Hydrophobic Contribution of Amino Acids in Peptides Measured by Hydrophobic Interaction Chromatography

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The adsorption behaviors of amino acids in short chain peptides were examined. Each amino acid, aliphatic or charged, was inserted between the two tryptophans of a peptide, GWWG. The capacity factors of these peptides on an Ocytl-Sepharose column were measured. The adsorption enthalpies, entropies, and the number of repelled water molecules after adsorption were estimated to analyze the contribution of each different amino acid to its hydrophobic adsorption. The peptides inserted with aliphatic amino acids owned the highest capacity factors but released the least amount of adsorption heat among all the peptides under examination. It was found that the hydrophobic contribution of aliphatic amino acids was derived from the entropy gain by repelling the ordered water surrounding them. The insertion of negatively charged amino acids greatly reduced the capacity factors but still repelled a significant number of water molecules after adsorption. This indicated that the water molecules surrounding ionic amino acids were not orderly aligned. The dehydration cost energy but the water repelling did not offer enough entropy to drive the adsorption. Subsequently, lower retention was obtained from the peptides inserted with negatively charged ionic amino acids. The insertion of lysine increased the adsorption enthalpy but repelled no water molecules after adsorption. It was speculated that the inserted lysine still interacted with hydrophobic ligands but disturbed the interaction between ligands and adjacent tryptophans. Therefore, the adsorption enthalpy increased and the capacity factors decreased. Different amino acids contributed to hydrophobic interaction in different ways. The simultaneous analysis of capacity factor, adsorption enthalpy, adsorption entropy, and the number of repelled water molecules facilitated the understanding of the adsorption processes.

Introduction

Hydrophobic interaction generally describes the association between apolar or the apolar parts of amphipathic molecules in aqueous medium. Generally speaking, electrostatic interaction, hydrogen bonding, and van der Waals interactions all contribute to the hydrophobic interaction. It was believed that hydrophobic interaction played an important role in many biosystems. For example, hydrophobic interaction is usually a determinant factor in the binding between cell surface receptor and its respective ligand;[1] the intramolecular hydrophobic interaction is also recognized as the most important factor in stabilizing the protein structure;[2−5] the interaction between peptides and the cell membrane is also related to hydrophobic interaction.[6] Therefore, the hydrophobic contribution of amino acid has long been evaluated through various means. Nozaki and Tanford [7] measured the solubilities of free amino acids in water−ethanol and water−dioxane mixtures to scale the amino acid hydrophobicity. Fauchere and Pliska [8] measured the partition coefficients of *N*-acetylamino acid amides in an octanol−water two-phase system. Gehas and Wetlaufer[9] studied the retention of dansyl derivatized amino acids on hydrophobic interaction chromatography to estimate the solute's nonpolar contacting area. Guo et al.[10] used a RP-C18 column to measure the retention of 20 synthetic peptides with specifically substituted amino acids and calculate the contribution of each amino acid. Devido et al.[11] measured the retention of derivatized amino acids on C18 columns to calculate the free energy of transfer of the solute from an aqueous mobile phase to a nonpolar stationary phase. Makhatadze and Privalov[12] measured the partial molar heat capacities of various peptides by scanning microcalorimetry to scale the hydrophobicity of the amino acid side chains based on the temperature dependences of their heat capacities.

Controversy has been observed among the hydrophobicity studies. For example, the amino acid leucine has been found more hydrophobic than isoleucine in some studies but less in others. Glycine has been ranked as the most hydrophobic amino acid.[13] The controversy might arise from the differences in measuring methods. It could also be due to the differences in testing samples. Some studies used free amino acids, while some used capped amino acids. More and more studies used peptides to study the hydrophobicity of amino acids.[10,14,15]

The use of chromatographic methods to measure the hydrophobicity has several advantages: it is easy to operate; it does not need ultrapure samples; and the required sample volume is small. Therefore, the chromatographic method has become the most popular way for measuring solute hydrophobicity. Compared to reverse phase chromatography, hydrophobic interaction chromatography appears to be a more appropriate method since it actually measures the hydrophobicity in an aqueous environ-

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TABLE 1: Molecule Weights and Sequences of Peptides Used in This Study

no.	sequences	symbol	M_r
1	glycyl-tryptophanyl-leucyl-tryptophanyl-glycine	GWLWG	617.71
2	glycyl-tryptophanyl-isoleucyl-tryptophanyl-glycine	GWIWG	617.71
3	glycyl-tryptophanyl-valyl-tryptophanyl-glycine	GWVWG	603.68
4	glycyl-tryptophanyl-alanyl-tryptophanyl-glycine	GWAWG	575.62
5	glycyl-tryptophanyl-glycine-tryptophanyl-glycine	GWGWG	561.604
6	glycyl-tryptophanyl-aspartyl-tryptophanyl-glycine	GWDWG	619.64
7	glycyl-tryptophanyl-glutamyl-tryptophanyl-glycine	GWEWG	633.67
8	glycyl-tryptophanyl-histidyl-tryptophanyl-glycine	GWHWG	641.693
9	glycyl-tryptophanyl-lysyl-tryptophanyl-glycine	GWKWG	632.73
10	glycyl-tryptophany-tryptophanyl-glycine	GWWG	504.552

ment rather than in organic solvents. However, most of the studies still used reverse phase liquid chromatography (RPLC).

Although the relative hydrophobicity can be evaluated by various methods, molecular understanding of how different amino acids interact with hydrophobic surface may not be easily obtained. According to Lin et al.,[16] the hydrophobic interaction can be divided into three sequential subprocesses: (a) dehydration or deionization of the biomolecules and the adsorbent, i.e., water or ion molecules surrounding the biomolecules and hydrophobic ligands are excluded, hence, enthalpy is necessary and the process would have entropic gain; (b) hydrophobic interaction between the biomolecules and hydrophobic adsorbent (this interaction is usually accompanied by the system enthalpy release and entropy loss); and (c) rearrangement of the excluded water or ion in a bulk solution (this step usually involved in enthalpy necessary and entropy loss of the system). How different amino acids participate in each subprocess needs elucidation.

In this study, we try to evaluate the hydrophobicity of short chain peptides. Each different amino acid, aliphatic or charged, was inserted between the two tryptophans in peptide GWWG. Ten peptides containing different amino acids, GWWG, GWGWG, GWAWG, GWVWG, GWIWG, GWLWG, GWHWG, GWEWG, GWDWG, and GWKWG, were studied in hydrophobic interaction chromatography (HIC). We have previously demonstrated that tryptophan-containing peptides had strong interaction with Octyl-Sepharose.[17] The insertion of amino acid in GWWG ensures accurate estimation of the retention times of these peptides in the isocratic elution mode. The temperature and salt dependences of the capacity factors are measured. The adsorption enthalpies can be calculated by linear van't Hoff plot. The hydrophobic area participating in hydrophobic interaction can be estimated by the number of repelled water molecules, which can be evaluated by a simplified Preferential Interaction Model. The hydrophobic behavior of each amino acid and how it participates in each subprocess of adsorption can be understood by comparing the capacity factor, the adsorption enthalpy, the adsorption entropy, and the number of repelled water molecules of these peptides with those of peptide GWWG.

Materials and Methods

Materials. Ammonium sulfate and tris(hydroxymethyl)-aminomethane were purchased from Merck (Germany). Glycine-glycine-glycine-glycine (GGGG) and glycine-glycine-glycine-glycine-glycine (GGGGG) were purchased from Sigma (St. Louis, U.S.A.), The short chain peptides (amino acid sequences are listed in Table 1) were synthesized by Digital GENE Biosciences (Taipei, Taiwan). Octyl-Sepharose gel (ligand concentration: 40 μmol/mL) was purchased from Pharmacia Biotech. (Uppsala, Sweden).

Chromatographic Operation. Hydrophobic interaction chromatography was carried out by an HPLC system that included two high-pressure pumps (Model pu-610 pump, GL Sciences, Tokyo, Japan), a UV monitor (Model 500, Lab Alliance, PA), and an autosampler (Model 816, Spark, Emmen, Holland). The signals were processed by a Chem-Lab data station (SISC, Taipei, Taiwan). The volume of the sample loop was 100 μL. Hydrophobic beads were packed in a 10 $\times$ 1 cm^2 jacketed column and the final bed height was 5.5 cm. The column temperature was controlled by a Firstek B402-D (Shinjuang, Taiwan) circulation water bath, and the column was equilibrated with 20 mM Tris buffer (pH 6) containing various amounts of $(NH_4)_2SO_4$. Each peptide sample was injected to the column at a concentration of 0.1 mg/mL and was eluted isocratically at a flow rate of 0.4 mL/min. The eluent was monitored with a Lab Alliance Model 500 UV monitor at 220 nm. The investigated temperature ranged from 10 to 35 °C. Four measurements were taken at each temperature. Two of them were taken while we increased the temperature from 10 to 35 °C. The other two were taken when the temperature was decreased from 35 °C back to 10 °C. The maximum deviation was found to be less than 5% from the average.

Estimation of the Thermodynamic Parameters. The apparent adsorption reaction takes place as follows:

$$\mathrm{M} + \mathrm{L} \rightarrow \mathrm{ML} \tag{1}$$

where M represents the free solute, L represents the ligand on the matrix, and ML is the adsorbed solute. Then the equilibrium constant can be calculated as follows:

$$K_{eq} = \frac{C_{ML}}{C_M C_L} \tag{2}$$

where C_M denotes the concentration of free solute and C_L and C_{ML} denote the concentration of free ligand in the matrix and the concentration of adsorbate on the ligand, respectively. The equilibrium constant can be related to the capacity factor, k', by eq 3

$$K_{eq} = \frac{n_s}{n_m}\frac{1}{C_L} = \frac{k'}{C_L} \tag{3}$$

where n_s and n_m represent the amount of solute in the stationary phase and mobile phase, respectively. The capacity factor (k') is measurable by chromatography according to the following equation:

$$k' \equiv \frac{t_R - t_0}{t_0}$$

where t_R is the retention time of the sample and t_0 is the retention time of nonretained molecules (GGGGG). Since C_M is usually small, C_L is very close to the maximum concentration of ligand in matrix (L_m) in elution chromatography, and the relation

between k' and K_{eq} can be expressed as

$$k' = K_{eq}C_L \approx K_{eq}L_m \tag{4}$$

L_m can be calculated by the ligand density based on total volume (solid phase + mobile phase). Since

$$\Delta G^0 = -RT \ln K_{eq} \tag{5}$$

and

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \tag{6}$$

$$\ln K_{eq} = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \tag{7}$$

so that

$$\ln k' = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln L_m \tag{8}$$

where ΔG° denotes the standard Gibbs free change, and ΔH° and ΔS° are the standard enthalpy and entropy changes.

Preferential Interaction Theory. Perkins et al.[18] have utilized the preferential interaction model to analyze the effect of salt by the following relationship:

$$\ln k' = c + \frac{(\Delta\nu_+ + \Delta\nu_-)}{g}\ln m_3 - \frac{n\Delta\nu_1}{m_1 g}m_3 \tag{9}$$

where

$$g = \left(\frac{\partial \ln m_3}{\partial \ln a_\pm}\right)_{T,P}$$

$\Delta\nu_1$, $\Delta\nu_+$, and $\Delta\nu_-$ are the stoichiometrically weighted change in the number of moles of water, salt cations, and salt anions, respectively, in the local region of the protein−ligand complex and reactants of the process, and n is the total number of cations and anions per formula unit of the salt. m_3 is the molal concentration of ammonium sulfate and m_1 is the molal concentration of water. We simplify the derivation as follows:

If the adsorption of peptides is assumed to be accompanied by the release of salt and water molecules, the adsorption can be represented by the following reaction

$$P + A \overset{K_a}{\longleftrightarrow} C + wW + sS \tag{10}$$

where P denotes the peptide, A denotes the adsorbent, C is the peptide−surface complex, W and S denote water and salt. The coefficients w and s represent the number of repelled water and salt molecules upon peptide adsorption. The equilibrium constant, K_a, can then be represented by

$$K_a = \frac{a_C a_W{}^w a_S{}^s}{a_P a_A} \tag{11}$$

The relation between capacity factor and equilibrium constant K_a becomes

$$\ln K_a = \ln k' - \ln \phi + w \ln a_W + s \ln a_S - \ln a_A \tag{12}$$

or

$$\ln k' = \ln K_a + \ln \phi - [w \ln \gamma_W + s \ln \gamma_S] - [w \ln(1 - x_S) + s \ln x_S] \tag{13}$$

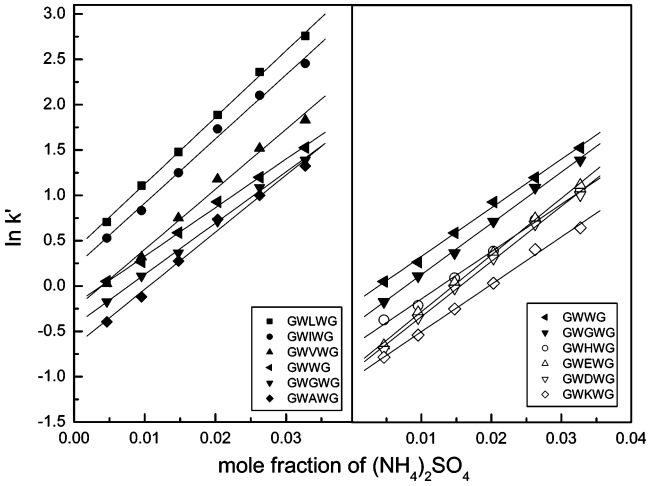

Figure 1. Dependence of the capacity factor on salt concentration: the capacity factors of peptides on an Octyl-Sepharose column at various ammonium sulfate concentrations at 298 K.

where γ_W and γ_S denote the activity coefficients of water and salt, and x_S is the mole fraction of salt in aqueous solution. If the activity coefficients can be regarded as constants, ln k' can be approximated by

$$\ln k' = \ln k_0 + wx_S - s \ln x_S \tag{14}$$

According to the study of Perkins,[18] Diogo,[19] and Esquibel-King,[20] the number of repelled salt molecules was negligible in hydrophobic interaction chromatography. The equation can be simplified as the following form, which is identical with the form derived from solvophobic theory [19,21] and from the effect of salt concentration on the retention of eluites in hydrophobic interaction chromatography:[22]

$$\ln k' = \ln k_0' + wx_S \tag{15}$$

By plotting ln k' versus the molar fraction of the salt, the number of repelled water molecules after the adsorption of each peptide can be estimated.

Results

Capacity Factors of Peptides at Different Salt Concentrations. Tryptophan had the highest hydrophobicity among all the amino acids.[7,8,23] We had previously shown that the peptide GWWG could be moderately retained on an Octyl-Sepharose column and the interation between tryptophan and octyl ligand was exothermic.[17] Peptides were synthesized by inserting different amino acids between the two tryptophans in GWWG and reliable retention times could be obtained on the Octyl-Sepharose column. The capacity factors (k') of the peptides, GWWG, GWGWG, GWLWG, GWIWG, GWVWG, GWAWG, GWHWG, GWEWG, GWDWG, and GWKWG, on an Octyl-Sepharose column were measured isocratically at different temperatures and salt concentrations. The effects of salt concentration on peptide retention at 298 K were shown in Figure 1. It was found that the peptide GWGWG had a similar capacity factor to that of peptide GWWG at 1.5 M $(NH_4)_2SO_4$. However, the capacity factor of GWGWG became slightly lower than that of GWWG at lower salt concentrations. The capacity factors of those peptides inserted with aliphatic amino acids were mostly higher than that of the peptide GWWG except for alanine, and the capacity factor increased with the number of methylene groups in the side chain. The capacity factors of peptides inserted with ionic amino acids were all lower than

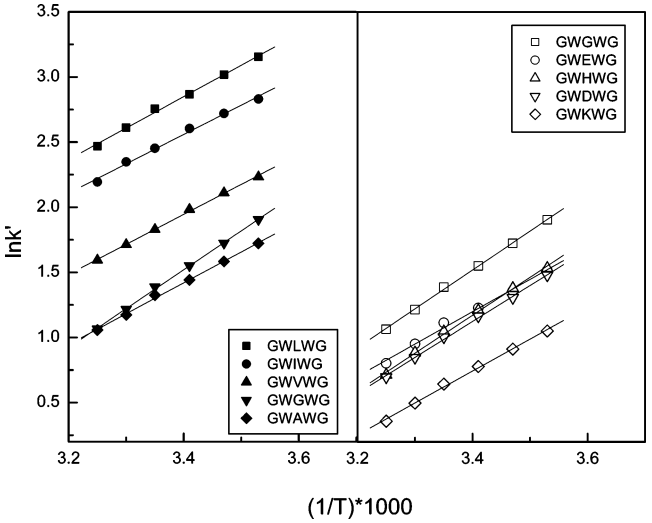

Figure 2. Van't Hoff plot for the adsorption of peptides on the Octyl-Sepharose column at 1.5 M ammonium sulfate.

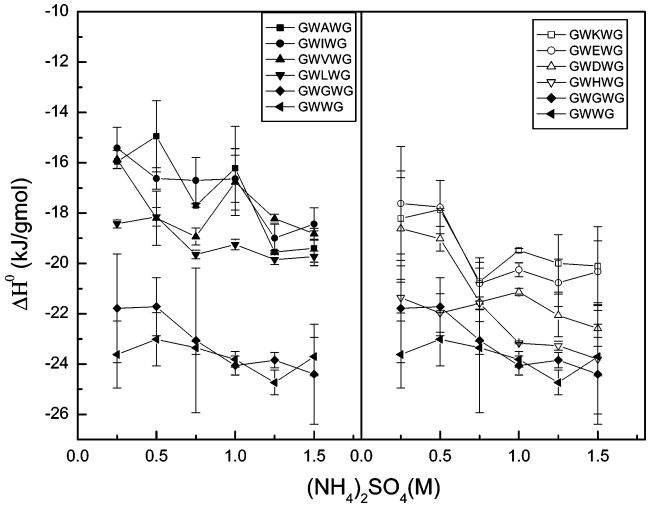

Figure 3. Standard adsorption enthalpies of peptides on the Octyl-Sepharose column at different ammonium sulfate concentrations. Standard deviations are calculated by $[\sum_i(X_i - \bar{X})^2/(n-1)]^{1/2}$.

that of GWWG. Among all the peptides, the lysine inserted peptide appeared to be the least retentive. Obviously, the insertion of aliphatic amino acid in GWWG increased the hydrophobic interaction between peptides and Octyl-Sepharose, but the ionic amino acids reduced it. However, it surprised us that the alanine insertion was less retentive than the glycine one.

Adsorption Enthalpy. The capacity factors were also measured at various temperatures. The natural logarithm of k' at 1.5 M $(NH_4)_2SO_4$ was plotted against the inverse temperature and shown in Figure 2. It was found that the logarithm of k' increased linearly with the increase in inversed temperature. The regression parameters were all higher than 0.99. Therefore, the adsorption enthalpies can be calculated from the slopes according to eq 8. The calculated enthalpies at different $(NH_4)_2SO_4$ concentrations were shown in Figure 3. It was found that the adsorption enthalpy fluctuated quite a bit with salt concentration. Similar phenomena were also observed in the studies of Byun et al.[24] and Esquibel-King et al.[20] The retention time measurements were usually very accurate. The deviations were mostly smaller than 5%. However, this led to a possible 10% deviation in k' estimation. The adsorption enthalpies were obtained from a linear van't Hoff plot. Although the regression showed good

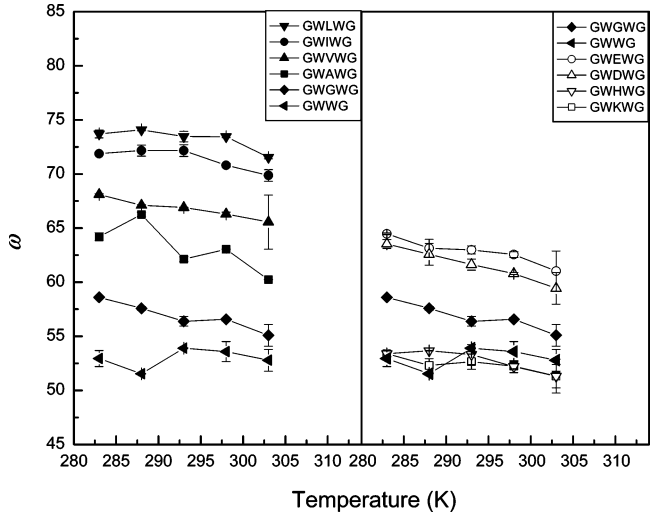

Figure 4. Number of water molecules released after peptide adsorption. The error bar indicates the variation between two measurements during ascending and descending temperatures.

TABLE 2: Average Number of Water Molecules Released after Peptide Adsorption

sequence	ω^a	$\delta\omega^b$
GWLWG	73.5 ± 0.10	19.9 ± 1.02
GWIWG	70.8 ± 0.17	17.2 ± 1.09
GWVWG	66.3 ± 0.16	12.7 ± 1.08
GWAWG	62.0 ± 0.02	9.5 ± 0.94
GWGWG	56.6 ± 0.16	3.0 ± 1.08
GWDWG	60.8 ± 0.09	7.2 ± 1.01
GWEWG	62.6 ± 0.27	9.0 ± 1.09
GWHWG	52.2 ± 0.55	
GWKWG	52.2 ± 0.24	
GWWG	53.6 ± 0.92	

[a] The number of repelled water molecules at 298 K. [b] The ω value of each peptide subtracts that of peptide GWWG.

linearity in the plot, the adsorption enthalpies could vary by 18% between the results obtained from ascending and descending temperatures. All the peptides had negative heat of adsorption, that is, all the adsorptions were exothermic. Compared to the peptide GWWG, the insertion of glycine increased the adsorption enthalpy little, but the insertion of either aliphatic or ionic amino acids elevated the adsorption enthalpy.

Number of Repelled Water Molecules. The number of repelled water molecules after adsorption could be calculated according to eq 15. As shown in Figure 1, $\ln k'$ increased linearly with x_S. The regression parameters were all higher than 0.99. Figure 4 showed the number of repelled water molecules after peptide adsorption within 283 to 308 K. It was found that the number of repelled water molecules slightly decreased as the temperature increased. The ω values of each peptide and the value contributed by each inserted amino acid at 298 K were listed in Table 2. The variations of estimated values were mostly within one water molecule. The peptide GWWG had the number of repelled water molecules around 53.6. The insertion of glycine repelled 3 more water molecules upon adsorption. That reflected each tryptophan repelled 24−27 water molecules. The peptide GWLWG had the highest number of repelled water molecules of 73.5 at 298 K. It meant that the insertion of leucine increased the number of repelled water molecules by 19.9. The insertion of aliphatic amino acids apparently repelled more water molecules after hydrophobic interaction. And the increase in repelled water molecules correlated roughly with the aliphatic chain length. The inserted isoleucine repelled 17.2, valine repelled 12.7, and alanine repelled 9.5 water molecules. The

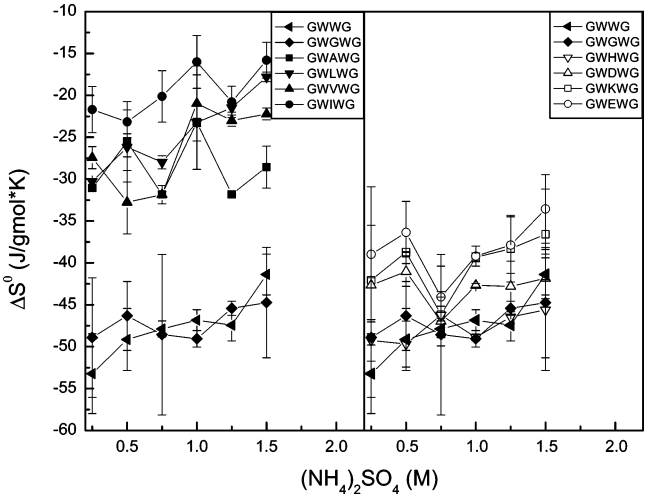

Figure 5. Standard adsorption entropies of peptides on the Octyl-Sepharose column at different ammonium sulfate concentrations at 298 K. Standard deviations are calculated by $[\sum_i(X_i - \bar{X})^2/(n-1)]^{1/2}$.

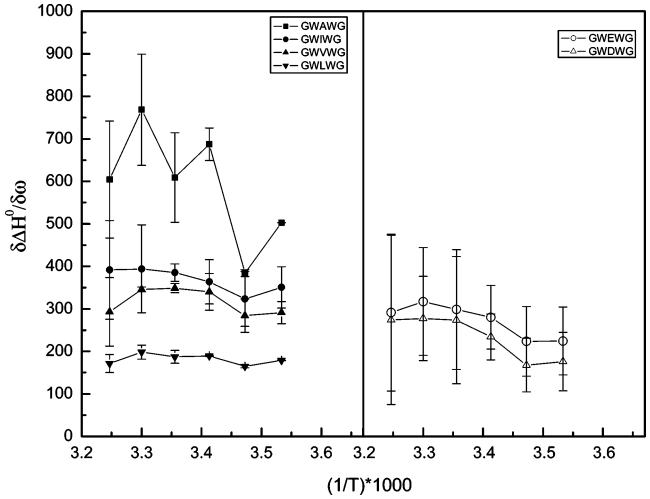

Figure 6. Enthalpy increase after adsorption by repelling each water molecule at 0.75 M $(NH_4)_2SO_4$. The error bar indicates the variation between two measurements during ascending and descending temperatures.

insertion of positively charged amino acid did not increase the number of repelled water molecules, but the insertion of negatively charged amino acids did. The insertion of glutamic acid increased the number of repelled water molecules by 9 and the insertion of aspartic acid increased the number of repelled water molecules by 7.2.

Perkins et al.[18] had also applied the preferential interaction model to calculate the number of repelled water molecules after hen egg white lysozyme and ovalbumin adsorbed onto an Octyl-Sepharose column. They reported that the number of repelled water molecules were 120 and 101 of ovalbumin and hen egg white lysozyme, respectively. Considering their huge sizes, it seemed that Perkins' measurements were low. The number of repelled water molecules reflected the actual contact area between the peptide and the ligand. Therefore, Perkins' measurements might suggest that the contacting area between the two proteins and octyl ligands was not that large.

Adsorption Entropy. Once the adsorption enthalpies were obtained the adsorption entropy could be calculated from eq 8. As shown in Figure 5, peptides GWWG and GWGWG had the lowest adsorption entropy. The adsorption entropies of peptides inserted with ionic amino acids were higher and of those inserted with aliphatic amino acids were higher still. The reason all the peptides had higher adsorption entropy than GWWG was partly due to the repelling of ordered water on the hydrophobic surface. As indicated in Figure 4 the peptides inserted with aliphatic amino acids had the highest number of repelled water molecules, followed by the peptides inserted with ionic amino acids.

Increase in Adsorption Enthalpy per Repelled Water Molecule. The number of repelled water molecules by each amino acid insert revealed the hydrophobic contacting area of each inserted amino acid. If we divide the adsorption enthalpy increase by the number of repelled water molecules, that is, $\delta(\Delta H°)/\delta\omega$ of each amino acid insert, the value indicated the specific adsorption enthalpy of each amino acid insert. As shown in Figure 6, among all the aliphatic amino acids, the value $\delta(\Delta H°)/\delta\omega$ of alanine was the highest, followed by isoleucine and valine, while that of leucine was the lowest. This result might indicate that the side chain of leucine had the strongest van der Waals interaction with the octyl ligand per unit contacting area. The heat release from van der Waals interaction could partly compensate for the enthalpy increase from water repelling. Subsequently, the stronger van der Waals interaction per unit contacting area resulted in a lower value of $\delta(\Delta H°)/\delta\omega$. The values of $\delta(\Delta H°)/\delta\omega$ can help us look in more details about the hydrophobic interaction. However, both the $\Delta H°$ and ω estimations contained a certain degree of variation, and larger variation could occur in the calculation of $\delta(\Delta H°)/\delta\omega$. The $\delta(\Delta H°)/\delta\omega$ values of negatively charged amino acids were also shown in Figure 6. It was found that the calculated $\delta(\Delta H°)/\delta\omega$ values were comparable to those of aliphatic amino acids.

Discussion

Peptides Inserted with Glycine. Glycine has only one hydrogen atom in its side chain and was taken as a reference in the hydrophobicity scale of amino acids.[7] The insertion of glycine in peptide GWWG resulted in a decrease in capacity factor and an increase in repelled water molecules after adsorption. An increase in the number of repelled water molecules meant an increase in peptide−ligand contact and an increase in the system entropy. Since the side chain of glycine had only hydrogen atoms, the increased three repelled water molecules were primarily due to the increased backbone interaction. The repelling of ordered water from surface usually cost energy and offered entropy. But the adsorption enthalpy and entropy were found to increase slightly. This might suggest that the water surrounding glycine did not form a highly ordered water shell.

Peptides Inserted with Aliphatic Amino Acids. There is no doubt that aliphatic amino acids are considered a major group of amino acids contributing to the hydrophobicity of proteins. The insertion of aliphatic amino acids in peptide GWWG resulted in increases in capacity factors, increases in adsorption enthalpies, and considerable increases in the number of repelled water molecules. The high increase in the number of repelled water molecules indicated that aliphatic amino acids strongly enhanced the direct interactions between peptides and hydrophobic ligands. The van der Waals interaction released heat to lower the enthalpy increase by disturbing the ordered water on surface. Although the increase in peptide−ligand interaction still led to increases in adsorption enthalpies, the reduction of adsorption enthalpies by van der Waals interaction allowed the entropy increases from repelled water molecules to overpower the enthalpy increases and resulted in decreases in Gibbs free energies. The only exception was the amino acid alanine. The insertion of alanine in GWWG repelled 9 more water molecules than GWWG at 298 K. The entropy gain from releasing the

ordered water did not overcome the price paid by the enthalpy increase. Therefore, the capacity factor of alanine peptide was actually lower than that of GWWG. More surprisingly, the capacity factor of peptide inserted with alanine was even lower than that with glycine. The possible reason might be the large number of repelled water molecules. The peptide GWAWG repelled 9, but GWGWG repelled only 3 more water molecules than GWWG. It seemed that the strong interaction between tryptophans and octyl ligands brought alanine in GWAWG close to the ligand. However, the van der Waals interaction between alanine side chain and octyl ligand was not strong enough to bring down the enthalpy increase because of repelling ordered water. This was partly evidenced by the highest $\delta(\Delta H^\circ)/\delta w$ value of alanine than other aliphatic amino acid inserts. It seemed that the entropy gain from repelling ordered water was not enough to pay for the accompanying enthalpy increase. Subsequently the free energy of adsorption of GWAWG was higher than those of GWWG and GWGWG.

Hydrophobic Contribution of Leucine and Isoleucine. Among the aliphatic amino acid containing peptides, the hydrophobic contribution of aliphatic amino acids followed the order leucine > isoleucine > valine > alanine. This result was similar to most hydrophobic scales except that leucine was found more hydrophobic than isoleucine. In this study, the hydrophobic contribution of leucine was found to be always larger than that of isoleucine, whether the peptide retention was measured at high or low salt concentrations. However, the result was different from that of Gehas et al.[9] They measured the retention time of the dansyl derivatives of 20 common amino acids by hydrophobic interaction chromatography and found the exposed hydrophobic surface area of isoleucine is larger than that of leucine. The hydrophobicity order of leucine and isoleucine actually varied with the methods used for measurement. For example, Fauchere and Pliska[8] examined the octanol/water partitioning data of 20 amino acids and found that the isoleucine was more hydrophobic than leucine. Guo et al.[10] measured the retention times of 20 synthetic peptides by reversed phase chromatography, but found that the amino acid leucine was more hydrophobic than isoleucine. Yoshida[25] published a hydrophilicity scale using normal phase liquid chromatography. They scaled leucine more hydrophobic than isoleucine.

The comparison between leucine and isoleucine was really disturbing. Different measuring methods gave different results. Studies adopting similar methods also brought in different results. The exposed hydrophobic surface area of isoleucine was larger than that of leucine by calculation.[9] However, seven out of thirteen chromatographic studies, according to Biswas et al.,[26] indicated that isoleucine was more hydrophobic than leucine. Five out of nine studies by the two-phase partitioning method showed the hydrophobicity of leucine was larger than that of isoleucine.

In this study, the capacity factor of leucine peptide was higher than that of isoleucine. The leucine peptide also owned 2.7 more repelled water after adsorption at 298 K. The repelling of water molecules cost energy; however, the adsorption enthalpy of leucine peptide was lower than that of isoleucine one by roughly 2 kJ/gmol. It was suspected that the heat release from stronger interaction between leucine and octyl ligand compensated for the heat lost from the extra repelled water molecules. However, considering the possible variations in the measurements, the differences between leucine and isoleucine, 2.7 in repelled water and 2 kJ/gmol in adsorption enthalpy, were still not large enough to make a strong statement. More accurate measurements are needed.

Peptides Inserted with Negatively Charged Amino Acids. The insertion of glutamate and aspartate in GWWG resulted in the reduction of capacity factor. There was still direct contact between negatively charged amino acids and octyl ligands. The insertion of glutamic acid increased the number of repelled water molecules by 9 and the insertion of aspartic acid by 7.2 at 298 K. Although the insertion of negatively charged amino acids increased the adsorption enthalpy, the $\delta(\Delta H^\circ)/\delta w$ values were no higher than those of most of the aliphatic amino acids. Furthermore, despite the significant number of additional repelled water molecules, the adsorption entropies of peptides inserted with negatively charged amino acids were not significantly higher than that of GWWG. It was suspected that the water surrounding the side chains of ionic amino acids was not orderly aligned. Therefore, the repelling of water did not provide enough entropy elevation. This may be the reason the peptides inserted with negatively charged amino acids had the lower capacity factors.

Peptides Inserted with Positively Charged Amino Acids. The adsorption of peptides inserted with positively charged amino acids repelled even fewer water molecules than GWGWG. The peptide inserted with histidine and lysine almost had the same number of repelled water molecules as GWWG upon adsorption. However, the enthalpies of GWKWG increased after adsorption. One of the possible explanations was that the inserted lysine still had some interaction with the ligand. But the interaction reduced tryptophan−ligand interaction. The number of repelled water molecules gained from the inserted amino acid was equivalent to that lost from the reduction of tryptophan−ligand interaction. As a result, the total number of repelled water molecules remained the same. Since the interaction between tryptophan and ligand was highly exothermic, the reduction of tryptophan−ligand interaction led to an increase in adsorption enthalpy. The increase in adsorption enthalpy resulted in an increase in Gibbs free energy and subsequently the capacity factor decreased. Lysine had a higher adsorption enthalpy than histidine. Therefore, lysine peptide had a lower capacity factor.

Conclusion

The capacity factors of peptides containing aliphatic amino acids were larger than that of peptide GWWG except for the alanine peptide. The higher capacity factors were contributed by entropy gain from the higher number of repelled water molecules.

The capacity factors of peptides containing charged amino acids were all lower than that of peptide GWWG. There were still significant numbers of repelled water molecules of the negatively charged amino acids. It was suspected that the water molecules surrounding the ionic amino acids were not orderly aligned. Therefore, the repelling of water did not provide enough entropy gain.

The capacity factor of lysine peptide was even lower. No extra repelled water molecules were found compared with that of peptide GWWG. Since the adsorption enthalpy was higher than that o f GWWG, it was suspected that there was still interaction between lysine and the octyl ligand. The interaction reduced tryptophan−ligand interaction and resulted in the lower capacity factors. Simultaneous analysis of the capacity factor, the adsorption enthalpy, the adsorption entropy, and the number of repelled water molecules deepened our understanding of the hydrophobic contribution of different amino acids in small peptides.

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