Guanidinium Protonation Equilibria of L-Canavanine in Different Ionic Media

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The guanidinium protonation constant pK_{a2} of L-canavanine has been determined in aqueous solution at different temperatures and different ionic strengths, using a potentiometry technique. The effect of an organic solvent added to the aqueous solution on the protonation processes is also reported and explained. The organic solvents used were methanol, dimethylformamide (DMF), dimethylsulfoxide (DMSO), acetone, and dioxane. The pK_{a2} values for the ionization in a water (1) + dioxane (2) mixture have been determined at four different temperatures from T = (298.15 to 328.15) K. Finally, the thermodynamic quantities ($\Delta H°$ and $\Delta S°$) associated with this protonation process were calculated and discussed.

Introduction

L-(+)-(*S*)-Canavanine, the δ-oxa analogue of the proteinogenic amino acid (L-arginine), is the 2-amino-4-(guanidinooxy) butyric nonproteinogenic amino acid present in various beans, clovers, onions, seeds and sprouts of alfalfa, and certain leguminous plants.[1] L-Canavanine works as a potent antagonist that exhibits antimetabolic activity in many living systems in vitro and in vivo.[2−4] Also, L-canavanine can compete with L-arginine when cellular enzymes such as arginyl tRNA synthetase, induced nitric oxide synthase, and arginase target free arginine.[2−4] More importantly, L-canavanine is a substrate for arginyl tRNA synthetase in which L-canavanine can be charged by arginyl tRNA synthetase to replace L-arginine during protein synthesis, creating aberrant canavanyl proteins.[5] The known functions of arginyl residues in enzymes and in other proteins depend on the positive charge of its side chain.[4] The guanidinium cation, whose normal pK_a value is ca. 12.5, exists throughout the usual pH range of protein stability.[4] It participates in recognition of anionic substrates, binds cofactors, forms internal hydrogen bonds and salt bridges, and enhances canavanyl protein hydrophilicity.[4−6] L-Canavanine substitutions disrupt the tertiary and/or quaternary structure that is responsible for the three-dimensional conformation unique to the protein.[4−6]

The study of protonation and solvation processes in solutions of L-canavanine is important to determine the connection between its chemical structure and biological activity,[7,8] as polarity and activity of water are expected to be lower in an active site cavity of an enzyme than in bulk water. In this work, the protonation processes of L-canavanine were examined in water containing different organic solvents, from which the thermodynamic data obtained would be useful to researchers in biomedicine.[7,8] Thus, it is worthwhile to study systematically amino acids, peptides, and proteins in solvents having a different number of hydroxyl groups. These studies may shed some light on the mechanism about how organic solvents affect the stability of proteins. Recently, the acid base behavior of the essential 20 amino acids, 10 hydroxamic acids, 10 phenolic compounds, and non-protein L-norvaline amino acid in aqueous solution and in different solvent mixtures has been studied by us.[9−12] The literature data show that there are no reports available on the determination of protonation constants of L-canavanine in aqueous−organic solvent mixtures. Due to the presence of the hydroxyl group, L-canavanine has high hydrogen-bonding capability. Thus, solvent effects on the ionization of L-canavanine in various (water + organic solvent) systems are of particular interest, especially for specific (solute + solvent) interactions. The present work reports thermodynamics of the protonation equilibria of L-canavanine in water at different ionic strengths and different solvent mixtures.

Experimental Section

Materials and Solutions. L-Canavanine of analytical grade (Sigma-Aldrish, USA) was used without further purification. This L-canavanine material was assayed in triplicate by titration with a carbonate-free solution of standard NaOH. This assay showed that the mass fraction purity of the L-canavanine was (0.99 ± 0.05). The organic solvents, methanol, DMF, DMSO, acetone, and dioxane, were of high purity (A.R. or spectro grade products). A carbonate-free sodium hydroxide solution was prepared by dissolving the Analar pellets in ultra pure water, and the solution was standardized potentiometrically with potassium hydrogen phthalate (Merck, AG). Nitric acid, sodium hydroxide, and sodium nitrate were from Merck p.a.

Apparatus. pH-potentiometric titrations were performed using a Metrohm 796 titroprocessor with a 685 dosimate, a 728 magnetic stirrer, coupled with a dosino buret model 700. The precision of the instrument was ± 0.001 pH units. pH titrations were carried out in an 80 cm^3 commercial double-walled glass vessel. The ionic strength of the solutions was maintained at a constant level by using the desired concentration of $NaNO_3$ solution as supporting electrolyte, and the temperature was adjusted inside the cell at the desired value by circulating thermostatted water using an oil-thermostatted setup. During the course of the titrations, a stream of oxygen-free nitrogen was passed through the reaction cell to eliminate the adverse effect of atmospheric carbon dioxide.

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10.1021/je100292g
Published on Web 07/27/2010

Calibration of Glass Electrode Cell. A computer program (GLEE, glass electrode evaluation)[13,14] has been used for the calibration of the glass electrode by means of a strong acid−strong base titration. This program provided an estimate of the carbonate contamination of the base, the pseudo-Nernstian standard potential, and slope of the electrode and, optionally, the concentration of the base and pK_W. It uses a (nonlinear) least-squares refinement to fit a modified Nernst eq 1

$$E = E^0 + s \log[H^+] \quad (1)$$

where E is a measured electrode potential; E^0 and s are parameters of the refinement and represent the standard electrode potential and slope; and $[H^+]$ represents the hydrogen ion concentration.

In acid solutions, the hydrogen ion concentration is obtained from the mineral acid concentration, T_H, as calculated from eq 2, that is, $\lg[H^+] = \lg(T_H)$.

$$T_H = \frac{a_H v_0 + \gamma b_H v}{v_0 + v_1 + v} \quad (2)$$

where a_H is the concentration in mol·dm^{-3} of acid of which v_0 cm^3 were added to the titration vessel; b_H is the concentration in mol·dm^{-3} of base in the buret (by convention given a negative sign); v_1 is the volume in cm^3 of background electrolyte solution added to the titration vessel; and v in cm^3 is the volume of base added from the buret. γ is a correction factor for the base concentration: if γ is refined, the calculated base concentration is γb_H.

In alkaline solutions, the effective concentration of the base is usually reduced by the presence of a small amount (preferably < 1 %) of carbonate contamination. The extent of this contamination can be estimated by means of a Gran plot.[13,14] Initially, E^0 is estimated from the acid region,[13,14] and s is taken as the ideal Nernstian slope (T/5.0399 mV). Then eqs 3 and 4 are fitted by linear least-squares.

Acid region:

$$(v_0 + v_1 + v)10^{E-E^0/s} = m^a v + c^a \quad (3)$$

Alkaline region:

$$(v_0 + v_1 + v)10^{(E^0-E/s)-pK_a} = m^b v + c^b \quad (4)$$

A typical Gran plot is designed. From the slopes and intercepts of the fitted lines, two estimates are obtained of the volume of base consumed at the equivalence point $v_e^a = -c^a/m^a$ from the acid region and $v_e^b = -c^b/m^b$ from the alkaline region. Assuming that the difference is due to carbonate, the effective base concentration is reduced by the factor v_e^a/v_e^b in the alkaline region. The mineral acid concentration in the alkaline region is then given by eq 5.

$$T_H = \frac{a_H v_0 + \gamma \frac{v_e^a}{v_e^a} b_H v}{v_0 + v_1 + v} \quad (5)$$

T_H is negative, and $\log[H^+] = -pK_W - \log(-T_H)$.

With these estimates of $\log[H^+]$, the standard potential and slope can be obtained by least-squares fitting of eq 1. The whole process, including the Gran plot, is repeated with the refined values of E^0 and s.

Briefly, the standardization of the electrode system was carried out, each time in organic solvent−water mixtures studied by Gran's method.[13,14] An estimated amount of solution, at the same conditions of temperature, ionic strength, and solvent composition, was placed in a double-walled, thermostatted vessel. The potential was allowed to stabilize after each addition of acid or base, and then the value was used to obtain the standard potential of the cell, E^0. The electrode was immersed in background solution, and it was titrated with a strong base in the same experimental conditions of ionic strength and solvent composition. Usually, about 10 or 12 additions are enough to verify E^0 is accurately determined, which provides change of initial pH of the background solution from pH∼ which is a value about two units lower than the pK_a of the compounds studied. In a second step, a suitable amount of L-canavanine was added to the pretitrated background solution. From the pair of potential values and volume added, pK_a values were calculated using the ESAB[15,16] and PKPOT programs.[17] These programs allow the thermodynamic acid−base constants in aqueous and nonaqueous media to be determined, taking into account the activity coefficient of the species.

Procedure for Equilibrium Titration. The following solutions were prepared (total volume of 50 cm^3) and titrated potentiometrically against a standard carbonate-free NaOH (0.1 mol·dm^{-3}) solution:

(a) 0.003 mol·dm^{-3} HNO_3 + 0.1 mol·dm^{-3} $NaNO_3$.

(b) Solution a + 0.001 mol·dm^{-3} L-canavanine.

The pH-metric titrations were carried out at the desired temperature in a purified nitrogen atmosphere. The temperature was controlled by circulation of water through the jacket, from the ultrathermostat bath, and maintained within ± 0.1 K.

All the test solutions contained an appropriate proportion (w/w) of the different organic solvents studied. The total volume was adjusted to 50 cm^3 by adding double-distilled water. At each solvent percentage, at least four titrations were performed under carefully controlled experimental conditions. Typically, more than 60 pH readings (points of potentiometric measurements) were collected and taken into account for each titration.

Calculations. To account for the differences in acidity, basicity, dielectric constant, and ion activities for partially aqueous solutions relative to pure aqueous ones, pH values of the former solutions were corrected by making use of the procedure described by Douheret.[18,19] The pK_a values were calculated adopting the Irving and Rossotti technique as described.[20,21]

Computations related to the estimation of dissociation constants were performed by regression analysis of titration curves using the least-squares computer ESAB[15,16] and PKPOT programs.[17] The least-squares computer program PKPOT has been developed to run on a PC-compatible computer, for the study of ionic equilibria from potentiometric data. It allows for the refinement of equilibrium constants in systems described by up to 5 components and 20 complex species $A_pB_qC_rD_sH_t$. Standard potentials of electrode and reactant concentrations were refined. The program can deal with up to ten titration curves. It provides several statistical tests, as was as graphics presentations of data and residuals distribution. The program allows for the determination of stoichiometric formation constants (at fixed ionic strength) or thermodynamic constants. The data analyzed were given

Scheme 1. Chemical Structure of L-Canavanine and Its Protonation Equilibria

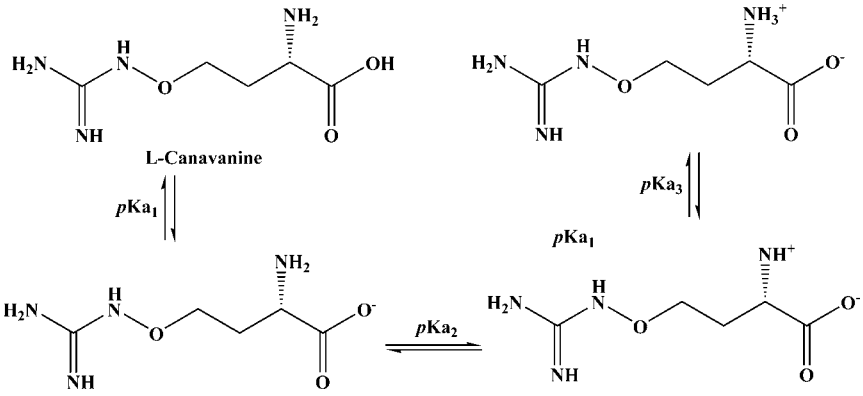

Table 1. Protonation Constants (pK_{a2}) of L-Canavanine at Different Ionic Strengths (I/mol·dm^{-3}) and Different Temperatures (T/K)

I	pK_{a2}			
mol·dm^{-3}	T/K = 298.15	T/K = 308.15	T/K = 318.15	T/K = 328.15
0	7.030 ± 0.002	6.961 ± 0.007	6.758 ± 0.007	6.567 ± 0.003
0.1	7.010 ± 0.007	6.943 ± 0.008	6.703 ± 0.003	6.512 ± 0.006
0.15	7.008 ± 0.003	6.864 ± 0.003	6.632 ± 0.005	6.423 ± 0.007
0.2	6.897 ± 0.005	6.731 ± 0.007	6.512 ± 0.007	6.384 ± 0.003
0.25	6.542 ± 0.007	6.516 ± 0.007	6.397 ± 0.008	6.196 ± 0.006

as volume/emf or volume/pH for several application examples including titrations in aqueous and nonaqueous media.

The adequacy of a proposed regression chemical model with experimental data and the reliability of the parameter pK_a can be estimated and examined by the goodness-of-fit test.[14]

The pK_{a2} values were calculated from the relationship[20,21]

$$\bar{n}_H = \beta[H^+](1 + \beta[H^+])^{-1} \quad (6)$$

where β is the proton-L-canavanine formation constant and $\bar{n}_H$ is the average number of protons associated per mole of L-canavanine at several pH values. The following equation[20,21] was used for calculation of the $\bar{n}_H$ values from the titration curves corresponding to solutions a and b.

$$\bar{n}_H = \left\{ yC_L + \frac{(V_a - V_b)C_b}{V_0} \right\}(C_L) - 1 \quad (7)$$

where y is the number of dissociable protons ($y = 1$ in the case of L-canavanine). V_a and V_b are the volumes of NaOH consumed to reach the same pH values in titration curves a and b,

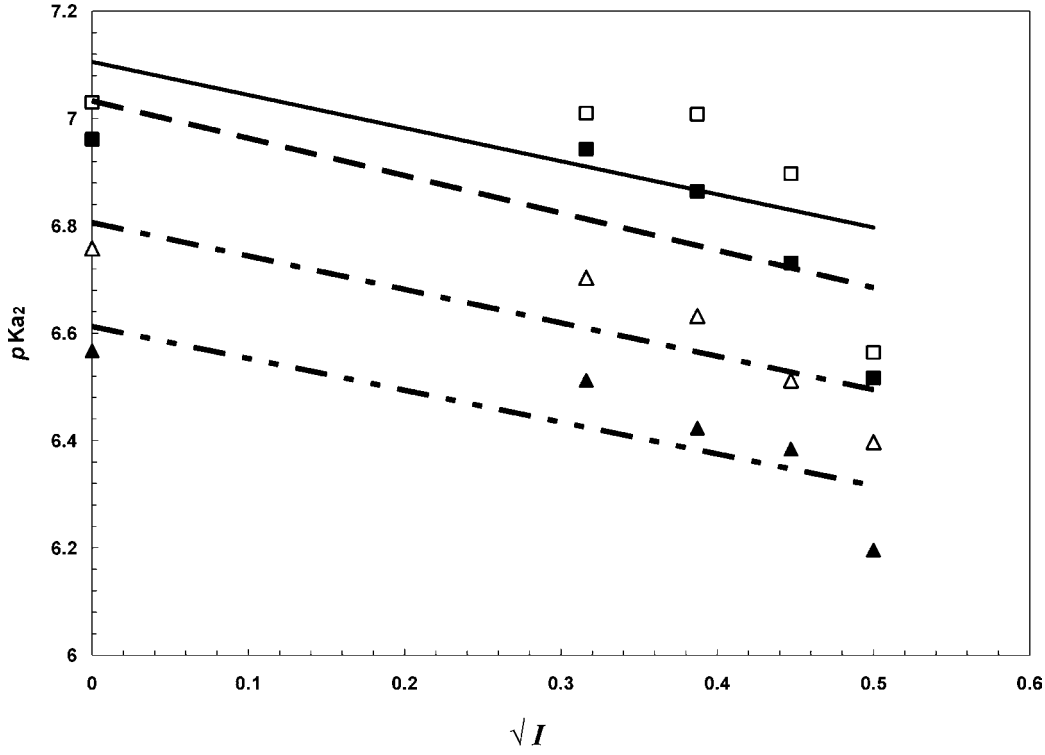

Figure 1. Plot of experimental (symbols) and correlated pK_{a2} (lines) values of L-canavanine versus $\sqrt{I}$/mol·dm^{-3} at different temperatures: □, — —, 298.15 K; ■, - - -, 308.15 K; Δ, ——, 318.15 K; ▲, — - —, 328.15 K.

Table 2. Thermodynamic Quantities (Enthalpy, ΔH°, and Entropy, ΔS°, Changes) for the Protonation Equilibria of L-Canavanine at Different Ionic Strengths (I/mol·dm^{-3})

I	ΔH^o	$-\Delta S^o$
mol·dm^{-3}	kJ·mol^{-1}	J·mol^{-1}·K^{-1}
0	12.878 ± 0.044	15.600 ± 0.078
0.1	14.031 ± 0.092	11.606 ± 0.113
0.15	16.118 ± 0.12	4.4297 ± 0.09
0.2	14.310 ± 0.10	9.374 ± 0.124
0.25	9.8629 ± 0.09	21.826 ± 0.142

respectively. C_b and C_L are the concentration of NaOH and L-canavanine, respectively, and V_0 is the original volume (50 cm^3).

Thermodynamic quantities (ΔH^o and ΔS^o) associated with the protonation equilibria of L-canavanine were evaluated from the temperature dependence of the protonation constants by applying a linear least-squares analysis according to the van't Hoff equation

$$pK_{a_2} = \frac{\Delta H^o}{RT} - \frac{\Delta S}{R} \quad (8)$$

In rigorous thermodynamic calculations, the equilibrium constant (β_t) should be expressed in terms of activities of the component ions at equilibrium. However, for convenience, concentrations are generally used, where, for a particular species i, $a_i = C_iF_i$, where C_i is the concentration of the ion i, a_i its activity, and F_i its activity coefficient.

Results and Discussion

Certain functional groups found in biological molecules, in particular, carboxylic acids or amino groups, can gain or lose H^+ depending on the availability of hydrogen ions (or protons) in the solution. It is worth mentioning that the pK_{a1} value of L-canavanine investigated can be associated with carboxylic acid function. This value is low ($\leq$ 2.40), exists in acidic solutions, and is not important in the physiological region and hence is not of interest. Therefore, this value is not used in our calculations since the pH-metric data are measured in the range $2.5 \leq$ pH ≤ 11. From the chemical structures shown in Scheme 1 and the second proton dissociation constant (pK_{a2}) of L-canavanine calculated potentiometrically and listed in Table 1, one can conclude that protonation equilibrium constants are controlled by the electronic effects of substituent groups.

The second proton dissociation constant (pK_{a2}) value of L-canavanine obtained was found to be 7.03 (Table 1), at $T =$ 298.15 K and $I = 0$ mol·dm^{-3} $NaNO_3$, and a comparison with those of other workers in aqueous medium shows that the result agrees within a very reasonable range.[22] From the experimental data shown in Table 1, it was noted that the pK_{a2} values decreased when the ionic strength medium increased. The plot of correlated pK_{a2} versus $\sqrt{I}$ is shown in Figure 1, and this is in full agreement with the Debye−Hückel equation.[23]

The thermodynamic quantities (ΔH^o and ΔS^o) associated with the dissociation of L-canavanine were calculated at each ionic strength, and the values given in Table 2 indicate that the dissociation processes are temperature dependent. It was observed that the positive values of enthalpy changes (ΔH^o)

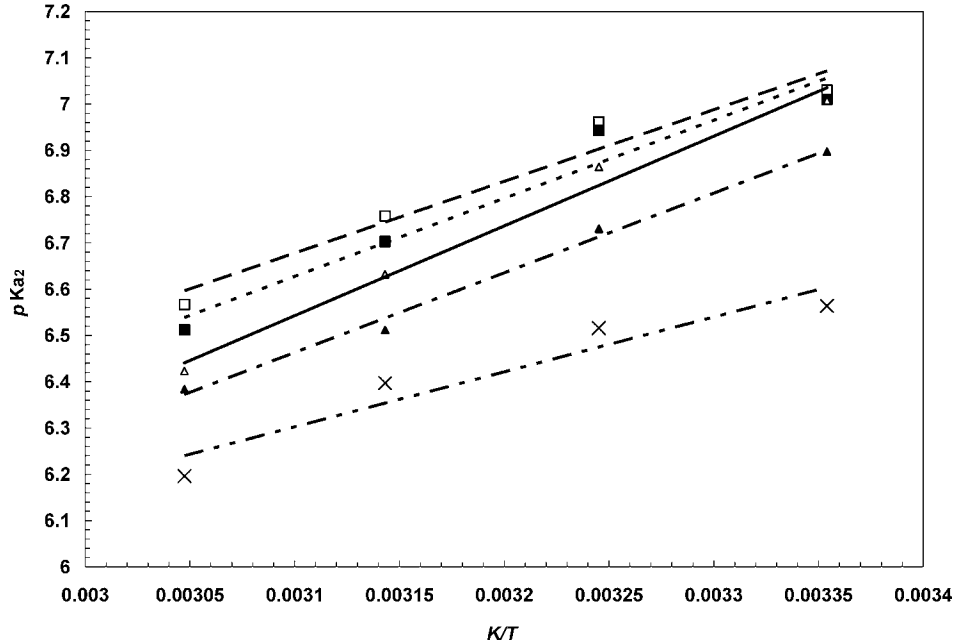

Figure 2. Plot of experimental (symbols) and correlated pK_{a2} (lines) values of L-canavanine versus $1/T$/K^{-1} at different ionic strengths (I/mol·dm^{-3}): □, — —, 0.00 mol·dm^{-3}; ■, - - -, 0.10 mol·dm^{-3}; Δ, ——, 0.0.15 mol·dm^{-3}; ▲, — - —, 0.20 mol·dm^{-3}; ×, — - - —, 0.25 mol·dm^{-3}.

Table 3. Protonation Constants (pK_{a2}) of L-Canavanine in a Water (1) + Organic Solvent (2) Mixture at $T = 298.15$ K and $I = 0.1$ mol·dm^{-3} $NaNO_3$

	pK_{a2}				
organic solvent % (v/v)	water−methanol	water−DMF	water−DMSO	water−acetone	water−dioxane
10	6.822 ± 0.006	6.881 ± 0.008	6.923 ± 0.002	6.968 ± 0.004	6.988 ± 0.004
20	6.634 ± 0.005	6.752 ± 0.004	6.836 ± 0.004	6.926 ± 0.005	6.937 ± 0.007
30	6.534 ± 0.002	6.661 ± 0.005	6.768 ± 0.007	6.834 ± 0.005	6.894 ± 0.004
40	6.321 ± 0.004	6.494 ± 0.003	6.671 ± 0.004	6.789 ± 0.005	6.811 ± 0.007
50	6.023 ± 0.007	6.404 ± 0.004	6.575 ± 0.003	6.634 ± 0.003	6.708 ± 0.003

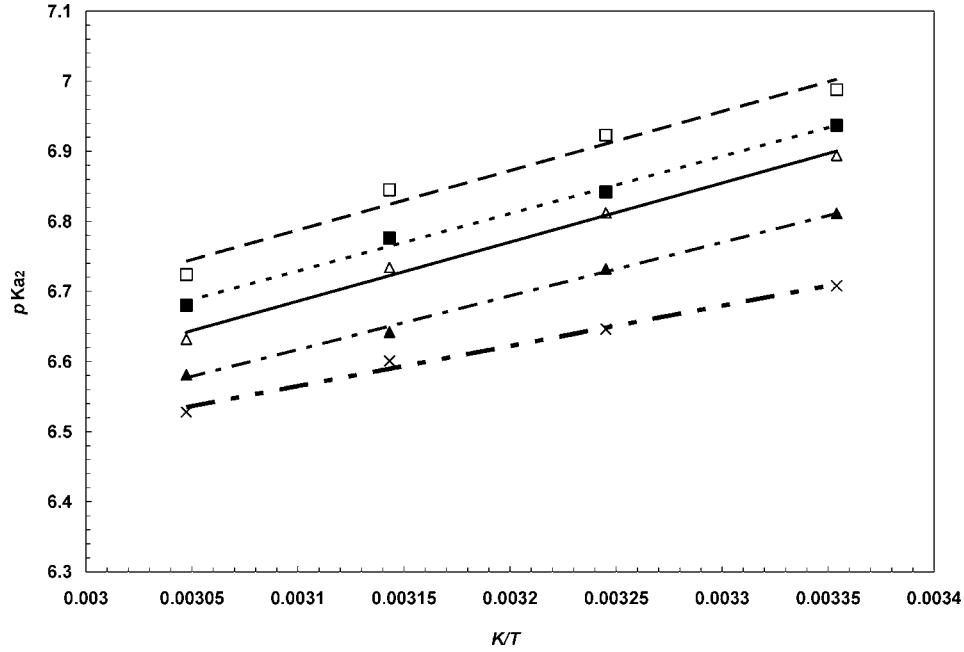

Figure 3. Plot of experimental (symbols) and correlated pK_{a2} (lines) values of L-canavanine versus $1/T/K^{-1}$ in a water (1) + dioxane (2) mixture: □, — —, 10 %; ■, - - -, 20 %; Δ, ——, 30 %; ▲, — - —, 40 %; ×, — - - —, 50 %.

Table 4. Protonation Constants (pK_{a2}) of L-Canavanine in a Water (1) + Dioxane (2) Mixture at Different Temperatures, I = 0.10 mol·dm^{-3} $NaNO_3$

	pK_{a2}			
organic solvent, % (v/v)	T/K = 298.15	T/K = 308.15	T/K = 318.15	T/K = 328.15
10	6.988 ± 0.006	6.923 ± 0.008	6.845 ± 0.002	6.724 ± 0.004
20	6.937 ± 0.005	6.842 ± 0.004	6.776 ± 0.004	6.68 ± 0.005
30	6.894 ± 0.002	6.812 ± 0.005	6.734 ± 0.007	6.632 ± 0.005
40	6.811 ± 0.004	6.732 ± 0.003	6.642 ± 0.004	6.581 ± 0.005
50	6.708 ± 0.007	6.646 ± 0.004	6.601 ± 0.003	6.528 ± 0.003

Table 5. Thermodynamic Quantities (Enthalpy, ΔH°, and Entropy, ΔS°, Changes) for the Protonation Equilibria of L-Canavanine in a Water (1) + Dioxane (2) Mixture at I = 0.1 mol·dm^{-3} $NaNO_3$

	ΔH°	−ΔS°
organic solvent % (v/v)	kJ·mol^{-1}	J·mol^{-1}·K^{-1}
10	7.044 ± 0.044	34.596 ± 0.078
20	6.804 ± 0.034	34.852 ± 0.113
30	7.013 ± 0.053	33.846 ± 0.094
40	6.350 ± 0.092	35.335 ± 0.113
50	4.750 ± 0.109	39.907 ± 0.124

indicate that the protonation processes are accompanied by the absorption of heat and the processes are endothermic and follow the general pattern for ionization processes for amino acids and non-protein amino acids. The negative values of entropy changes (ΔS°) point to increased ordering due to association and indicate that the total number of solvent molecules bound with the dissociated L-canavanine is greater than that originally accompanying the undissociated form. A plot of correlated pK_{a2} versus $1/T$ gives a straight line as shown in Figure 2. The correlation is fully acceptable and shows a good linear dependence of pK_{a2} upon temperature.

The effects of different organic solvents (methanol, dimethylformamide (DMF), dimethylsulfoxide (DMSO), acetone, and dioxane) on the pK_{a2} values of L-canavanine (Table 3) can be interpreted using the solvate chromic quantitative values of Kamlet−Taft hydrogen bond acidity and basicity (α,β) and dipolarity polarizability π* of the solvent.[23,24] These solvate chromic parameters may be used to determine multiple interacting solvent effects on the dissociation equilibria of L-canavanine. For simplicity, the effect of the solvents on the protonation process can be attributed to the decrease in hydrogen bonding in water by the organic solvent, the dielectric constant of the mixed solvent, and the protonation of the organic solvent molecules.[23−25] In the case of different solvents, the protonation process is affected by both the dielectric constants of the solvents and the extent of hydrogen bonding to the organic solvent.[23−26]

The empirical changes in the pK_{a2} values of L-canavanine as the solvent is enriched in methanol (Table 3) can be interpreted as resulting from the relatively high stabilization of the conjugate bases by donor hydrogen bonds in a pure aqueous medium relative to that in the presence of methanol. This is due to the greater tendency of water molecules to donate a proton in a solvent-to-solute hydrogen bond (α = 1.17).[23−25] In view of this effect, an increase in the methanol proportion in the aqueous medium will result in an increase in the activity coefficient of the conjugate base, thereby causing a slight increase in the pK_{a2} values.

The observed slight changes in pK_{a2} values of L-canavanine in the presence of varying amounts of DMF and DMSO can be explained as resulting from the following two opposing effects: (i) (DMF + water) or (DMSO + water) mixture is considered to be more basic than water.[23,24] This behavior is based on the building up of a strong acceptor hydrogen bond (β = 0.69 for DMF and 0.76 for DMSO) from the ($-^{+}$NH) group of L-canavanine in the former medium as compared to that in the latter one, thus facilitating the ionization process of the cationic ($-^{+}$NH) group, i.e., a low pK_{a2} value. (ii) The expected low stabilization of the conjugate L-canavanine free base by a hydrogen bond donates from solvent molecules in DMF or (DMSO + water) mixture compared to that obtained in pure

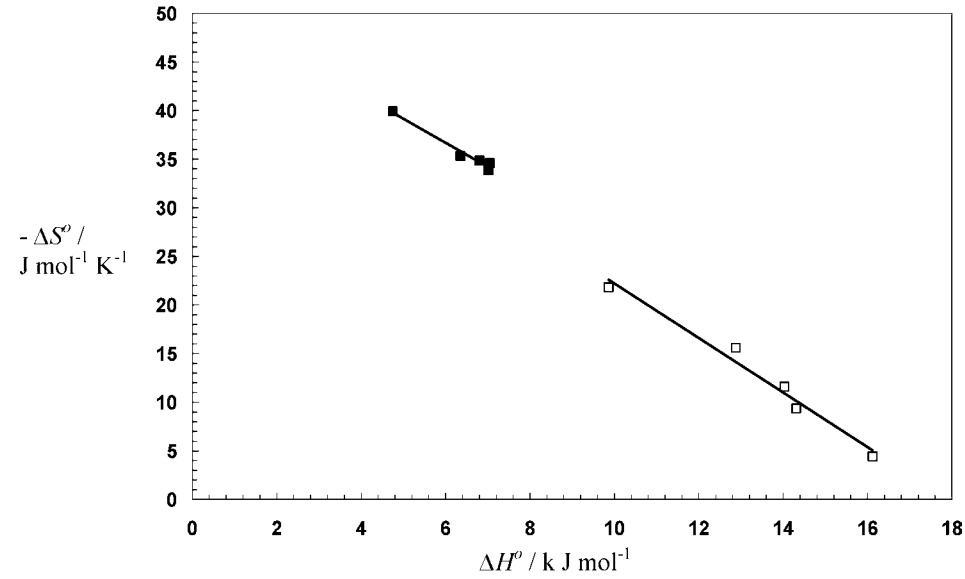

Figure 4. Enthalpy−entropy compensation plots for the protonation equilibria of L-canavanine in water solutions at different ionic strengths (□), and in a water (1) + dioxane (2) mixture at $I = 0.1$ mol·dm^{-3} $NaNO_3$ (■). The data are taken from Tables 2 and 5.

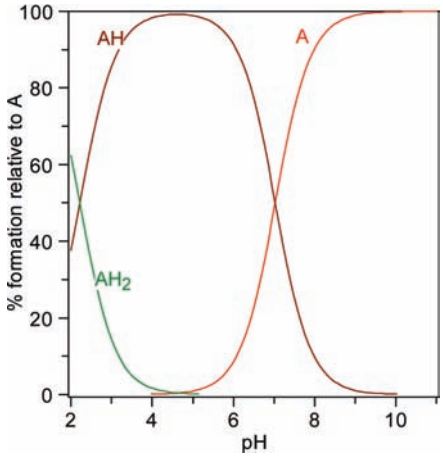

Figure 5. Distribution diagram of the different species of L-canavanine in water at 25 °C and an ionic strength of 0.15 mol·dm^{-3} HNO_3 (A = canavanine, AH = canavanineH, AH_2 = canavanineH_2).

aqueous medium. This in turn results in a high pK_{a2} value. The experimental small increase in the pK_{a2} values of L-canavanine when the amount of the organic cosolvent acetone (low basic aprotic solvent) in the medium is increased can be mainly attributed to a low stabilization of the free conjugate bases of L-canavanine by hydrogen bonding interaction. The observed increase in the pK_{a2} of L-canavanine as the medium is enriched in the aprotic nonionizing dioxane solvent may be attributed to the fact that the release of the proton is more difficult in the presence of this cosolvent.[25] This behavior is probably attributed to the lower β values of dioxane ($\beta = 0.37$).

The second dissociation constants of L-canavanine have been determined in water containing (+20, +30, +40, and +50) % (v/v) dioxane at four different temperatures from $T = (298.15$ to 328.15) K at intervals of 10 K. A plot of correlated pK_{a2} versus $1/T$ gives straight lines (Figure 3). The correlation is acceptable and shows a good linear dependence of pK_{a2} upon temperature. The values of pK_{a2}, together with their standard deviations, are listed in Table 4.

As can be seen from Table 5, the similarity between ΔH^o values of the dissociation processes of L-canavanine in water and (water + dioxane) indicates a similar pattern of solvation in these media. The negative values of ΔS^o are expected to be due to the fact that the degree of reorientation and partial immobilization of the dioxane and water molecules near the L-canavanine ions are greater in (water + dioxane) than in pure water.[25] Figure 4 shows the plot of ΔH^o values against ΔS^o. It seems that solvation controls not only ΔH^o but also ΔS^o because the absolute value of ΔH^o increases as the entropy change, ΔS^o, decreases.

The equilibrium distribution of various species of L-canavanine in water is shown as a function of pH (Figure 5). The calculations are based on the protonation constant values given in Table 1 and in the literature.[22,27]

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Received for review March 25, 2010. Accepted July 17, 2010.

JE100292G