolecules, and the free water molecules are extracted, being associated with polymer upon the gel cooling and swelling. Other researchers have shown that the adsorption of proteins increases considerably at a temperature above the LCST.[5,6] PNIPA-based sorbents were utilized in temperature-modulated chromatography, to separate mixtures of steroids,[7,8] peptides,[9] amino acids,[10] nucleotide analogues, and proteins.[11] Most researchers claim that the binding of biochemicals is mostly determined by their hydrophobic interactions with the stationery phase, and thus, the variations near the LCST result from changes in the sorbent hydrophobicity. Others suggested that the phenomenon of temperature-modulated binding may be due to the changes in the pore size resulting from the PNIPA volume transition.[12,13] A possible route to distinguish between an interaction-based mechanism and a size exclusion mechanism, or to discern the relative balance of these mechanisms, is a direct measurement of the binding enthalpies and entropies. To the best of our knowledge, such a measurement was never described in the literature. Moreover, even the interactions between commercial hydrophobic chromatography supports and proteins were studied only recently.[14−17]

Here, we present the first direct measurement of the adsorption enthalpy between amino acids and PNIPA microgels, using isothermal titration calorimetery (ITC).[18] Two amino acids with different hydrophobicities were selected as model biochemicals. The results presented in this paper provide a better insight into the binding mechanisms and may be used as a basis for further research involving more complicated systems.

* To whom correspondence should be addressed. E-mail: bianco@techunix.technion.ac.il.

† The Inter-Departmental Program for Biotechnology, Technion—Israel Institute of Technology.

‡ Department of Chemical Engineering, Technion—Israel Institute of Technology.

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10.1021/la025735r CCC: $22.00
Published on Web 10/05/2002

Experimental Section

Materials. *N*-isopropylacryl amide (NIPA; Sigma) was recrystallized from a mixture of toluene and petrol ether 40−60. Ammonium persulfate (Carlo Erba) was recrystallized from MiliQ water. All other chemicals were analytical grade and were used as received. Tetramethylethylenediamine (TEMED) and valine were purchased from Sigma. Aspartic acid was purchased from Fluka. 2-Methoxy ethanol, ninhydrin, and tin chloride were purchased from Riedel DeHaen. *n*-Propanol and phosphate buffer (as precalibrated ampule) were purchased from Carlo Erba. Citric acid was purchased from Bio Lab. Pluronic L-61 was obtained from BASF. All solution were prepared using MiliQ water. Amino acid solutions for binding isotherm and ITC measurements were prepared in phosphate buffer at pH 7.

Synthesis. Cross-linked PNIPA microgels were synthesized using inverse polymerization technique, according to the procedure given by Lin et al.[19] The monomer (*N*-isopropylacrylamide, 3 g), cross-linker (*N*,*N*′-methylenebisacrylamide, 0.125 g), and initiator (ammonium persulfate, 0.6 g) were dissolved in 30 mL of distilled water and bubbled with nitrogen to remove the dissolved oxygen. This solution was poured into 150 mL of paraffin oil containing 1% of pluronic L-61, which was previously purged with nitrogen. After aqueous droplets were formed in the oil phase, 3 mL of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (accelerator) were added to the continuous phase to the initiate redox polymerization, which is then performed for 3 h at 4 °C. After polymerization, the beads were separated by excess water, washed several times with a mixture of acetone and water (1:1) to remove the monomer, and filtered to obtain PNIPA microgel beads. The UV adsorption of the wash water was measured to ensure that all unreacted monomer was removed from the gel.

Equilibrium Binding Isotherms. PNIPA microgel particles were separated from access water, and a weighed amount of swollen particles (ca. 0.5 g at 25 °C) was transferred into a glass tube and heated to the experiment temperature. A total of 5 mL of amino acid solution was added, and the mixture was shaken in a temperature-controlled water bath at 150 rpm for 2.5 h. The solution was then separated from the gel by vacuum filtering through a glass fiber filtering paper (GF-A, Whatman). Amino acids in the remaining solution were dyed using the ninhydrin procedure.[20] Their concentration C' was determined from adsorption measurements at 570 nm, using a UV Unico 2100 spectrophotometer. This procedure was repeated three times for each concentration at 25 °C and at least five times at for each concentration 37 °C, where the measurements were more scattered. Instead of averaging the data, all data points are presented on the binding isotherms shown in Figures 1 and 2. We estimated the experimental error to be 15% at 37 °C and 10% at 25 °C.

To calculate the binding isotherms, as described below, the amount of water squeezed from the gel upon collapse was determined using light microscopy; micrographs of gel particles verified their spherical shape both at 25 and 37 °C. The swelling ratio was determined by comparing the diameter of the particles above and below LCST. The amount of water squeezed from the gel was assumed identical to the difference between the particle's volume at 25 °C and its volume at 37 °C. More than 100 particles were observed at both temperatures. We found that the gel loses 90% of its volume during heating process from 25 to 37 °C. A similar swelling ratio was previously reported for submicron gels.[21,22] The swelling ratio was identical for beads immersed in buffer and in amino acid solution.

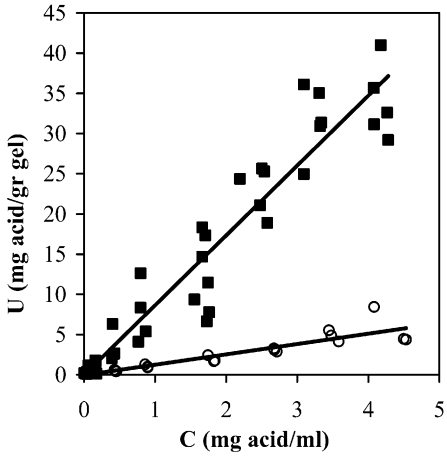

Figure 1. Equilibrium binding isotherm for valine (○) at 25 and (■) at 37 °C.

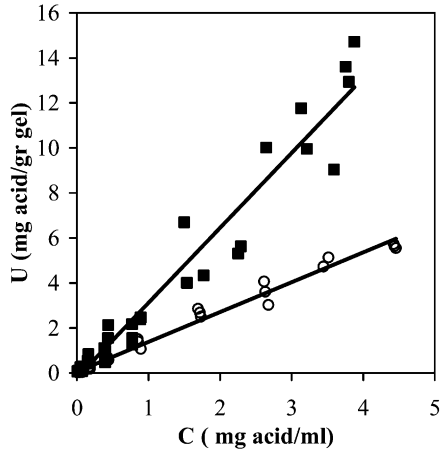

Figure 2. Equilibrium binding isotherm for aspartic acid (○) at 25 and (■) at 37 °C.

The binding isotherm, in terms of weight of bound acid per gram gel U, was calculated from a mass balance:

$$U = \frac{V_0' C_0' - V' C'}{w_g} \qquad (1)$$

where w_g is the weight of the wet gel at the experiment temperature (meaning, the weight of swollen or collapsed gel introduced into the system), V_0' and V' are the volume of the amino acid solution at the beginning of the experiment and the volume of the filtrate, respectively, and C_0' and C' are the corresponding amino acid concentration. At 25 °C, V' and V_0' are equal. At 37 °C, the volume V' also includes water squeezed from the collapsed gel, because the solutions initially were mixed at room temperature and then heated to 37 °C.

DSC Measurements. Gel suspension was mixed with dry amino acid to a concentration of about 5 mg/mL of

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amino acid and placed into a VP-DSC (Microcal). Scanning was performed from 20 to 60 °C in a scan rate of 1.5 °C/min. Data analysis was carried out using the software "Origin lab" supplied with the instrument.

ITC Measurements. ITC measurements were performed using a VP−ITC apparatus (Microcal). An amino acid solution was titrated serially (14 injections, 20 μL each, at 12 min time intervals) into the calorimetric cell containing gel suspension (ca. 0.1 g gel/mL). Measurements involving gel were taken in low gain mode, for enhanced accuracy. Reference measurements (i.e., amino acid titrated into buffer and buffer titrated into gel suspension) were taken in high gain mode. All ITC measurements were conducted after full degassing of the solution. It should be noticed that the accessible concentration range for aspartic acid is limited because of its lower solubility in water.

ITC Data Analysis. The VP−ITC software is designed for systems such as ligand-protein interactions, which are characterized by s-shape titration curves, where the top of the curve is achieved at saturation**.** Our preliminary experiments have shown that the s shape is not observed, and the software provided with the instrument was incapable of performing the data analysis reasonably. Therefore, all calculations were performed using a user-defined script.

For the calculations of the binding enthalpy, we need first to derive a general equation describing the enthalpy change ΔH associated with injection of volume ΔV into the cell. The system enthalpy H can be written as a function of the partial enthalpies h_i of the components[23] and their weights w_i:

$$H = w_s^w h_s^w + w_s^g h_s^g + w_g h_g \tag{2}$$

where the subscripts "s" and "g" stand for the solute (amino acid) and the gel, respectively, and the superscripts "w" and "g" refer to the solute environment (water or gel), respectively.

Let us consider the reference experiments. The first reference experiment involves injection of buffer into a gel suspension. We have observed negligible heat release in this experiment and thus concluded that the term $\Delta(h_g w_g)$, the enthalpy of dilution of the gel, may be neglected. The second reference experiment is a measurement of the amino acid heat of dilution of solute in the absence of gel where $w_s^g = w_g = 0$. In such experiment, a volume ΔV of solution is injected into the titration cell, causing a displacement of the same volume into the access tube. We have assumed that the enthalpy change upon injection can be divided into two stages. First, ΔV mL of solution is ejected from the cell, without a change in the concentration. The enthalpy change ΔH_1^d associated with this stage is given by:

$$\Delta H_1^d = h_s^w\{(V_c - \Delta V)C_0 - V_c C_0\} \tag{3}$$

where C_0 is the solute concentration and V_c is the volume of the titration cell. The second stage is the introduction of ΔV ml solution from the syringe, which contains a solution with a concentration of C_{syr}. The enthalpy change ΔH_2^d associated with this stage is

$$\Delta H_2^d = h_s^w\{\ V_c C - (V_c - \Delta V)C_0\} \tag{4}$$

where C is the final concentration in the cell, which may be calculated from a mass balance:

$$C_{syr}\Delta V + C_0(V_c - \Delta V) = CV_c \tag{5}$$

By combining eqs 3−5, we obtain the following relationship for the heat of dilution ΔH^d

$$\Delta H^d = \Delta H_1^d + \Delta H_2^d = h_s^w \Delta V(C_{syr} - C_0) \tag{6}$$

In a titration experiment, gel beads are also present in the cell, but no gel is injected. We consider again two stages, the first of which involves ejection of gel suspension from the cell without concentrations change and the second involves injection. We assume that the following relations apply:

$$V_c = V_g + V_0 \tag{7a}$$

$$\Delta V = \Delta V_g + \Delta V_0 \tag{7b}$$

where the subscript "g" and "0" correspond to gel and solution volumes (or volume changes during the first stage), respectively. We further assume that the gel suspension is well mixed at all times; that is, the volume changes during the first stage are given by

$$\Delta V_0 = \frac{V_0}{V_c}\Delta V \tag{8a}$$

$$\Delta V_g = \frac{V_g}{V_c}\Delta V = \frac{\Delta w_g}{\rho_g} \tag{8b}$$

where Δw_g is the change in the gel weight during ejection and ρ_g is the gel density. Thus, the enthalpy changes ΔH_1^t and ΔH_2^t associated with the two stages are given by

$$\Delta H_1^t = -h_s^w C_0 \Delta V_0 - h_s^g U_0 \Delta w_g - h_g \Delta w_g \tag{9a}$$

$$\Delta H_2^t = h_s^w\{(V_0 - \Delta V_0 + \Delta V)C - (V_0 - \Delta V_0)C_0\} + h_s^g\{U(w_g - \Delta w_g) - U_0(w_g - \Delta w_g)\} \tag{9b}$$

where U_0 and U are the amino acid concentration in the gel (in terms of weight of bound acid per gram gel) before and after the current injection, respectively. C_0 and C are the solution concentration of the unbound amino acid before and after the current injection, respectively. By combining eqs 7−9 and after some algebraic manipulation, we obtain the total enthalpy change ΔH of the process:

$$\Delta H = h_s^w\left\{\left(V_0 + \frac{V_g \Delta V}{V_c}\right)C - C_0 V_0\right\} + h_s^g\{(w_g - \Delta w_g)U - w_g U_0\} - \Delta w_g h_g \tag{10}$$

Finally, to calculate ΔH we need also to consider the mass balance for the solute:

$$\Delta V C_{syr} + C_0(V_0 - \Delta V_0) + U_0(w_g - \Delta w_g) = U(w_g - \Delta w_g) + C(V_0 - \Delta V_0 + \Delta V) \tag{11}$$

and the relations between U and C, obtained from the binding isotherms.

We note that the reference experiment have detected a concentration-dependent heat of dilution. Because the gel volume affects the acid concentration, the heat of dilution could not be directly subtracted from the experimental data. Thus, we have calculated h_s^w from the

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dilution experiment and used its value in eq 10. As mentioned before the gel dilution reference experiment have shown that the term $\Delta w_g h_g$ can be neglected.

Results and Discussion

Equilibrium Binding Isotherms. The equilibrium binding isotherms for valine and aspartic acid, at two temperatures, are shown in Figures 1 and 2, respectively. All curves show a linear behavior and do not reach saturation in the examined concentrations range. Thus, the binding isotherm may be described using the following relation:

$$U = K_p C \tag{12}$$

where K_p is a constant partition coefficient. From the data for valine shown in Figure 1, we obtained values of K_p = 1.0 and 8.5 mL/g gel at 25 and 37 °C, respectively. From the data for aspartic acid shown in Figure 2, we obtained values of K_p = 1.3 and 3.3 mL/g gel at 25 and 37 °C, respectively.

For both amino acids, the partition coefficient is higher at 37 °C. Near the LCST, there is a sharp decrease in the hydrogen bonding between the PNIPA and the water.[24] As a result, the number of free amide groups on the polymer gel increases. This process is associated with an increasing hydrophobicity of the gel. The enhanced binding of both amino acids may be attributed either to an increase the hydrophobic interactions or to formation of hydrogen bonds between the amine groups and the free amide on the PNIPA. Moreover, the hydrophobic nature of valine explains its higher binding, because of hydrophobic interactions. More explicit information could be gleaned from an appropriate magnetic resonance study. At 25 °C, there are no specific interactions between the valine and the gel, leading to a partition coefficient of approximately 1, indicating that there is no preference for the gel phase. Aspartic acid has a negative net charge at pH = 7. Because it is known that PNIPA gels may be negatively charged, we have conducted a simple experiment to confirm this point. We have applied a 30 V potential on a gel sample and observed the direction of movement, confirming that the gel is indeed negatively charged. Surprisingly, the partition coefficient of aspartic acid at 25 °C is higher than 1 although electrostatic repulsion may be expected. Yet, aspartic acid has three groups that are capable of forming hydrogen bonds, and its preference to the gel phase may be due to formation of hydrogen bond with the PNIPA.

DSC Measurements. Figures 3−5 show the VP-DSC measurements taken from gel samples immersed in buffer (Figure 3), in a 5 mg/mL valine solution (Figure 4), and in 5 mg/mL aspartic acid solution (Figure 5). It can be seen that introducing amino acids causes only minor changes, lowering the transition temperature by 0.4 °C (from 32.6 to 32.2 °C) with both acids. The total enthalpy change during the PNIPA collapse (endothermic peak) was nearly identical (ca. 0.4 cal/g) for all three systems, indicating that there is no major energetic change associated with the addition of the acids. The temperature shift may be due to changes in the amount of the hydrogen bonds or with its strength. During the phase transition, the amount of hydrogen bonds between the water and the gel decreases and the internal hydrogen bond between amid groups increases.[24] Because no enthalpy changes were observed upon adding amino acids, DSC cannot be used to assess the heat of amino acids binding. The heat released during phase change is probably much larger than the expected contribution of the binding heat of binding, and these minor changes could not be detected using DSC.

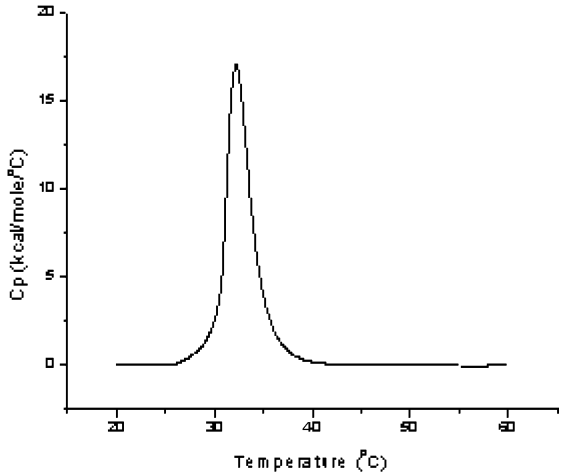

Figure 3. DSC thermograph for PNIPA microgel immersed in buffer.

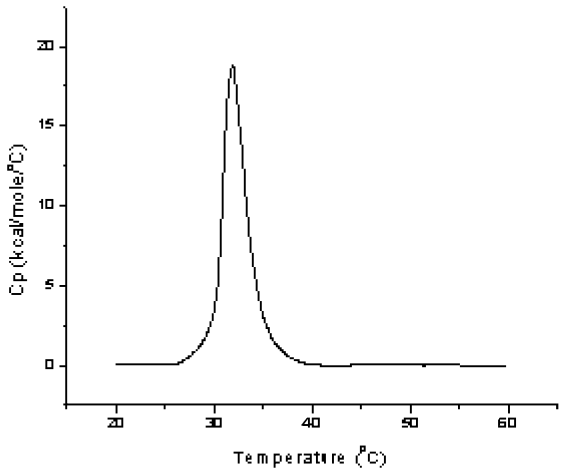

Figure 4. DSC thermograph for PNIPA microgel immersed in a 5 mg/mL valine solution.

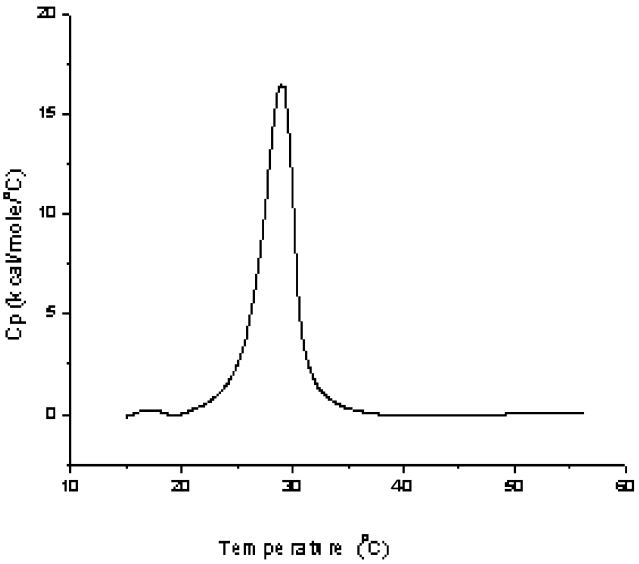

Figure 5. DSC thermograph for PNIPA microgel immersed in a 5 mg/mL aspartic acid solution.

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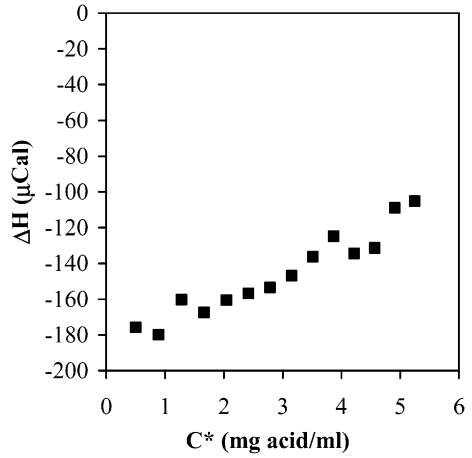

Figure 6. Processed ITC data set for valine at 25 °C.

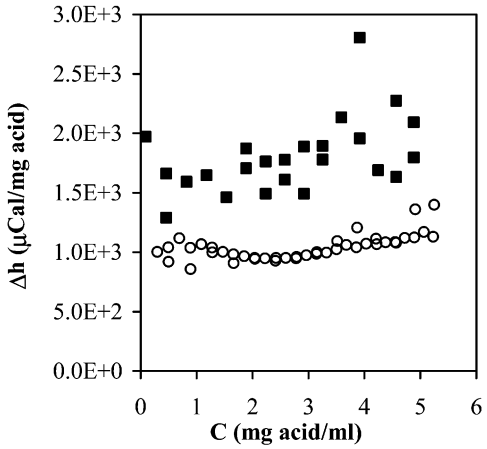

Figure 7. Enthalpy of binding between PNIPA gel and valine (○) at 25 and (■) 37 °C.

ITC Measurements. ITC measurements were conducted in order to determine the enthalpy of binding between amino acids and PNIPA gel. As a measure to the strength of interactions, we have calculated the enthalpy change associated with the transfer of 1 mg amino acid from the aqueous phase to the gel phase, which is given by $\Delta h = h_s^g - h_s^w$. However, it should be noted that this analysis is approximated, because it is impossible to separate the thermal effect caused by the binding, from the one caused by the water desorption from the hydrophobic PNIPA surface.[25]

Figure 6 shows a typical example of a processed data set (for valine at 25 °C) as calculated using the instrument's software. *C** in this Figure refers to an apparent concentration which is calculated from a mass balance, without taking into account the gel volume or preferred binding to the gel. The enthalpy of transfer Δh was calculated from these data using eqs 6 and 10−12.

Figures 7 and 8 show the concentration dependence of Δh for valine and aspartic acid respectively, at two temperatures. In Figure 8, a clear and a sudden change in the enthalpy values can be seen. This transition occurs at a concentration of about 1.25 mg/mL for aspartic acid at both temperatures.

The binding of valine to the PNIPA gel is an endothermic process both at 25 °C and at 37 °C, as suggested by the positive values of Δh. This finding implies that the preferred binding observed at 37 °C is probably entropy-

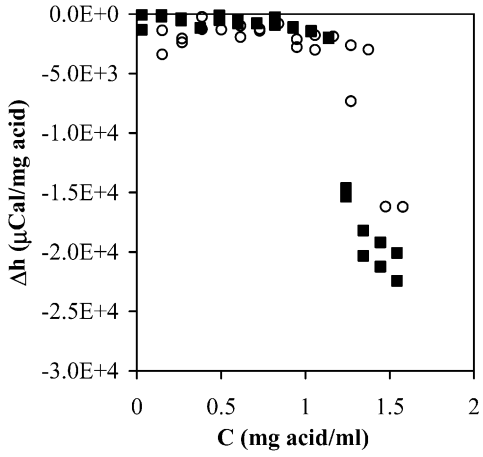

Figure 8. Enthalpy of binding between PNIPA gel and aspartic acid (○) at 25 and (■) 37 °C.

driven. A possible explanation could be that the rotational entropy loss of the solute molecules because of their binding to the gel phase is excessively compensated by the decrease in the hydrophobic interactions between the gel and the water. An entropy-driven adsorption of proteins on common hydrophobic sorbents was previously described.[16] Another observation is that at both temperatures there is a monotonic, almost linear dependence of the enthalpy of binding on the concentration. At 37 °C, the slop of the curve and the total values are higher than at 25 °C. Two possible mechanisms may account for concentration-dependent binding enthalpy:[15] First, repulsion forces between the adsorbed molecules, arising from electrostatic interactions or steric hindrance have an endothermic effect, which increases with surface density. Second, the water structure near hydrophobic surfaces becomes less ordered upon increasing the surface concentration of adsorbed molecules. Thus, the heat required for dehydration decreases with surface concentration. At 25 °C, the surface density was lower; thus, the hindrance effect was lower. As the temperature was raised, more acid was adsorbed; the surface density increased and so did the enthalpy needed to bring more valine molecules closer to the surface of gel.

For the aspartic acid, we observed very low exothermic enthalpies below the transition concentration. This suggests that the enthalpy plays a role in the binding process that may be associated with formation of hydrogen bonding between the gel and the amino acid. Above the transition concentration, the enthalpy change associated with the aspartic acid binding decreases considerably, indicating changes in the amount of the hydrogen bonds or their strength. To this end, the origin of this transition is not clear. A possible mechanism that may be suggested for the transition is that above a critical surface density the PNIPA gel is practically covered with aspartic acid and a formation of a second layer begins. This layer is formed because of intermolecular hydrogen bonding and thus is an exothermic process. Yet, it should be noted that the binding isotherms do not show any transition at this concentration.

Conclusions

The binding of valine and aspartic acid to PNIPAM microgels was studied using equilibrium binding isotherms, DSC and ITC, at 25 and at 37 °C. The binding isotherms were linear, indicating constant adsorption coefficients. The binding of the two acids was enhanced with a temperature elevation from 25 to 37 °C, due to the

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more hydrophobic nature of the gel at 37 °C. At 37 °C, the binding of valine is higher due to its more hydrophobic nature compare to aspartic acid. This finding implies that the binding of valine is due to hydrophobic interactions. The ITC measurements that revealed an endothermic binding process (namely, enthropy-driven binding) support this conclusion. The binding of aspartic acid was higher than that of valine at 25 °C, possibly due to better hydrogen bonding with the gel at this temperature. ITC results showed that, contrary to valine, the binding of aspartic acid was exothermic, supporting the suggestion that the binding mechanism is based on hydrogen bonds or ion pair formation. The sharp change in the enthalpy values that was observed at a transition concentration of about 1.25 mg/mL is discussed. DSC measurements show no effect of amino acids addition on the collapse enthalpy, but the 0.4 °C lowering of the phase transition temperature may imply that weaker or fewer hydrogen bonds between water and the gel are formed. Our results show that a direct measurement of the binding enthalpy gives insight into the binding mechanism between biochemicals and PNIPA gel. Further work dealing with more complicated systems (such as proteins) is currently on its way.

Acknowledgment. This research was supported by Technion V.P.R. Fund—E. and J. Bishop Research Fund and by the Fund for the Promotion of Research at the Technion. We thank the Otto Meyerhof Center for Biotechnology established by Minerva Foundation (Munich, Germany) for the financial contributed in purchasing the VP-ITC and VP-DSC instrument.

LA025735R