Amino Acid Side-Chain Contributions to Free Energy of Transfer of Tripeptides from Water to Octanol

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The location of amino acids in soluble or membrane proteins is related to the hydrophobicity of the side chains. Amino acid hydrophobicity values are based upon the thermodynamics of transfer from an aqueous to a nonaqueous environment. However, for certain hydrophilic residues uncertainty exists on the appropriate hydrophobicity values. We have measured the octanol-water partition coefficients ($P_{o/w}$) of tripeptides of the sequence N-^{14}C-acetyl-Ala-X-Ala-NH-tButyl (AcAlaXAlaNHtButyl), where the central residue X was either Gly, Ala, Phe, Trp, Pro, His, Asp, or Glu. The $P_{o/w}$ for the tripeptides agreed reasonably well with values calculated by the fragment method of D. J. Abraham and A. J. Leo (*Proteins Struct. Func. Gen.* 2, 130–152, 1987). The log $P_{o/w}$ of the uncharged form was 1.6, 2.7, and 2.5 greater than the log $P_{o/w}$ of the ionized form for the His, Asp, and Glu peptide, respectively. The new data on the pH dependence of the ionizable side chains, His, Asp, and Glu, should result in better prediction of the partition coefficient of peptides as a function of pH. The thermodynamic parameters were determined from the temperature dependence of partitioning. In the temperature range studied (2 to 65°C) the transfer of tripeptides from water to octanol was entropy governed except for the ionized peptides. A heat capacity term was necessary to account for the transfer of tripeptides containing non polar residues. The heat capacity change for transfer from water into octanol was −45, −73, −81, and −88 cal/mol K for Ala, Phe, Trp, and Pro peptides, respectively. Peptides containing Gly, His (pH 7.2), and the uncharged forms of Asp, Glu, and His did not show a significant change in heat capacity. The side-chain contribution of the central residue X (ΔG_x) to the free energy of transfer was obtained from the difference between the free energy of transfer of the peptide containing the central residue X and the Gly peptide; $\Delta G_x = \Delta G_{(AcAlaXAlaNHtButyl)} - \Delta G_{(AcAlaGlyAlaNHtButyl)}$. The relative order of hydrophobicity of the side chains correlated well with previous studies. However, a significant difference was found for the absolute hydrophobicity between the present study and experimental data on N-acetyl amino acid amide derivatives (J. Fauchere and V. Pliska, *Eur. J. Med. Chem.* 18(4), 369–375, 1983). The ΔG_x values at pH 7.2 were 0, −0.13, −2.19, −2.52, −0.29, −0.16, 3.50, and 3.12 kcal/mol for Gly, Ala, Phe, Trp, Pro, His, Asp, and Glu, respectively. These hydrophobicity values in a tripeptide environment provide suggested values for a hydrophobicity scale.

KEY WORDS: amino acid side chains; free energy of transfer; hydrophobicity; octanol–water partition coefficient; peptide; pH.

INTRODUCTION

The prediction of protein structure or protein–membrane interactions from amino acid sequence depends to a large extent on the hydrophobicity values assigned to the amino acid side chains (1–3). Our interest in this problem stems from engineering peptides that can interact with membranes in a pH-dependent fashion (4–6). To accomplish this requires hydrophobicity values for ionizable side chains as a function of pH. Although several hydrophobicity scales have been proposed, hydrophobicity values for ionizable residues are inconsistent among studies (2,7–9) and values at other than neutral pH are rare.

Hydrophobicity is a measure of the relative affinity of a solute for a hydrophobic phase compared to an aqueous phase. The published hydrophobicity scales for amino acids have been compiled in one of three ways: (i) experimental measurements of free energy of transfer between an aqueous and a nonaqueous phase (10–13); (ii) statistical analysis of amino acid distribution in proteins of known structure using the criteria that hydrophobic residues are found more often in the interior of globular proteins (14,15) or in contact with the bilayer in the case of membrane proteins (16); and (iii) computation based upon a Hansch-type analysis where the amino acids are subdivided into chemical fragments and the sum of the fragment constants is used to estimate the hydrophobicity of the side chains (8,9).

The first approach measures the partitioning of amino acids between an aqueous and a nonaqueous phase. Various nonaqueous phases such as ethanol (10), vapor phase (11), octanol (7,12), or a C18 reverse-phase column (13) have been employed in the measurement of the partitioning. Early work by Yunger and Cramer (12) measured the octanol–water partition coefficients ($P_{o/w}$) of the 20 amino acids. The hydrophobicity scale based upon these measurements has a number of anomalies due to the charge and potential for hydrogen bonding of the α amino and α carboxylate groups. To account more accurately for the influence of a peptide backbone on the hydrophobicity values of the side chains, Fauchere and Pliska (7) measured the $P_{o/w}$ of N-acetyl amino acid amides. They calculated the contribution (π) of the side chains by subtracting log $P_{o/w}$ of N-acetyl glycine amide from the log $P_{o/w}$ of other derivatives [$\pi = \log P_{o/w(x)} - \log P_{o/w(G)}$]. These values are widely used because they are a self-consistent set derived from experimental measurements of all 20 amino acid derivatives. It is not known whether the hydrophobicity contribution of side chains in longer peptides would be the same as the N-acetyl amino acid amides. This would be true if intramolecular interactions were absent in the peptides and hence the additivity rule holds.

Abraham and Leo (8) suggested that the fragment method could be used to calculate the $P_{o/w}$ of new compounds or to reassess experimental values of doubtful quality. In applying this method to peptides, (i) the assignment of a fragment value for the peptide bond and the appropriate fragment values for ionizable residues as a function of pH and (ii) the role of proximity effects between peptide bonds and polar residues have yet to be resolved. Moreover, there is a discrepancy between the values measured by Fauchere and Pliska (7) for Glu and Asp and values computed by the fragment method of Leo and Abraham (8).

To establish the pH-dependent hydrophobicity values for the ionizable residues and to resolve some of the differences between the existing hydrophobicity data, we determined the $P_{o/w}$ of a series of N- and C-termini blocked

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tripeptides. Secondary structure formation is not significant in short peptides and blocking of the N and C terminal minimizes complications from ionic interactions between the end groups. The first and third residues are alanine and the central residues are Gly, Ala, Phe, Trp, Pro, His, Asp, and Glu. This series of peptides covers a wide range of hydrophobicity values and permits the effect of charge on partitioning behavior to be studied.

Thermodynamic parameters of the transfer of tripeptides from water to octanol were obtained from the temperature dependence of partitioning. The data were analyzed using both a linear and a multiple-linear van't Hoff equation. The first analysis assumes the temperature independence of ΔH and ΔS, whereas the second method treats them as a function of temperature and includes a heat capacity term.

A comparison of the side-chain contribution (π_X) to the free energy of transfer measured using the tripeptides with those obtained with *N*-acetyl amino acid amides (7) reveals the effect of the peptide bond on the additivity assumption. The measurements provide suggested values for a hydrophobicity scale.

MATERIALS

The blocked amino acids were purchased from Bachem Biochemicals (Torrance, CA), Peninsula Laboratories (Belmont, CA) or US Biochemical (Cleveland, Ohio) and used without further purification. 1-Hydroxybenzotriazole (HOBT), anhydrous dimethylformamide (DMF), pyridine, methyl-d_3 alcohol-d (MeOD), chloroform-d ($CDCl_3$), and trifluoroacetic acid (TFA) were from Aldrich (Milwaukee, WI). HPLC-grade *n*-octanol from Aldrich was used without further purification. HPLC-grade methanol and acetonitrile and all other ACS-grade solvents were from Fisher Scientific (Pittsburgh, PA). All other reagents were analytical grade. Acetic anhydride [1-^{14}C]-$(CH_3CO)_2O$ (10 mCi/mmol) in 80% benzene was purchased from NEN Research Products (Boston, MA). Scintillation cocktail was Ready Value from Beckman.

METHODS

Peptide Synthesis

Conventional solution phase methods were employed for the peptide synthesis. The mixed anhydride (MA) or DCC/HOBT method (17) was used for the coupling reaction. Removal of the Boc group from the intermediates was done with concentrated HCl/ethyl acetate (1:2/*V*:*V*) at room temperature. Side-chain blocking groups (benzyl) of Asp, Glu, and His were removed by catalytic hydrogenation after completion of the Fmoc tripeptide synthesis (17). The formyl group of Trp was removed by 10% diethylamine in DMF. For positive identification of peptides, proton NMR spectra and fast atom bombardment (FAB) mass spectra were obtained.

To obtain the requisite sensitivity for measuring a wide range of partition coefficients, the Fmoc was removed by 10% diethylamine in DMF (17) and the tripeptides were radiolabeled using ^{14}C-acetic anhydride. Radiolabeled acetylated peptides were purified by preparative TLC (20 × 20 cm, 500 μm, silica gel GF, Analtech). More information of the synthesis and characterization is available to the interested reader (A. Kim, Ph.D. thesis, 1990).

To ensure the purity of the radiolabeled peptides, a series of multiple extractions was performed. First, the partition coefficients between octanol and water ($P_{o/w-1}$) of the peptides were measured using glass tubes (16 × 150 mm) containing 5 ml of octanol, 5 ml of buffer (2 m*M* Tes, pH 7.2, 150 m*M* NaCl), and aliquots of ethanolic peptide solution. The mixture was vortexed for 1 min. The phases were separated by centrifugation and 1 ml of each phase was transferred into glass vials using disposable pipettes and the radioactivity was determined in a beta scintillation counter (Beckman LS 3801). The $P_{o/w-1}$ was calculated from the ratio of disintegrations per minute (dpm) in the two phases. From the remaining octanol phase, 3 ml was transferred into a clean tube and reequilibrated with 3 ml of new buffer. After vortexing and centrifugation, radioactivity in 1 ml of each phase was determined and the partition coefficient was calculated ($P_{o/w-2}$). The remaining aqueous phase (3 ml) from the first partitioning was reequilibrated with fresh octanol and the partition coefficient was measured ($P_{o/w-3}$).

If there were impurities which preferred either the octanol or the aqueous phase, $P_{o/w-2}$ or $P_{o/w-3}$ would be different from $P_{o/w-1}$. $P_{o/w}$ of AcAlaGlyAlaNHtButyl were all the same, indicating that this peptide was pure. For AcAlaAspAlaNHtButyl and AcAlaGluAlaNHtButyl, partitioning was performed at pH 2 because at pH 7 the concentration in the octanol phase would be too low to perform a second partitioning. The partition coefficient remained the same after the extraction for these two peptides. Therefore AcAlaGlyAlaNHtButyl, AcAlaAspAlaNHtButyl, and AcAlaGluAlaNHtButyl were used without further purification. $P_{o/w-2}$ of AcAlaAlaAlaNHtButyl was significantly larger than $P_{o/w-1}$ (0.33 vs 0.085), implying the presence of water soluble impurities. The partition coefficient of AcAlaProAlaNHtButyl also changed after extraction.

To determine the percentage impurity of peptides, reverse-phase HPLC was done on a Vydac C18 analytical column (0.46 × 15 cm, 10 μm). Ethanolic solution (10 to 50 μl) of peptide was injected and a linear gradient of 20–50% methanol in water was run with a flow rate of 1.5 ml/min for 30 min. Radioactivity was determined with the fractions collected every minute. Reverse-phase HPLC of AcAlaAlaAlaNHtButyl showed that about 30% of the radioactivity was associated with the solvent peak and the rest of the radioactivity was found at the retention time corresponding to the peptide peak. After repurification of this peptide by preparative TLC, the solvent peak on HPLC chromatogram showed no radioactivity and the three partition coefficients measured by the multiple extraction protocol were the same. AcAlaPheAlaNHtButyl, AcAlaTrpAlaNHtButyl, AcAlaHisAlaNHtButyl, and AcAlaProAlaNHtButyl were analyzed by the HPLC procedure and the percentage impurity was determined to be 2.2, 0.4, 50, and 15%, respectively. AcAlaTrpAlaNHtButyl was used for the partition experiment without further purification. The partition coefficient of unlabeled AcAlaTrpAlaNHtButyl obtained by the fluorescence measurement was the same as the result of radiolabeled peptide.

Since the impurity was most likely an acetate or a carboxylate, an ethanolic solution of peptides were passed through anion-exchange resin (AG1-X8, formate form; Bio-Rad) to absorb the impurity. Typically, 0.5 g of resin dispersed in ethanol was packed into a disposable polypropylene column (bed volume, 1 ml; Bio-Rad) and rinsed with ethanol. The peptide solution was added to the column and the eluate was collected. The column was washed with an additional 1 ml of ethanol. After the ion-exchange chromatography, there was no radioactivity associated with the solvent peak in HPLC. For the partition coefficient measurement described below, AcAlaPheAlaNHtButyl, AcAlaProAlaNHtButyl, and AcAlaHisAlaNHtButyl were purified by elution through an ion-exchange column.

Partition Coefficients

Partition coefficients were measured using the conventional shake flask method (18). Samples were prepared in triplicate and the average of the three values was taken for the partition coefficient. Octanol (5 ml) and 5 ml of buffer (pH 7.2, 2 m*M* Tes, 150 m*M* NaCl) were placed in glass screw-cap tubes (16 × 150 mm). Preliminary experiments done after mutual presaturation of octanol and water gave the same results as those of experiments done without presaturation. Typical experiments were done without presaturation of solvents. Ethanolic solutions of peptides (10 to 30 μl) were added into tubes using a Hamilton syringe to yield a total radioactivity of 1.5×10^4 to 2×10^4 dpm per tube except for the ionized peptides. For the latter case up to 1×10^5 dpm per tube was used. Peptide concentration in the aqueous phase ranged from 10^{-2} to 10^{-4} m*M* depending on the partition coefficients of the peptides. Tubes were securely capped with teflon lined caps.

Equilibration was obtained by one of the following two methods. The first method consisted of the rotation on a rotator (Sepco Tube Rotator, Scientific Equipment Products, Baltimore, MD) and centrifugation after equilibrium had been reached. In the case of the 2°C measurement, samples were equilibrated while rotating for 2 days in a cold room, and at higher temperatures (35, 45, 55°C), the rotator was placed in an incubator which maintained the desired temperature ±1°C. After 1 day of rotation, tubes were centrifuged for 15 min at room temperature, 1 or 1.5 ml of both phases was transferred into 20-ml scintillation vials, and the radioactivity was determined. The second method was to vortex the samples for 30 sec after 1 hr of incubation in a water bath at the desired temperature. The sample tube was incubated for another hour and vortexed again. After vortexing, samples were left overnight in the water bath to allow the phases to separate. The centrifugation step was omitted. Radioactivity was determined in both octanol and the buffer phase. The partition coefficient ($P_{o/w}$) was calculated from the ratio of dpm in the octanol phase to dpm in the buffer phase.

At room temperature the difference between partition coefficients measured by the two methods was less than 5% for all the peptides studied. Partition coefficients of AcAlaTrpAlaNHtButyl measured by the two methods were the same up to 65°C. At 45 and 55°C, the difference was almost twofold for the ionized peptides such as AcAlaAspAlaNHtButyl (pH 9), AcAlaGluAlaNHtButyl (pH 9), and AcAlaHisAlaNHtButyl (pH 3). The differences found for the ionized peptides might be due to the very low partition coefficients (1×10^{-4} to 10^{-3}), which would be much more sensitive to the amount of water present in octanol phase. Curve fitting of the data to estimate the thermodynamic parameters for these ionized peptides was not attempted. Details of the data analysis for other peptides are described under Results and Discussion.

Recovery of peptides from both phases was always in the range of 97 to 107%, indicating no significant peptide adsorption onto the glass. Similar peptide recoveries were obtained using silanized glassware.

Partition Coefficients as a Function of pH

For peptides containing ionizable side chains, partition coefficients at room temperature were measured as a function of pH. A preliminary experiment using an aqueous phase which was titrated with 1 *N* HCl or NaOH showed a significant pH change after partition measurements. For example, the pH of the aqueous phase (pH 8) dropped to 6 after the partitioning measurement. Therefore aqueous phases were prepared using appropriate buffers: pH 2, titrated with 1 *N* HCl; pH 3, glycine/HCl; pH 4 and pH 5, acetic acid/sodium acetate; pH 6, 7, and 8, Tes buffers titrated with 1 *N* HCl or NaOH; and pH 9 and pH 10, glycine/NaOH. All buffers contained 150 m*M* NaCl and the total concentration of buffer species in each buffer ranged between 2 and 3 m*M*. Although the buffers differed among pH studies, the predominant ions were sodium and chloride in all cases to standardize the effect of ions on partitioning (19).

To measure the partition coefficient of AcAlaHisAlaNHtButyl, octanol (5.5 ml) and aqueous phase (3 ml) at each pH and 30 μl of peptide solution were equilibrated in tubes which were rotated at room temperature for 1 day. After centrifugation, the radioactivity in 5 ml of octanol and 1 ml of aqueous phases was determined. The pH of the remaining aqueous phase was measured using an Orion Ross combination pH electrode connected to Corning pH meter 145. For AcAlaAspAlaNHtButyl and AcAlaGluAlaNHtButyl, octanol (6 ml) and aqueous phase (2 ml) at each pH and 10 or 30 μl of peptide solution were equilibrated. Octanol (5.5 ml) and buffer (0.5 ml) were transferred into counting vials and the radioactivity determined. The pH of the remaining aqueous phases was measured. Three independent experiments were performed for each peptide.

The temperature dependence of $P_{o/w}$ of these peptides was examined at pH 3 and 9 for AcAlaHisAlaNHtButyl and at pH 2 and 9 for AcAlaAspAlaNHtButyl and AcAlaGluAlaNHtButyl in the appropriate buffers as described above.

Calculation of log *P* Using the Fragment Method

Abraham and Leo used the fragment method to calculate the partition coefficients of the amino acids and their derivatives (8). The log *P* of the tripeptide backbone was calculated using the fragment values (*f*) and other factors (*F*) given by Abraham and Leo (8). Fragment constants for side chain (f_R) were those calculated by Abraham and Leo (8) according to the equation $f_R = \pi + f_H(0.23)$. They were 0.23, 0.55, 2.10, 2.11, and 1.18 for Gly, Ala, Phe, Trp, and Pro, respectively. The fragment constants of His, Asp, and

Glu differed depending on how the polar proximity effects between the peptide bonds and the side chains were calculated. When the polar proximity effect was calculated from the sum of the fragment constants of two peptide bonds (full polar proximity effect), the fragment constant of His was 0.48, and when the average of two peptide bonds was used it was 0.24. The fragment constants of protonated Asp and Glu obtained using the full polar proximity effect were 0.63 and 0.22, respectively, and those for ionized Asp and Glu were −2.32 and −3.37, respectively.

RESULTS AND DISCUSSION

Thermodynamics of Tripeptide Partitioning Between Octanol and Water

The free energy of transfer (ΔG) of the various tripeptides from aqueous to octanol phase was calculated using the equation, $\Delta G = -2.3\ RT \log P$, where P is the partition coefficient calculated using the ratio of the mole fraction of peptide in the octanol to that in the aqueous phase. $P_{o/w}$, the ratio of the molar concentration in octanol to that in the buffer phase, can be converted to P by multiplying the molar volume ratio of the two solvents so that $P = P_{o/w}\ V_{oct}/V_{water} = 8.748 \times P_{o/w}$ assuming that the molar volume ratio of the two solvents remains the same over the temperature range studied.

The temperature dependence of P of six tripeptides at pH 7.2 is illustrated in Fig. 1. The error bars are smaller than the symbols. A nonlinearity above 45°C is obvious in the data for Phe and Trp peptides. To rule out the possibility of chemical degradation of AcAlaPheAlaNHtButyl and AcAlaTrpAlaNHtButyl at high temperatures, the partition coefficients of these two peptides were measured at 65°C and an aliquot of the buffer phase was reequilibrated with fresh octanol at room temperature. For both peptides P at room temperature, following incubation at 65°C, was the same as P measured at room temperature without preexposure to 65°C. This suggests that the peptides were intact after the 1-day equilibration at 65°C.

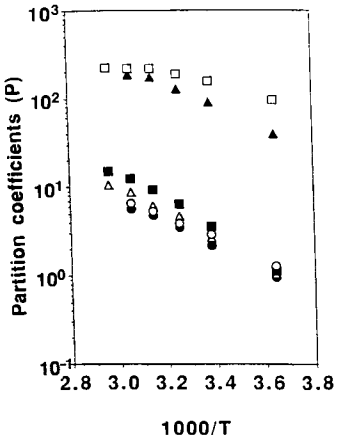

Fig. 1. Temperature dependence of partition coefficients (P) of tripeptides AcAla$X$AlaNHtButyl where X was Trp (□), Phe (▲), Pro (■), Ala (△), His (○), and Gly (●). P is the partition coefficient calculated from the ratio of the peptide mole fraction in the octanol versus the water phase at pH 7.2. Each symbol represents the mean of triplicate measurements and the standard deviation error bars are smaller than the symbols.

When the enthalpy (ΔH) and entropy (ΔS) of transfer are assumed to be temperature independent, the ΔH can be calculated using the linear van't Hoff relationship:

$$d \ln P/dT = \Delta H(T)/RT^2$$

When ΔH is independent of temperature,

$$d \ln P/d(1/T) = -\Delta H/R$$

Therefore, ΔH can be estimated from the slope of log P vs $1/T$. Once ΔG and ΔH are known, ΔS can be calculated from the relationship $\Delta G = \Delta H - T\Delta S$.

When curvature is evident in the van't Hoff plot, it has been diagnostic for a hydrophobic interaction (20). This indicates a heat capacity change (ΔC_p) and a temperature dependence of ΔH (20–24). The relationship between ΔG and T in this case is defined as follows (20–24):

$$\Delta G = \Delta H_o + \Delta C_p(T - T_o) - T\Delta S_o - T\Delta C_p \ln(T/T_o)$$

The second method of data analysis fits the parameters, ΔH_o, ΔS_o, and ΔC_p by a multiple linear fitting of ΔG and T in the above equation. The ΔH_o and ΔS_o are the enthalpy and entropy of transfer at the reference temperature, T_o (296 K).

The thermodynamic parameters obtained by the two methods are presented in Table I. The ΔH of transfer from water into octanol is positive for all the tripeptides listed in Table I, indicating that their transfer into octanol is entropy driven. The correlation coefficients were always better for the multiple linear fit than the linear van't Hoff fit. Standard deviations of ΔH and ΔS were less for the multiple linear fit than the linear fit for Ala, Phe, Trp, and Pro peptides. However, for the peptides containing Gly or His, the multiple linear fit to the data was not significantly better than the linear fit. For the AcAlaAspAlaNHtButyl (pH 2) and AcAlaGluAlaNHtButyl (pH 2), the linear van't Hoff fitting resulted in smaller standard deviations of ΔH and ΔS than the three-parameter fit.

The thermodynamic parameters obtained from the temperature dependence of partitioning may not be as precise as what one can obtain using a calorimetric method (25). However, in the absence of direct measurement of ΔC_p for the transfer of peptides from an aqueous to an organic solvent, these data are useful starting point for those interested in the transfer of amino acid side chains between phases such as occurs in protein denaturation and folding (20–25).

pH Dependence of $P_{o/w}$ of Charged Peptides

The pH dependence of $P_{o/w}$ was examined for AcAlaAspAlaNHtButyl, AcAlaGluAlaNHtButyl, and AcAlaHisAlaNHtButyl (Fig. 2). In these data $P_{o/w}$ is the apparent partition coefficient since no attempt was made to find the partition coefficient of the uncharged form. AcAlaAspAlaNHtButyl and AcAlaGluAlaNHtButyl are mostly ionized at pH 10 and uncharged at pH 2. AcAlaHisAlaNHtButyl is predominantly ionized at pH 3 and uncharged at pH 9. The $P_{o/w}$ values of uncharged and ionized peptides are summarized in Table II. As expected the $P_{o/w}$ becomes larger as the uncharged fraction increases. At all pH values, AcAlaGluAlaNHtButyl has a larger $P_{o/w}$ than

Table I. Thermodynamic Parameters of Transfer of Tripeptides from Water into Octanol

AcAla*X*AlaNHtButyl, *X* =	ΔG (kcal/mol)[a]	Fit[b]	ΔH [kcal/mol (SD)][c]	ΔC_p [cal/mol K (SD)][d]	ΔS [cal/mol K (SD)][e]	R^{2f}	SD of res.[g]
Gly	−0.46	lin	6.11 (0.33)	—	22 (1.1)	0.993	35
		mlt	6.32 (0.31)	−51 (34)	23 (1.1)	0.997	24
Ala	−0.59	lin	6.76 (0.25)	—	25 (0.9)	0.996	33
		mlt	7.17 (0.17)	−45 (12)	26 (0.6)	0.999	14
Phe	−2.65	lin	5.07 (0.39)	—	26 (1.3)	0.991	52
		mlt	5.74 (0.23)	−73 (17)	28 (0.8)	0.999	19
Trp	−2.99	lin	2.44 (0.41)	—	18 (1.3)	0.979	55
		mlt	3.19 (0.14)	−81 (10)	21 (0.5)	0.999	12
Pro	−0.75	lin	7.55 (0.46)	—	28 (0.5)	0.989	61
		mlt	8.36 (0.19)	−88 (14)	31 (0.5)	0.999	17
His (pH 7.2)	−0.63	lin	5.46 (0.23)	—	20 (0.7)	0.996	23
		mlt	5.61 (0.20)	−36 (22)	21 (0.7)	0.998	15
Asp (pH 2)	−0.27	lin	4.58 (0.27)	—	16 (0.9)	0.989	37
		mlt	4.82 (0.38)	−26 (28)	17 (1.3)	0.990	32
Glu (pH 2)	−0.36	lin	4.86 (0.27)	—	18 (1.3)	0.979	54
		mlt	5.31 (0.51)	−49 (38)	20 (1.7)	0.986	44
His (pH 9)	−0.66	lin	4.49 (0.30)	—	17 (1.0)	0.989	40
		mlt	4.92 (0.31)	−46 (23)	19 (1.4)	0.995	26

[a] Free energy of transfer at room temperature calculated from the partition coefficient P ($8.748 \times P_{o/w}$) according to $\Delta G = -RT \ln P$.
[b] Curve-fitting methods to estimate the thermodynamic parameters described under Results and Discussion. lin is the linear regression analysis using the van't Hoff equation $d \ln P/d(1/T) = -\Delta H/R$. mlt is using multiple linear regression analysis according to the equation $\Delta G = \Delta H_0 + \Delta C_p(T - T_0) - T\Delta S_0 - T\Delta C_p \ln(T/T_0)$.
[c] Enthalpy of transfer at room temperature. Standard deviations are shown in parentheses.
[d] Heat capacity change of transfer from water into octanol. Only the mlt method gives the estimates of ΔC_p. Standard deviations are shown in parentheses.
[e] Entropy of transfer at room temperature. Standard deviations are shown in parentheses.
[f] Correlation coefficients for each regression analysis.
[g] Standard deviation of residuals. The residuals are the difference between actual data points and the values estimated according to the regression curve.

does AcAlaAspAlaNHtButyl. This is due to the contribution of the additional methylene group in Glu.

The difference of log $P_{o/w}$ between the ionized and the uncharged forms (Δ log $P_{n/ion}$) for each peptide was calculated and included in Table II. For AcAlaHisAlaNHtButyl, AcAlaAspAlaNHtButyl, and AcAlaGluAlaNHtButyl, Δ log $P_{n/ion}$ was 1.57, 2.65, and 2.48, respectively. A significant difference was found for Δ log $P_{n/ion}$ between the tripeptides and the simple organic compounds. For aromatic amines Δ log $P_{n/ion}$ is 3.9, for aliphatic acids it is about 4.0, and for salicylic acid it is 3.1 (18). This discrepancy indicates that the effect of charge on the partition coefficient of tripeptides cannot be predicted from the behavior of simple organic molecules.

The contribution of one methylene group (π_{CH_2}) calculated by subtracting log P of AcAlaAspAlaNHtButyl from log P of AcAlaGluAlaNHtButyl differed depending on the pH. For the protonated peptides it was 0.067 and for charged peptides it was 0.24. The average of π_{CH_2} in hydrocarbons is 0.5 in hydrocarbons but is reduced when the CH_2 is between two very polar groups or the methylene chain folds back upon itself (18). The π_{CH_2} computed using Asp and Glu peptides is lower than that computed using a hydrocarbon chain. This is because the difference of log P between the two peptides is determined not only by one additional methylene in Glu but also by the difference in the polarity of the peptide bond and the carboxylate between AcAlaGluAlaNHtButyl and AcAlaAspAlaNHtButyl. The polar proximity effect between the peptide bond and the carboxylate in Glu is less than in Asp because of the longer distance between the polar groups in Glu. Therefore the peptide bond and carboxylate group in Glu are more polar than those in Asp. As a result, π_{CH_2} is less than the average values found in aliphatic compounds.

The difference of π_{CH_2} between the charged and the protonated forms is again due to the polar proximity effect. The effect propagates further in charged groups than uncharged polar groups. Therefore the polarity of these groups in Glu and Asp will be more similar in the charged form and the π_{CH_2} (0.24) in the charged form will appear to be greater than the π_{CH_2} (0.067) in the protonated form.

Ionizable peptides partition into the octanol phase in both the uncharged and the ionized forms. The $P_{o/w}$ of AcAlaGluAlaNHtButyl measured at pH 10 was orders of magnitude greater than $P_{o/w}$ calculated assuming that only the uncharged form can partition into octanol. $P_{o/w}$ can be expressed as

$$P_{o/w} = \frac{[HA]_o + [A^-]_o}{[HA]_w + [A^-]_w}$$

where [HA] is the protonated form and [A^-] is the ionized form of the peptide. If only HA can partition into the octanol phase, $P_{o/w}$ can be written as follows:

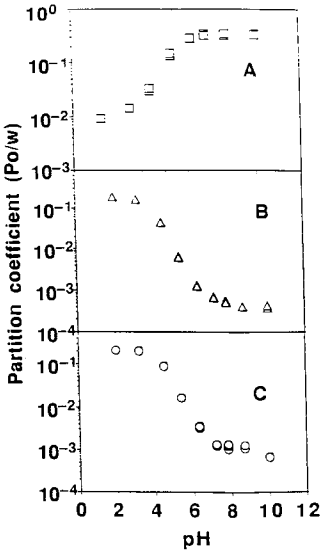

Fig. 2. pH dependence of partition coefficients ($P_{o/w}$) for peptides AcAlaHisAlaNHtButyl (A), AcAlaAspAlaNHtButyl (B), and AcAlaGluAlaNHtButyl (C). The partition coefficient was measured as described under Methods. Each symbol represents the data point from three independent measurements. The symbols overlap.

$$P_{o/w} = \frac{[HA]_o}{[HA]_w + [A^-]_w}$$

At pH 2.0 the Glu peptide (pK_a = 4.25, based upon the amino acid) is more than 99.7% protonated and the ratio $[HA]_o/[HA]_w$ is approximately the same as $P_{o/w}$. Therefore $[HA]_o/[HA]_w = 0.21$ and $[HA]_o = 0.21\ [HA]_w$. At pH 10, $[A^-]_w$ can be calculated using the Henderson–Hasselbach equation and $[A^-]_w = 5.6 \times 10^5\ [HA]_w$. Therefore $P_{o/w}$ at pH 10 will be

$$P_{o/w} = \frac{0.21[HA]_w}{[HA]_w + 5.6 \times 10^5[HA]_w} = 3.7 \times 10^{-7}$$

The measured value at pH 10 was 6.9×10^{-4}, i.e., three orders of magnitude greater than the calculated value. At this pH partitioning of the ionized peptides is the major contribution to the apparent partition coefficient.

The temperature dependence of $P_{o/w}$ of the ionizable peptides was measured at pH values where the peptides would be predominantly uncharged. The plot of P vs $1/T$ is shown in Fig. 3. The thermodynamic parameters of transfer of ionizable peptides are given in Table I. ΔH is positive for all cases and the ΔC_p values have a large standard deviation. When the ionized peptides are the dominant species, the measured partition coefficients were sensitive to the method used to separate the phases and the variability at elevated temperatures was high. Therefore curve fitting to obtain the thermodynamic parameters was not informative due to the absence of a data set over a sufficiently wide temperature range.

Calculation of log $P_{o/w}$ Using the Fragment Method

The apparent partition coefficients of the tripeptides can be computed using the fragment method of Abraham and Leo (8). Values of log $P_{o/w}$ computed in this fashion are presented in Table III. In most cases the computed values agree quite well with our experimentally measured log $P_{o/w}$ of peptides for Gly, Ala, Phe, and Trp peptides. The log $P_{o/w}$ of the tripeptides is determined mainly by the constituent groupings according to the group additivity rule. A large difference was found between the experimental and the calculated log $P_{o/w}$ of AcAlaProAlaNHtButyl. Even when the fragment constant of hydrogen, f_H (0.23), was added to account for the difference in the backbone structure, the difference between the experimental and the computed values was larger than for other nonpolar peptides. This indicates that the fragment method predicts a log $P_{o/w}$ for AcAlaProAlaNHtButyl that is inconsistent with our experimental measurements.

For peptides containing ionizable residues, His, Asp, and Glu, the calculation using the full polar proximity effect gave a better agreement between the experimental and the calculated log $P_{o/w}$ of the tripeptides. For AcAlaGluAlaNHtButyl, the experimentally obtained log $P_{o/w}$ at pH 10 is significantly greater than the calculated log $P_{o/w}$. This is because the fragment value for the side chain of Glu calculated

Table II. pH Dependence of Partition Coefficients ($P_{o/w}$) of Ionizable Peptides

AcAla*X*AlaNHtButyl, *X* =	$P_{o/w(n)}$ [a]	$P_{o/w(ion)}$ [b]	$\Delta\log P_{o/w(n/ion)}$ [c]
His	0.35	9.3×10^{-3}	1.57
Asp	0.18	4×10^{-4}	2.65
Glu	0.21	6.9×10^{-4}	2.48
π_{CH_2} [d]	0.067	0.24	

[a] The partition coefficients ($P_{o/w}$) of peptides at pH values where they are predominantly uncharged: pH 9 for His and pH 2 for Asp and Glu.

[b] The partition coefficients ($P_{o/w}$) of peptides at pH values where they are predominantly ionized: pH 3 for His and pH 10 for Asp and Glu.

[c] $\Delta\log P_{o/w(n/ion)} = \log P_{o/w(n)} - \log P_{o/w(ion)}$.

[d] π_{CH_2} is the difference between partition coefficients of Asp and Glu peptides in log units. $\pi_{CH_2} = \log P_{o/w(AcAlaGluAlaNHtButyl)} - \log P_{o/w(AcAlaAspAlaNHtButyl)}$.

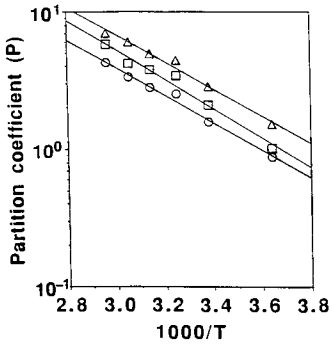

Fig. 3. Temperature dependence of the partition coefficients (P) of ionizable peptides, AcAlaHisAlaNHtButyl (△), AcAlaGluAlaNHtButyl (□), and AcAlaAspAlaNHtButyl (○) at a pH where the peptides are predominantly uncharged. Each symbol represents the mean of triplicate measurements and the standard deviation error bars are smaller than the symbols.

Table III. Comparison of Experimental and Calculated log $P_{o/w}$ of Tripeptides

AcAlaXAlaNHtButyl, X =	Gly	Ala	Phe	Trp	Pro
log $P_{o/w(expt)}$	−0.60	−0.51	1.01	1.25	−0.39
log $P_{o/w(calc)}$	−0.75	−0.43	1.12	1.13	0.20 (−0.03)[b]
Δlog $P_{o/w}$	0.15	−0.08	−0.11	0.12	−0.59 (−0.36)[b]
	His (pH 7)	Asp (pH 2)	Asp (pH 10)	Glu (pH 2)	Glu (pH 10)
log $P_{o/w(expt)}$	−0.48	−0.74	−3.40	−0.67	−3.15
log $P_{o/w(calc)}$	−0.74 (−0.50)	−0.98 (−0.35)	−3.93 (−3.3)	−1.0 (−0.76)	−4.59 (−4.35)
Δlog $P_{o/w}$[a]	0.26 (0.02)	0.24 (−0.39)	0.53 (−0.10)	0.33 (0.09)	1.44 (1.2)

[a] Δlog $P_{o/w}$ = log $P_{o/w(expt)}$ − log $P_{o/w(calc)}$, where P_{expt} is the measured octanol–water partition coefficient of the tripeptides and P_{calc} is the partition coefficient calculated using the fragment method as described in the method. The values in the parentheses are those calculated using full polar proximity effect for charged residues (8).

[b] log $P_{o/w}$ of Pro peptide was calculated from the equation log $P_{o/w}$ = −0.98 + f_R − f_H(0.23) to account for the difference in the backbone structure.

by Abraham and Leo is too low; according to their calculation (8), Glu is more hydrophilic than Asp. However, our and others' (7,11,13) experimental evidence indicates that Glu is more hydrophobic than Asp due to the additional methylene. The difficulty in predicting log $P_{o/w}$ of polar residues seems to be in assigning a polar proximity effect. Abraham and Leo added only a 10% proximity effect to obtain the log $P_{o/w}$ of the Glu residue because there are three carbons between the peptide bond and the carboxylate. For the Asp residue, however, where two carbons exist between peptide bond and carboxylate, a 26% proximity effect was added to obtain the log $P_{o/w}$. As a result, Glu is computed to be less hydrophobic than Asp, which is inconsistent with the experimental data. This points out a current limitation of such calculations as applied to peptides.

Hydrophobicity of Amino Acid Side Chains

The log $P_{o/w}$ of tripeptides can be used to derive the amino acid side chain hydrophobicity in a manner analogous to that of Fauchere and Pliska (7). The hydrophobicity value π of the central residue in AcAla*X*AlaNHtButyl is determined by subtracting log $P_{o/w}$ of AcAlaGlyAlaNHtButyl from log $P_{o/w}$ of the peptide; π = log $P_{o/w(AcAlaXAlaNHtButyl)}$ − log $P_{o/w(AcAlaGlyAlaNHtButyl)}$. A more general term to express the side-chain hydrophobicity is the contribution of residue X (ΔG_x) to the free energy of transfer of the peptides and can be calculated by the equation, $\Delta G_x = -RT2.3\,\pi$ or $\Delta G_x = \Delta G_{(AcAlaXAlaNHtButyl)} - \Delta G_{(AcAlaGlyAlaNHtButyl)}$, where the ΔG terms on the right-hand side are the free energies of transfer of tripeptides from water to octanol as summarized in Table I.

Comparison with Other Hydrophobicity Data

The hydrophobicity values of the residues examined in this study and those selected from various published data are presented in Table IV. Hydrophobicity data shown in the first section of Table IV are the original values reported as either π (7–9,19) or ΔG_x (2,27). The second section presents the data converted to the same unit, ΔG_x. Correlation coefficients between the results of the present study and others with or without Asp and Glu residues are included in the last two columns. The π values obtained from the present study as described in the preceding section are presented in the first row.

The relative order of hydrophobicity of nonpolar residues agreed well among the studies (Table IV). However, a detailed comparison between our data and the various studies reveals some significant differences. Differences are to be expected since the reference state, model compounds, choice of phases, and details of the computation varied from study to study. The correlation of our data to the F&P scale was 0.86 and improved when Asp and Glu were not included (the last column in Table IV). The difference between the hydrophobicity values of Asp and Glu obtained in the present study and those of Fauchere and Pliska (7) is more than 2 kcal/mol in terms of free energy of transfer. Abraham and Leo (8) suggested that the interaction between the terminal amide group and the carboxyl group in *N*-acetyl aspartyl amide or *N*-acetyl glutamyl amide might contribute to the increased hydrophobicity measured for Asp and Glu by Fauchere and Pliska. A similar interaction such as a hydrogen bond between the peptide backbone and the carboxylate group of Asp or Glu is sterically possible in the tripeptides containing Asp and Glu. Whether such an interaction is less favorable in tripeptides than in *N*-acetyl aspartyl amide or *N*-acetyl glutamyl amide is not known.

The hydrophobicity values of Ala, Phe, Trp, and Pro residues determined from the partitioning of tripeptides were less than those obtained from *N*-acetyl amino acid amides (7). The π_x value is a true measure of the hydrophobicity of the side chain X only when the model compounds are the same in all aspects except the substitution of hydrogen in the parent compound with the group of interest X. If the substituent group influences the conformation of the model compounds, the π value may reflect the influence of conformation on steric effects, hydrogen bonding, or electronic effects (18). In the case of nonpolar *N*-acetyl amino acid amides, conformation is unlikely to differ significantly among the derivatives. The tripeptide AcAlaGlyAlaNHtButyl, which served as the reference compound in this study, might form a folded structure more easily than other tripeptides due to the inherent flexibility of Gly residue. The first

Table IV. Comparison of Hydrophobicity Parameters from Tripeptides with Published Data

Peptides (AcAlaXAlaNHtButyl)	Gly	Ala	Phe	Trp	Pro	His	Asp	Glu	R1[o]	R2[p]
π (tripeptides)[a]	0	0.09	1.61	1.86	0.44	0.12	−2.57	−2.29	1.00	1.00
π (F&P)[b]	0	0.31	1.79	2.25	0.72	0.13	−0.77	−0.64	0.86	0.96
K&D (kcal/mol)[c]	−0.4	1.8	2.8	−0.9	−1.6	−3.2	−3.5	−3.5	0.45	0.45
ESG (kcal/mol)[d]	−1.0	−1.6	−3.7	−1.9	0.2	3.0	9.2	8.2	0.87	0.39
π (A&L)[e]	0	0.32	1.87	1.88	0.95	0.01 (0.25)	−3.18 (−2.55)	−3.84 (−3.60)	0.95	0.83
π (Roseman)[f]	0	0.39	2.27	2.13	—	−0.64	−3.81	−2.91	0.97	0.89
π (Akamatsu)[g]	0	0.32	1.95	1.92	0.86	—	—	—	0.92	—
ΔG_x (kcal/mol)[h]	0	−0.13	−2.19	−2.52	−0.29	−0.16	3.50	3.12		
ΔG_x (kcal/mol) (F&P)[i]	0	−0.42	−2.43	−3.06	−0.98	−0.18	1.05	0.87		
K&D (kcal/mol)[j]	0	−2.2	−3.2	0.5	1.2	2.8	3.1	3.1		
ESG (kcal/mol)[k]	0	−0.6	−2.7	−0.9	1.2	4.0	10.2	9.2		
A&L (kcal/mol)[l]	0	−0.43	−2.54	−2.56	−1.29	0.46	4.32	5.22		
Roseman (kcal/mol)[m]	0	−0.49	−3.09	−2.90	—	0.87	5.18	3.96		
Akamatsu (kcal/mol)[n]	0	−0.43	2.65	2.61	1.17	—	—	—		

[a] The hydrophobicity data obtained in this study from the octanol–water partition coefficients of tripeptides at pH 7.2; $\pi = \log P_{o/w(AcAlaXAlaNHtButyl)} - \log P_{o/w(AcAlaGlyAlaNHtButyl)}$.

[b] Data of Fauchere and Pliska (7) determined from the octanol–water partition coefficients of *N*-acetyl amino acid amides; $\pi = \log P_{o/w(N\text{-acetyl amino acid amide})} - \log P_{o/w(N\text{-acetyl glycyl amide})}$.

[c] Kyte and Doolittle's hydropathy scale (27).

[d] Engelman and co-worker's hydrophobicity scale (2).

[e] The π values calculated by Abraham and Leo (8) using the fragment method. The values in parentheses are those calculated using full polar proximity effect.

[f] The π values calculated by Roseman (9) using the fragment method.

[g] The π values calculated by Akamatsu and co-workers (19) using the fragment method.

[h] The free energy of transfer of side chains from water into octanol was calculated according to the equation, $\Delta G_x = -2.3\,RT\,\pi$ using the π values of this study.

[i] The free energy of transfer of side chains from water into octanol was calculated according to the equation, $\Delta G_x = -2.3\,RT\,\pi$ using the π values of Fauchere and Pliska (7).

[j] K&D data were scaled to set value for Gly to be 0. The sign was changed to be consistent with other data.

[k] ESG data were scaled to set value for Gly to be 0.

[l] The free energy of transfer of side chains from water into octanol was calculated according to the equation, $\Delta G_x = -2.3\,RT\,\pi$ using the π values of Abraham and Leo (8). For His, Asp, and Glu, π values obtained using the full polar proximity effect were used.

[m] The free energy of transfer of side chains from water into octanol was calculated according to the equation, $\Delta G_x = -2.3\,RT\,\pi$ using the π values of Roseman (9).

[n] The free energy of transfer of side chains from water into octanol was calculated according to the equation, $\Delta G_x = -2.3\,RT\,\pi$ using the π values of Akamatsu and co-workers (19).

[o] Correlation coefficient between the indicated data and the π values obtained in this study from the octanol–water partition coefficients of tripeptides.

[p] Correlation coefficients between the indicated data and the present result with data for Asp and Glu omitted.

and the last peptide bonds in the folded tripeptide could then participate in a hydrogen bond, which would increase the log *P* of the peptide AcAlaGlyAlaNHtButyl. Hence the π for other residues would be reduced, resulting in the discrepancy observed for the π values for nonpolar residues between the present study and Fauchere and Pliska (7). Although we did not examine the peptide conformation in this work, NMR studies of tetrapeptide (26) Gly–Gly–*X*–Ala in water indicated that the tetrapeptides adopted a random coil structure regardless of the nature of residue *X*. So we think it is unlikely that the folded conformation of AcAlaGlyAlaNHtButyl is a major factor in the discrepancy in the π values between our values and those of Fauchere and Pliska.

NMR indicates that the bulky side chains are located near the peptide backbone rather than directed away from the peptide backbone into the solvent. The nonrandom spatial arrangement of the bulky hydrophobic residues was attributed to intramolecular short-range interactions independent of chain length (26). The nonpolar side chains of tripeptides used in the present study would prefer to decrease contact with water similar to the tetrapeptides discussed above. An intramolecular hydrophobic interaction between adjacent nonpolar residues would stabilize the peptide in water and this would be reflected as lower partitioning into octanol. Therefore the apparent hydrophobicity of individual residues in tripeptides calculated from the difference of log *P* between AcAla*X*AlaNHtButyl and AcAlaGlyAlaNHtButyl would be lower than π values obtained from *N*-acetyl amino acid derivatives. Lower π values due to the folding or intramolecular hydrophobic interaction have been reported for other organic compounds (18).

The experimental data of log $P_{o/w}$ of tripeptides reported by Akamatsu and coworkers (19) also suggest that the hydrophobic contribution of bulky side chains in tripeptides appear to be less than that in *N*-acetyl amino acid amide derivatives. The side-chain contribution of residues in

Table V. Comparison of Hydrophobicity Contribution of Side Chains in Tripeptides and *N*-Acetyl Amino Acid Amides

Ala	$\log P_{o/w(Phe\text{-}Val\text{-}Ala)} - \log P_{o/w(Phe\text{-}Val\text{-}Gly)} = 0.14^a$
	$\log P_{o/w(AcAlaAlaAlaNHtButyl)} - \log P_{o/w(AcAlaGlyAlaNHtButyl)} = 0.09^b$
	$\log P_{o/w(N\text{-}Ac\text{-}Ala\text{-}amide)} - \log P_{o/w(N\text{-}Ac\text{-}Gly\text{-}amide)} = 0.31^c$
Phe	$\log P_{o/w(Phe\text{-}Phe\text{-}Phe)} - \log P_{o/w(Gly\text{-}Phe\text{-}Phe)} = 1.31^a$
	$\log P_{o/w(AcAlaPheAlaNHtButyl)} - \log P_{o/w(AcAlaGlyAlaNHtButyl)} = 1.61^b$
	$\log P_{o/w(N\text{-}Ac\text{-}Phe\text{-}amide)} - \log P_{o/w(N\text{-}Ac\text{-}Gly\text{-}amide)} = 1.79^c$
$CHCH_3$	$\log P_{o/w(Leu\text{-}Val\text{-}Leu)} - \log P_{o/w(Leu\text{-}Ala\text{-}Leu)} = 0.46^a$
	$\log P_{o/w(N\text{-}Ac\text{-}Val\text{-}amide)} - \log P_{o/w(N\text{-}Ac\text{-}Ala\text{-}amide)} = 0.91^c$

[a] Partition coefficients ($P_{o/w}$) of tripeptides measured by Akamatsu and co-workers (19).

[b] Partition coefficients ($P_{o/w}$) of tripeptides measured in the present study.

[c] Partition coefficients ($P_{o/w}$) of *N*-acetyl amino acid derivatives measured by Fauchere and Pliska (7).

tripeptides is compared to the corresponding *N*-acetyl amino acid amide data (Table V). The difference of log $P_{o/w}$ between Phe–Val–Gly and Phe–Val–Ala was only 0.14, compared to 0.31 in the *N*-acetyl amino acid amide. The difference of log $P_{o/w}$ between Phe–Phe–Phe and Gly–Phe–Phe was 1.31, compared to 1.79 in the *N*-acetyl amino acid amide. The difference between Val and Ala in tripeptides was only half that in *N*-acetyl amino acid amides. The di- and tripeptides used by Akamatsu and co-workers were composed of rather bulky side chains such as Val, Leu, Phe, or Trp. It is likely that intramolecular interactions between adjacent residues would decrease the total hydrophobic surface area available to water molecules and decrease the apparent hydrophobicity of individual residues.

Our results and those of Akamatsu and co-workers (19) support the idea that the apparent hydrophobicity of side chains in peptides is lower than that determined using *N*-acetyl amino acid amides due to the interaction between adjacent residues in the peptide sequence. Therefore the π values obtained using peptides should represent a better estimate of side chains hydrophobicity in peptides. The magnitude of the intramolecular hydrophobic interaction in peptides would depend upon the nature of the residues involved. For the blocked tripeptides, both the tButyl group and the Ala residues could be involved in such hydrophobic interaction. The interaction with the tButyl group might be similar to that of a valine due to their similar size. If the π values were measured using peptides of the sequence Gly–*X*–Gly instead of AcAla*X*AlaNHtButyl, they might be a little greater than the values obtained in this study. Whereas if Leu–*X*–Leu were used, π values might be smaller than the present results.

Two major findings from the above comparison can be summarized as follows. (i) The hydrophobicity of nonpolar side chains in a peptide structure is less than in amino acid analogues. This is attributed to intramolecular hydrophobic interactions. (ii) There are other discrepancies which cannot be explained by intramolecular interactions. The differences between our data and the widely used Kyte and Doolittle scale (27,28) or Engelman and co-workers' scale (2) are about 1 kcal/mol for Pro, 2 kcal/mol for Trp, and 3 kcal/mol for His. For Asp and Glu, the difference between our data and those of Fauchere and Pliska was 2 kcal/mol and it was about 6 kcal/mol when compared to Engelman and co-workers' value.

A 2-kcal/mol difference in side-chain hydrophobicity results in a 600-fold difference in octanol–water partition coefficients (P) between the Gly- and the Trp-containing tripeptides. Its effect is also clearly evident in the measured partition coefficient of the tripeptides into dimyristoylphosphatidylcholine liposomes. The measured partition coefficients of AcAlaPheAlaNHtButyl and AcAlaTrpAlaNHtButyl into dimyristoylphosphatidylcholine liposomes were 80 and 400, respectively, while partitioning of the other tripeptides was not detectable (unpublished data). If the values of Kyte and Doolittle for Gly and Trp are used to predict the partition coefficients (Table IV), one would not expect such a large difference. The impact of using inappropriate hydrophobicity values for residues will of course depend upon the length and composition of the peptides. However, in a short peptide or when a large fraction of the residues is assigned inappropriate hydrophobicities, the final prediction could be considerably misleading.

Computational Methods to Establish Amino Acid Hydrophobicities

An alternative approach to establishing amino acid hydrophobicities is to divide or fragment the side chain into chemical groups and to sum the hydrophobic contribution from each group to obtain a π value for the side chain. The amino acid hydrophobicity calculated using the fragment method by three independent workers are listed in Table IV. The π values for Ala and other aliphatic side chains (not shown in Table IV) do not show significant difference. However, π values of the aromatic side chains differ depending upon the analogues or fragments chosen by each author. Histidine, aspartic acid, and glutamic acid also showed large differences between the A&L (8) and Roseman (9) scales. Roseman calculated the π values for protonated Asp (-0.71) and Glu (-0.18) from the log $P_{o/w}$ of protonated acetic acid and propionic acid, respectively. The π values obtained in our study for Asp and Glu at pH 2 were -0.14 and -0.08, respectively. Roseman calculated the π values of Asp and Glu at pH 7 assuming that the difference between the ionized and the protonated form is 4.06, which is the average value for an aliphatic acid. However, our data on the pH dependence of log $P_{o/w}$ of tripeptides containing Asp or Glu showed that the difference of log $P_{o/w}$ between ionized and protonated forms was about 2.6. As a result the difference between our data and the Roseman π values at pH 7 was larger than for the protonated forms. Abraham and Leo (8) calculated the hydrophobicity of these residues by summing the contribution of each fragment and correcting for the polar proximity effect. In their π values, aspartic acid is more hydrophobic than glutamic acid. As discussed above, this is due to the difficulty in assigning an appropriate value for the polar proximity effect.

Akamatsu and colleagues used a number of different equations to predict the log $P_{o/w}$. Equation (7) in their paper include the sum of π values of Fauchere and Pliska, steric parameters, and the correction factors for each residues: log

$P' = 1.067\ \Sigma\pi + 0.647\ E_s^{c'}(R_N) + 0.454\ E_s^{c'}(R_M) + 0.322\ E_s^{c'}(R_C) + 0.345\ I_Y - 0.159\ I_W + 0.231\ (I_s + I_T) - 4.744$. The exact coefficients for the steric parameter and the correction factor for each residue depended upon the π values used. The equation for the prediction of log P using π values of Akamatsu and co-workers is shown in Table VI. The log $P_{o/w}$ values of tripeptides experimentally determined and those calculated by Akamatsu's equation with or without steric effect (log $P_{o/w} = \Sigma\pi_i - 4.7$) are shown in Table VI. The $\Sigma\pi_i$ represents the sum of contributions of the side chains; therefore -4.7 can be regarded as the log $P_{o/w}$ of the tripeptide backbone alone. The last three columns show that calculated log $P_{o/w}$ values differed significantly depending upon the π values used. The log $P_{o/w}$ values calculated using the π values obtained in this study (the last column in Table VI) were as good as the log $P_{o/w}$ obtained by Akamatsu using the steric parameter and the correction factor in addition to $\Sigma\pi$.

In summary, the fragment method is useful to predict the log P of compounds where complicated intramolecular interaction are absent. In peptides or aromatic or ionizable compounds intramolecular interactions and the polarity of the compound may present obstacles to the accurate prediction of ΔG of transfer.

CONCLUSIONS

Although there was a good correlation in the relative order, the hydrophobicity values of amino acid side chains in tripeptides were different from those measured in *N*-acetyl amino acid amides (7) or calculated using the fragment method (8,9,19). Nonpolar amino acids such as Ala, Phe, Trp, and Pro had a lower apparent hydrophobicity in the tripeptide than as *N*-acetyl amino acid amides. In addition, the temperature dependence of transfer was best described by the inclusion of a heat capacity term for the nonpolar residues. The ionizable residues Asp and Glu in the tripeptide were about 2 kcal more hydrophilic than as the *N*-acetyl amino acid amide (7). The π values obtained in the present study using blocked tripeptides should represent a better estimate of the hydrophobicity of amino acid side chains in peptides than previous studies because they incorporate interactions between adjacent residues.

Based upon these studies, we propose that the free energy of transfer from water to octanol for the eight amino acid side chains at pH 7.2 are 0, -0.13, -2.19, -2.52, -0.29, -0.16, 3.50, and 3.12 kcal/mol for Gly, Ala, Phe, Trp, Pro, His, Asp, and Glu, respectively.

Table VI. Partition Coefficients ($P_{o/w}$) of Tripeptides

Peptides	log P_{expt} [a]	log P_{calc} [b]	log P_{calc} [c]	log P_{calc} [d]	log P_{calc} [e]
Gly–Phe–Phe	−1.33	−1.34	−0.84	−1.16	−1.52
Phe–Phe–Phe	−0.02	0.03	1.11	0.63	−0.09
Trp–Gly–Gly	−2.72	−2.83	−2.78	−2.45	−2.84
Trp–Phe–Ala	−1.00	−1.01	−0.55	−0.39	−1.18
Phe–Val–Ala	−2.19	−2.14	−1.20	−1.42	−1.82
Tyr–Gly–Phe	−1.86	−2.09	−1.59	−1.99	—

[a] Partition coefficients ($P_{o/w}$) experimentally measured by Akamatsu and co-workers (19).

[b] log $P_{o/w}$ estimated from Eq. 6 of Akamatsu and co-workers; log $P' = 0.960\ \Sigma\pi + 0.561\ E_s^{c'}(R_N) + 0.338\ E_s^{c'}(R_M) + 0.255\ E_s^{c'}(R_C) + 0.164\ I_Y + 0.351\ I_W + 0.637\ I_M + 1.666\ (I_s + I_T) - 4.788$.

[c] log $P_{o/w}$ calculated from the equation log $P_{o/w} = \Sigma\pi - 4.7$ using the π values of Akamatsu and co-workers (19).

[d] log $P_{o/w}$ calculated from the equation log $P_{o/w} = \Sigma\pi - 4.7$ using the π values of Fauchere and Pliska (7).

[e] log $P_{o/w}$ calculated from the equation log $P_{o/w} = \Sigma\pi - 4.7$ using the π values obtained in this study.

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