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Protonation Constants of Some Alanyl Dipeptides in Mixed Aqueous Organic Solvents

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Abstract The stochiometric protonation constants of ($\log_{10}K_1$ and $\log_{10}K_2$) some alanyl dipeptides (Ala–Tyr, Ala–Phe, Ala–Val, Ala–Leu, Ala–Ala and Ala–Met) were determined in binary mixtures of water with 1,4-dioxane and dimethylsulfoxide containing 20, 40 and 60 % (v/v), using a potentiometric titration method at 25 ± 0.1 °C and constant ionic strength of 0.10 mol·L^{-1} sodium chloride. The autoprotolysis constant values (K_{ap}) of the media were also determined for each solvent mixture. The protonation constants of the dipeptides were calculated with the computer program BEST and selection of the best fit chemical models was based on the statistical parameters. The effects of the different amino acids bound to the alanine on the acidity of the alanyl dipeptides have been investigated. Finally, the results are discussed in terms of the effect of the solvent composition on the protonation constants.

Keywords Dimethyl sulfoxide · 1,4-Dioxane · Protonation constants · BEST · Alanyl dipeptides

1 Introduction

A large number of the molecules of interest in biochemistry contain acidic or basic groups which govern many of their chemical, physical and biological properties. Most of these organic molecules are capable of gaining and/or losing a proton in aqueous solutions [1]. Among these organic molecules, peptides are naturally occurring biological substances and they are short chains of amino acid monomers linked by peptide (amide) bonds. The shortest peptides are dipeptides, consisting of two amino acids joined by a single peptide

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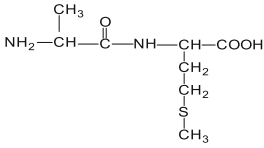
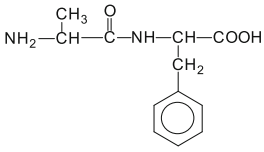
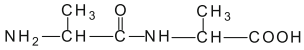
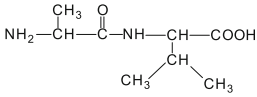
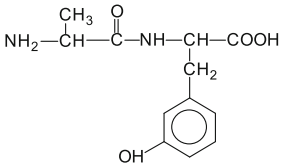
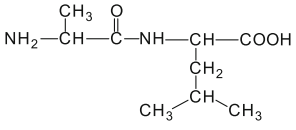
bond. The covalent chemical bonds are formed when the carboxyl group of one amino acid reacts with the amino group of another. They are found throughout every cell and tissue in the body and regulate the biological processes acting as antibiotics, food additives, poisons or pain-killers [2]. Thus, separation and analysis of peptides has become an increasingly important area of research [3].

Knowledge of protonation constants can provide valuable information for the quantitative treatment of systems involving acid–base equilibria in solution. So, they have been widely used in a number of research fields such as chemistry, biochemistry and pharmaceutical drug discovery and development. In pharmacology, ionization of a compound alters its physical behavior and macro properties such as solubility and lipophilicity. For example, ionization of any compound will increase the solubility in water, but decrease the lipophilicity. This is exploited in drug development to increase the concentration of a compound in the blood by adjusting the pK_a of an ionizable group [4]. Small dipeptides have acidic and basic functionalities and the extent to which the drugs enter the blood stream is controlled by their pK_a values. At the same time the pK_a values of proteins and amino acid side chains are of major importance for the activity of enzymes and the stability of proteins [5]. Many biological systems use proton transfer reactions to communicate between the extra-cellular and intra-cellular media. The rate of these reactions depends on

Table 1 Structures and abbreviations of the alanyl dipeptides studied

Name	Abbreviation	Structure
Alanyl–methionine	Ala–Met	
Alanyl–phenylalanine	Ala–Phe	
Alanyl–alanine	Ala–Ala	
Alanyl–valine	Ala–Val	
Alanyl–tyrosine	Ala–Tyr	
Alanyl–leucine	Ala–Leu	

the degree of dissociation of species in the solution phase. Solvent molecules act as proton acceptors and/or donors, therefore the solvent plays vital role in the reaction.

When a compound is sparingly soluble or insoluble in water it is the common practice to determine pK_a values in a solvent mixture such as water/dioxane or water/methanol in which the compound is more soluble [6]. The combination of pure solvents as mixed solvents substantially increases the diversity of reaction media and provides a good alternative for studying different properties of compounds [7, 8]. It has been suggested that organic solvent–water mixtures provide a better model for in vivo reactions [9]. Hence, the study of the dissociation and solvation processes in organic solvent–water mixtures will be very useful for obtaining information to elucidate the chemistry of in vivo processes like enzyme interactions, the assembly of lipids in biomembranes, surfactant aggregation, and so forth [10].

In the past few years, several experimental works dealing with the protonation and stability constants of amino acids and amino acid esters have been published [11–16]. However, a search in the literature showed few contributions on the acid–base behavior of dipeptides in aqueous–organic solvent mixtures [9, 13, 17–21].

This paper is concerned with the stochiometric protonation constants of some alanyl dipeptides determined potentiometrically in 20, 40 and 60 % (v/v) 1,4-dioxane–water and DMSO–water mixtures. The chosen dipeptides are alanyl–tyrosine (Ala–Tyr), alanyl–phenylalanine (Ala–Phe), alanyl–valine (Ala–Val), alanyl–leucine (Ala–Leu), alanyl–alanine (Ala–Ala) and alanyl–methionine (Ala–Met) and their structures and abbreviations are tabulated in Table 1. In the literature survey, it was found that the protonation constants of alanyl dipeptides have not been studied before in different aqueous solutions of 1,4-dioxane and DMSO. There is only one study on the determination of pK_a constants of L-alanyl–L-alanine dipeptides in water by using ab initio methods [22]. That is why, as a continuation of our previous works [11, 12, 14–17], in this study the protonation constants of alanyl dipeptides were determined and results are discussed in terms of the solvent–solvent and solute–solvent interactions for a better understanding of solvent effects. The effects of the different amino acids bound to the alanine on the acidity of the alanyl dipeptides have been investigated. The dipolar ionic form to neutral form ratio of these dipeptides in 1,4-dioxane–water and DMSO–water mixtures are also discussed.

2 Experimental

2.1 Materials and Solutions

Dipeptides used in this study were commercially available chemicals (Sigma−Aldrich), and were used as received. Doubly distilled conductivity water was used as the aqueous medium as well as for preparation of the 1,4-dioxane–water and DMSO−water mixtures. All other chemicals used in this study were of reagent grade purity. Stock solutions of strong acid and strong base were prepared using analytical reagent grade hydrochloric acid (Merck) and sodium hydroxide (Merck), respectively. Chemically pure sodium chloride (Merck) was used to maintain a constant ionic strength. Acid solutions prepared in water were standardized by titration against primary standard potassium hydrogenphthalate (Merck). Several 0.10 mol·L^{-1} sodium hydroxide solutions containing 0.10 mol·L^{-1} sodium chloride were prepared in 20, 40, and 60 % (v/v) aqueous 1,4-dioxane and aqueous DMSO solutions and stored in amber glass bottles protected against light. These solutions were standardized with

the use of a linear least squares fit of Gran plots for end-point determinations obtained by titrations of hydrochloric acid with these base solutions [23, 24].

2.2 Apparatus

The electromotive force, E, was measured using an Orion (Beverly, MA, USA) 960 model automatic titrator equipped with a combined pH electrode (Ingold, Andover, MA, USA). The combined glass pH electrode was modified by replacing its aqueous KCl solution with 0.1 mol·L^{-1} NaCl saturated with AgCl.

2.3 Cell Calibration

The potentiometric cell was calibrated before each experiment so that the hydrogen ion concentration was measured rather than the activity. The ionic strength of the sample solution used in this study was kept constant at 0.10 mol·L^{-1}, therefore the potential of the cell can be written as bellows;

$$E_{\text{cell}} = E^{\text{o}}_{\text{cell}} + E_{\text{j}} + k\log_{10}[\text{H}^+] = E^{\text{o}\prime}_{\text{cell}} + k\log_{10}[\text{H}^+] \quad (1)$$

where $E^{\text{o}}_{\text{cell}}$ represents a quantity independent of [H$^+$] but dependent on the activity of Cl$^-$ in the filling solution of the electrode and the activity coefficient of H$^+$ in the test solution. The activity coefficient of H$^+$ can be considered to be constant throughout the titration because the ionic strength of the test solutions is almost constant. E_{j} is the liquid junction potential and the constant k represent the Nernstian factor.

The calibration constants $E^{\text{o}\prime}_{\text{cell}}$ and k were determined by titration of hydrochloric acid solution with sodium hydroxide solution for each medium studied at 25.0 ± 0.1 °C. In all titrations, experimental points obtained in the acidic region were used for calibration. Within this range of pH, E_{j} is considered as constant [25].

2.4 Determination of K_{ap}

The standardization of combined pH electrode was also checked in the alkali range by the addition of an excess of NaOH. By assuming the $E^{\text{o}\prime}_{\text{cell}}$ and k values determined in the acidic range to be reliable in the alkali range and [OH$^-$] = concentration of base added in excess, we calculated reproducible values of the stoichiometric autoprotolysis constants (pK_{ap}) for the 20, 40 and 60 % (v/v) organic solvent–water mixtures by using $K_{\text{ap}} = [\text{H}^+][\text{OH}^-]$. Each p$K_{\text{ap}}$ value reported is the mean result of four series of measurements, and all the relative standard errors are less than 0.5 % [26, 27].

2.5 Measurements and Data Processing

All potentiometric measurements were performed in an 80 mL thermostatted (25.0 ± 0.1) °C double walled glass vessel. To exclude carbon dioxide from the system, a stream of purified nitrogen was passed through a sodium hydroxide solution. In the first step, the pH–ion meter calibration for all the solvent mixtures examined was performed by titration of hydrochloric acid with CO_2-free sodium hydroxide.

Then the protonation constants were evaluated from measurements of the electromotive force (emf) by titration of a 50.0 mL sample of a solution containing the 1.5×10^{-3} mol·L^{-1} dipeptide + 2.5×10^{-3} mol·L^{-1} HCl with standard 0.1000 mol·L^{-1} sodium

hydroxide solution, both in the same ionic strength and mole fraction of organic solvent of 1,4-dioxane and DMSO [(20, 40 and 60) % by volume]. During titrations, a potential reading was taken after waiting a suitable time (normally 2–3 min) for establishing the equilibrium. For each experiment, at least three replicates were performed. The protonation constants of the dipeptides were calculated by analyzing the titration data using the BEST computer program developed by Motekaitis and Martell [28, 29]. The program determines the protonation/or stability constants of ligands and complexes as well as the exact concentration of studied ligands. In general, the data necessary to set up the program are the pH values as a function of the base or acid volume added, the number of mmols of all reagents involved, the initial volume of solution, and the set of constants relative to all known equilibria present in the studied system. The protonation constants were evaluated by an iterative non-linear least squares fit of potentiometric equilibrium curves, through mass balance equations for all the components expressed in term of known and unknown equilibrium constants using the computer program BEST. The program was used to minimize the standard deviation of the fit (σ_{fit}) between observed and calculated pH values for the entire titration data set. All the models converged at $\sigma_{fit} < 0.03$ pH units of the observed pH values, which is considered to be an acceptable fit. The equilibrium constants reported in this paper were obtained as average values of three titrations. The selection of the equilibrium models was based on critical evaluation of the least squares fitting results, namely analysis of the statistical parameters.

3 Results and Discussion

3.1 Calibration and Autoprotolysis Constants of the Mixed Solvents

The autoprotolysis constants in all aqueous–1,4-dioxane and DMSO mixtures were determined at constant ionic strength (0.1 mol·L^{-1} NaCl) from titrations of 50 mL hydrochloric acid (0.01 mol·L^{-1}) with sodium hydroxide (0.1 mol·L^{-1}) solutions. The measured potential values were plotted against the logarithm of the hydrogen ion concentration in order to determine calibration constants ($E^{o/}_{cell}$ and k). The calibration constants obtained from these curves are tabulated in Tables 2 and 3 together with values reported in the literature for comparison.

As can be seen from the Tables 2 and 3, the glass electrode gave a Nernstian response with a slope of approximately 59 mV in both 1,4-dioxane–water and dimethylsulfoxide–water mixtures. It was thus concluded that these electrodes were suitable for the

Table 2 The calibration and autoprotolysis constants (pK_{ap}) in 1,4-dioxane–water mixtures

Medium	Calibration constants		Autoprotolysis constants	
	$E^{/}_{ort}$ (mV)	k (mV/pH)	pK_{ap}	pK_{ap} (literatüre)
20 % 1,4-dioxane–80 % water	300 ± 1	59.2 ± 0.1	14.23	14.22[a], 14.30[b], 14.31 ± 0.01[c]
40 % 1,4-dioxane–60 % water	304 ± 2	59.2 ± 0.1	14.81	14.86[a], 15.30[b], 14.89 ± 0.01[c]
60 % 1,4-dioxane–40 % water	311 ± 1	59.1 ± 0.1	15.60	15.62[a], 15.78[b], 15.54 ± 0.02[c]

[a] [27]

[b] [41]

[c] [16]

Table 3 The calibration and autoprotolysis constants (pK_{ap}) in dimethylsulfoxide–water mixtures

Medium	Calibration constants		Autoprotolysis constants	
	$E^{/}_{ort}$ (mV)	k (mV/pH)	pK_{ap}	pK_{ap} (literatüre)
20 % DMSO–80 % water	292 ± 1	59.7 ± 0.1	14.15	–
40 % DMSO–60 % water	286 ± 1	59.8 ± 0.1	14.85	–
60 % DMSO–40 % water	275 ± 2	59.2 ± 0.1	16.18	15.12 ± 0.08[a]

[a] [27]

determination of protonation constants for the media. The electrodes that gave a Nernstian response in acidic media were assumed to give the same type of response in basic media as well. The autoprotolysis constants (K_{ap}) for each medium were determined by the use of the same acid/base titration data. The autoprotolysis constant of a solvent is an important parameter in understanding acid–base equilibria in mixed solvents. The dielectric constants and the intrinsic acidic and basic strength of the solvent will influence the self ionization process in these media [30].

In the mixed media of water and organic solvent, the autoprotolytic reaction involved can be represented by

$$H_2O + H_2O \rightleftharpoons H_3O^+ + OH^- \quad (2)$$

$$S + H_2O \rightleftharpoons SH^+ + OH^- (S: 1,4-\text{dioxane or dimethylsulfoxide}) \quad (3)$$

The autoprotolysis constant expression is then

$$K_{ap} = ([H_3O^+] + [SH^+])[OH^-] \quad (4)$$

By assuming the concentration of $[SH^+]$ is, however, considerably smaller than $[H_3O^+]$ ($[SH^+] \ll [H_3O^+]$, the autoprotolysis constant expression can be formulated as shown Eq. 3.

$$K_{ap} = [H_3O^+][OH^-] \quad (5)$$

Since these K_{ap} values are the stoichiometric constants determined in a specified electrolyte medium, no data was available in literature for the same conditions. Considering the precision (standard deviation) of the results, it is also noteworthy that the study shows that the combined electrode system could be successfully used in order to determine autoprotolysis constants in water–organic solvent mixtures.

With some differences, the autoprotolysis constants obtained in this work are in agreement with those reported before [10, 24, 27]. The main differences are possibly due to the different background electrolytes used.

3.2 Protonation Constants of Alanyl Dipeptides

The protonation constant values of six alanyl-dipeptides were determined potentiometrically by titration of appropriate solutions of the dipeptides in the 1,4-dioxane–water and DMSO–water mixtures studied. In this way, dipeptides were fully protonated at the beginning of the titration by adding a certain amount of standardized hydrochloric acid and then titrated using sodium hydroxide solution (0.1000 mol·L^{-1}) as titrant. The calculation of the constants has been carried out using the BEST computer program. The values for

dipeptides in different 1,4-dioxane–water and DMSO–water mixtures are listed in Tables 4 and 5, respectively.

As can be seen from Tables 4 and 5, two protonation constants ($\log_{10}K_1$ and $\log_{10}K_2$) were found for all alanyl dipeptides investigated. The $\log_{10}K_1$ and $\log_{10}K_2$ values belong to the equilibria related to the attachment of H^+ to the $–NH_2$ and $–COO^-$ group, respectively, as follows:

$$L^- + H^+(S) \rightleftharpoons HL^{\pm} \text{and } (HL^0) + S; \quad K_1 = ([HL^0] + [HL^{\pm}])/[L^-][H^+] \quad (6)$$

$$HL^{\pm}(\text{and } HL^0) + H^+(S) \rightleftharpoons H_2L^+ + S; \quad K_2 = [H_2L^+]/([HL^{\pm}] + [HL^0][H^+] \quad (7)$$

where HL^0, $HL^{\pm}$, H_2L^+, and L^- are the neutral, zwitterion (dipolar ionic), cationic and anionic forms of the dipeptide respectively, and S refers to the mixed solvent.

To obtain more information about the effect of solvent composition on the corresponding equilibria, stoichiometric protonation constants were evaluated in detail. As is known, one of the most important parameters for determining the protonation constant is the reaction medium. The effect of solvent composition upon the protonation constants of the compounds has been the subject of investigations. Solvation of a solute in a mixed solvent is much more complicated than solvation in a pure solvent and in the literature there are various factors such as the dielectric constant of the medium, solvent structure, preferential solvation and some microscopic parameters [31, 32]. It is sometimes extremely difficult to assess how much each effect contributes to the protonation constants. For instance, Bates [33] are Chattopadhyay and Lahiri [34] have investigated the effect of changing solvent composition on the ionization of protonated base (BH^+) and the transfer Gibbs energies in mixed solvents. They found that the dielectric constant of the media were not the only parameter causing the solvent effect on the protonation constant. It strongly depends on the solute–solvent interactions of the different species in the mixtures [9].

Table 4 Stochiometric protonation constants of some alanyl dipeptides at 25.0 ± 0.1 °C for different 1,4-dioxane–water mixtures (μ = 0.10 mol·L^{-1} NaCl)

	Media					
	20 % D − 80 %W (v/v) (x = 0.02)		40 % D − 60 %W (v/v) (x = 0.05)		60 % D − 40 %W (v/v) (x = 0.09)	
Amino Acids*/ Dipeptides	$\log_{10}K_1$	$\log_{10}K_2$	$\log_{10}K_1$	$\log_{10}K_2$	$\log_{10}K_1$	$\log_{10}K_2$
Ala–Tyr	7.95 ± 0.01	3.39 ± 0.03	7.98 ± 0.02	4.01 ± 0.03	8.01 ± 0.02	4.67 ± 0.02
Phe*	9.10 ± 0.01	2.64 ± 0.01	9.15 ± 0.01	3.10 ± 0.01	9.15 ± 0.01	3.50 ± 0.01
Ala–Phe	7.84 ± 0.02	3.39 ± 0.02	7.98 ± 0.02	4.09 ± 0.02	7.96 ± 0.03	4.69 ± 0.03
Val*	9.60 ± 0.01	2.68 ± 0.01	9.60 ± 0.01	3.08 ± 0.01	9.66 ± 0.01	3.65 ± 0.01
Ala–Val	7.99 ± 0.02	3.49 ± 0.02	8.13 ± 0.02	4.12 ± 0.02	8.07 ± 0.02	4.76 ± 0.03
Leu*	9.64 ± 0.01	2.80 ± 0.01	9.70 ± 0.01	3.31 ± 0.01	9.75 ± 0.01	3.75 ± 0.01
Ala–Leu	8.04 ± 0.02	3.61 ± 0.02	8.09 ± 0.03	4.22 ± 0.02	8.09 ± 0.03	4.90 ± 0.02
Ala*	9.72 ± 0.01	2.64 ± 0.01	9.73 ± 0.01	3.10 ± 0.01	9.78 ± 0.01	3.63 ± 0.01
Ala–Ala	8.05 ± 0.02	3.63 ± 0.02	8.09 ± 0.03	4.17 ± 0.02	8.08 ± 0.02	4.78 ± 0.03
Ala–Met	8.10 ± 0.02	3.48 ± 0.01	8.07 ± 0.02	3.93 ± 0.02	8.09 ± 0.04	4.56 ± 0.02

Precisions are reported as standard deviation of the fit between observed and calculated values

* Stochiometric protonation constants of amino acids obtained from [11]

Table 5 Stochiometric protonation constants of some alanyl dipeptides at 25.0 ± 0.1 °C for different dimethyl sulfoxide–water mixtures (μ = 0.10 mol·L^{-1} NaCl)

	Media					
	20 % DMSO − 80 %W (v/v) (x = 0.02)		40 % DMSO − 60 %W (v/v) (x = 0.05)		60 % DMSO − 40 %W (v/v) (x = 0.09)	
Dipeptides	$\log_{10}K_1$	$\log_{10}K_2$	$\log_{10}K_1$	$\log_{10}K_2$	$\log_{10}K_1$	$\log_{10}K_2$
Ala–Tyr	7.76 ± 0.01	3.16 ± 0.02	8.06 ± 0.01	3.09 ± 0.02	7.22 ± 0.02	4.11 ± 0.02
Ala–Phe	8.03 ± 0.02	3.99 ± 0.02	8.16 ± 0.02	3.38 ± 0.02	5.09 ± 0.03	3.32 ± 0.04
Ala–Val	8.05 ± 0.02	3.47 ± 0.01	8.18 ± 0.02	3.42 ± 0.02	6.73 ± 0.02	4.15 ± 0.03
Ala–Leu	7.94 ± 0.02	3.44 ± 0.01	8.07 ± 0.03	3.36 ± 0.03	6.42 ± 0.03	4.09 ± 0.02
Ala–Ala	8.02 ± 0.02	3.41 ± 0.02	8.15 ± 0.03	3.31 ± 0.02	6.87 ± 0.02	3.74 ± 0.03
Ala–Met	8.15 ± 0.02	3.23 ± 0.02	8.39 ± 0.03	3.26 ± 0.03	7.03 ± 0.02	4.24 ± 0.03

Precisions are reported as standard deviation of the fit between observed and calculated values

Therefore, it is essential to elucidate the nature of solvent–solvent and solute–solvent interactions for a better understanding of solvent effects. With this in mind the results related to the variation of the protonation constants of alanyl dipeptides for each solvent media studied are considered as follows.

3.2.1 Protonation Equilibria of Alanyl-Dipeptides in 1,4-Dioxane–Water Mixtures

The numerical values of $\log_{10}K_1$ and $\log_{10}K_2$ of alanyl dipeptides suggest that the protonation of L^- and HL ($HL^{\pm}$) species take place in two distinct steps. In order to discuss the effect of solvent composition on the protonation constants of alanyl dipeptides, the variation of $\log_{10}K_1$ and $\log_{10}K_2$ values of ala–val with the reciprocal of dielectric constants ($1/\varepsilon$) of 1,4-dioxane–water mixtures is plotted in Fig. 1.

This figure shows that $\log_{10}K_1$ values are almost unaltered by the change in solvent composition. In contrast, $\log_{10}K_2$ values increase for each compound as the percentage of dioxane in the solvent mixtures increases. Regarding the variation of $\log_{10}K_1$ values with the solvent composition, we can say that the zwitterionic to neutral form ratio (K_z = $[HL^{\pm}]/[HL^0]$) decreases as the dioxane content increases. This can be inferred from

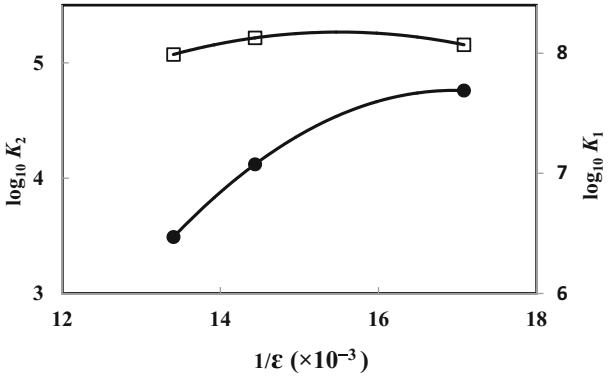


Fig. 1 Plots of $\log_{10}K_1$ (*square*) and $\log_{10}K_2$ (*filled circle*) values of ala–val versus the reciprocal of the dielectric constant ($1/\varepsilon$) of 1,4-dioxane–water mixtures

considerations on specific solute–solvent interactions and structural changes in dipeptides from water to dioxane–water media. In the related equilibrium (Eq. 6), the HL form would be more solvated than the L^- and $HL^{\pm}$ species in solvents containing a higher concentration of dioxane, whereas the opposite would hold for a solvent rich in water. If the zwitterionic to neutral form ratio was unaltered with increasing percentage of dioxane, then the $\log_{10}K_1$ values would be expected to decrease, which is not observed. If the neutral form became the predominant species when dioxane content is increased, then the $\log_{10}K_1$ values would increase as in the case of the neutral form, whereas they are not unaltered. Thus, even with the limitations, we can conclude that the K_z values for all alanyl dipeptides studied decrease as the concentration of dioxane increases.

Changes in $\log_{10}K_2$ values with increasing 1,4-dioxane content are due to increasing ion–ion interactions resulting from the decreasing dielectric constant and from changes in solvent–ion and solvent–solvent interactions. Hence, the $\log_{10}K_2$ values increase with the increasing dioxane content for all the alanyl dipeptides, suggesting that the zwitterion is the predominant species in dioxane–water mixtures as well as in water. This is based on the assumption that 1,4-dioxane solvates H_2L^+ better than $HL^{\pm}$. If the neutral form is the predominant species in 1,4-dioxane–water mixtures, then the $\log_{10}K_2$ values would decrease on addition of 1,4-dioxane since it would solvate HL better than H_2L^+. Thus, we can say that the $\log_{10}K_2$ values for these dipeptides increase as the concentration of 1,4-dioxane increases.

The almost linear relationship between the fraction of organic solvent and protonation constants of compounds has been studied by several authors [35–38] and the results obtained in this study also confirm this trend. The variation in $\log_{10}$ $\log_{10}K_1$ and $\log_{10}K_2$ values of all dipeptides with increasing 1,4-dioxane concentration is the same as for the corresponding free amino acids [11].

As a result, the solvent effect on the protonation constants of dipeptides can be utilized to predict whether the zwitterionic to neutral form is the predominant species and how the zwitterionic to neutral form ratio changes with the concentration of organic component in water–organic solvent mixtures.

The effect of peptide formation on the $\log_{10}K_1$ and $\log_{10}K_2$ values of the corresponding amino acids will also be discussed. For this purpose, the $\log_{10}K_1$ value of alanine, which is related to the protonation of $–NH_2$ group, and $\log_{10}K_1$ values of the corresponding dipeptides, were compared. When the change of $\log_{10}K_1$ of alanine given in Table 4 is examined, it is observed that this value decreases with the peptide formation. For instance, the $\log_{10}K_1$ value of alanine in 20 % 1,4-dioxane–80 % (v/v) water medium is 9.72, while the same values are: 7.95, 7.84, 7.99, 8.04, 8.05 and 8.10 for Ala–Tyr, Ala–Phe, Ala–Val, Ala–Leu, Ala–Ala and Ala–Met, respectively. This observation shows that, the electron density of the $–NH_2$ group in the dipeptide is lower than that of alanine, and the electron density of the $–COO^-$ group is higher than that of the corresponding amino acid because of the formation of a peptide bond. The same trend was observed for 40 and 60 % (v/v) 1,4-dioxane–water mixtures.

On the other hand, the $\log_{10}K_2$ values of the amino acids belong to the equilibria related to the attachment of H^+ to the $–COO^-$ group will be compared with those of dipeptides. According to the data given in Table 4, the $\log_{10}K_2$ values of amino acids increase with peptide formation. For instance, $\log_{10}K_2$ for alanine is 2.64 in 20 % 1,4-dioxane–80 % water while the same value is found as 3.63 for Ala–Ala. Similarly $\log_{10}K_2$ values for phenylalanine, valine, leucine are 2.64, 2.68, 2.80 and 2.64, respectively, and $\log_{10}K_2$ for alanyl–phenylalanine (Ala–Phe), alanyl–valine (Ala–Val) and alanyl–leucine (Ala–Leu) are 3.39, 3.49, 3.61 and 3.63, respectively, in the same medium. The differences between

the $\log_{10}K_2$ values of alanyl dipeptides and the $\log_{10}K_2$ values of the corresponding amino acids [$\Delta\log_{10}K_2 = \log_{10}K_{2(\text{alanyldipeptide})} - \log_{10}K_{2(\text{aminoacid})}$] range from 0.75 to 0.99 for 20 % 1,4-dioxane–80 % (*v/v*) water, from 0.91 to 1.19 for 40 % 1,4-dioxane–60 % (v/v) water, and from 1.11 to 1.19 for 60 % 1,4-dioxane–40 % (v/v) water mixtures, respectively.

This may be attributed to the fact that the peptide bond formation increases the electron density of the $-COO^-$ group and thus the proton bonding becomes easier. The same trend was observed for each 1,4-dioxane–water mixtures studied.

According to this discussion, we can say that the peptide bond formation cause a decrease in the $\log_{10}K_1$ values and an increase in the $\log_{10}K_2$ values of all amino acids studied.

3.2.2 *Protonation Equilibria of Alanyl-Dipeptides in Dimethylsulfoxide–Water Mixtures*

When the changes of $\log_{10}K_1$ and $\log_{10}K_2$ of alanyl dipeptides (Table 3) with the solvent composition are examined, it is observed that these values show different behavior. The protonation constant of the amino group of dipeptide, $\log_{10}K_1$, first increases in the range 20 to about 40 % (v/v) of DMSO and then decrease when the mixtures are richer in the organic solvent. However, the protonation constant of the carboxylic group, $\log_{10}K_2$, decreases with increasing percentages of DMSO in the same region and then increases up to 60 % DMSO. The different behavior of $\log_{10}K_1$ and $\log_{10}K_2$ observed in this study can possibly be attributed to the fact that in the protonation of the carboxylic acid group of the dipeptide, there is no change in the number of charges involved in the process ($HL^{\pm}$ (and HL^0) + H^+ (S) $\rightleftharpoons$ H_2L^+ + S), and therefore, change in polarity of the medium has a negligible effect on the protonation process and protonation depends only on the solvation of the different species by the solvents of the mixture. In contrast, in the protonation of the amino group of dipeptides charges are consumed (L^- + H^+ (S) $\rightleftharpoons$ $HL^{\pm}$ and (HL^0) + S), and therefore any variation in polarity of the medium exerts a strong influence on the process.

The numerical values of the protonation constants of the alanyl dipeptides show small changes when the mixtures are richer in water and larger increase or decrease when the mixtures are richer in DMSO. This variation with the percentage of the organic solvent is due to solute–solvent and solvent–solvent interaction effects. In fact, solvent–solvent interactions can be influenced by intermolecular forces between the solution components. This effect possibly changes the structure of the mixtures when the percentage of the organic solvents increases to higher values [39].

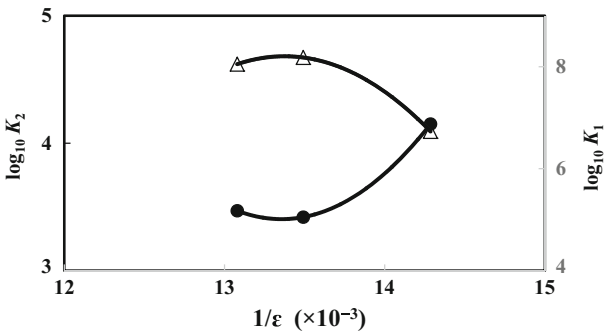


Fig. 2 Plots of $\log_{10}K_1$ (*triangle*) and $\log_{10}K_2$ (*filled circle*) values of ala–val against the reciprocal of the dielectric constant ($1/\varepsilon$) of DMSO–water mixtures

The variation of $\log_{10}K_1$ and $\log_{10}K_2$ values of ala–val with the reciprocal of the dielectric constant of the DMSO–water mixtures are given in Fig. 2 as a representative example of the alanyl dipeptides studied.

The $\log_{10}K_1$ values of the dipeptides show small increases initially and then decrease slightly (Fig. 2). This variation with the reciprocal of the dielectric constant of the DMSO–water mixtures may be due to the changing ratio of $HL^{\pm}$ and HL species and the specific solute–solvent interaction effect. However, the correlation between $\log_{10}K_2$ values and the reciprocal of the dielectric constant of the mixtures is not linear (Fig. 2) and deviates from the Born equation. This indicates that the protonation constants not only depend on electrostatic forces but also strongly depend on solute–solvent interactions of the different species in the mixtures. These results are also compatible with the reported data for glycine dipeptides in DMSO–water media [40].

There are no data related to the $\log_{10}K_1$ value of alanine and $\log_{10}K_2$ value of other amino acids forming the dipeptide in DMSO–water mixtures studied. So, it wasn't possible to compare the protonation constants of alanyl-dipeptides obtained in this work with literature value.

3.2.3 Variation of the Dipeptide Species with pH

Knowledge of the equilibrium constants of some compounds is necessary for the calculation of the concentrations of the ionized species at any pH. For this purpose, the corresponding protonation–deprotonation equilibria and pH ranges of existence of the various species of dipeptides in 1,4-dioxane–water and DMSO–water mixtures are shown as a function of pH in Figs. 3 and 4.

The curves clearly demonstrate that the predominant species is HL + ($HL^{\pm}$) (total concentration of the zwitterion and the neutral form) between pH = 5 and 8 in all DMSO–water and 1,4-dioxane–water media. The H_2L^+ species is predominant at low pH and its concentration decreases exponentially and becomes almost zero around pH = 5.0. The most predominant species is the $HL^{\pm}$ (zwitterionic) form of alanyl dipeptides present to an extent of 90 % in the pH range 4.0–8.0. Around pH = 6.0 formation of the free ligand (L^-) is observed, while its concentration progressively increases and attains its maximum at higher pH in both media.

Regarding the Figs. 3 and 4, we can see that the $\log_{10}K_2$ values of ala–val in 20 % v/v of organic solvent–water mixtures (3.41 and 3.45 for 1,4-dioxane and DMSO, respectively) correspond to the intercept between the distribution plots of ionic species H_2L^+ and HL + ($HL^{\pm}$). Likewise, the $\log_{10}K_1$ values (7.80 and 7.98 for 1,4-dioxane and DMSO, respectively) correspond to the intercept between the distribution plots of ionic species

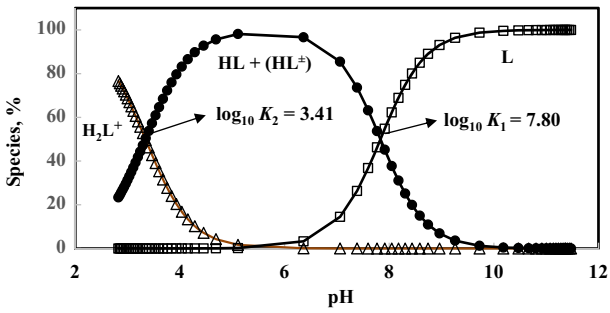


Fig. 3 Distribution diagrams of the different species of ala–val in 20 % v/v of 1,4-dioxane–water mixture at 25 °C and an ionic strength of 0.1 mol·L^{-1} (NaCl)

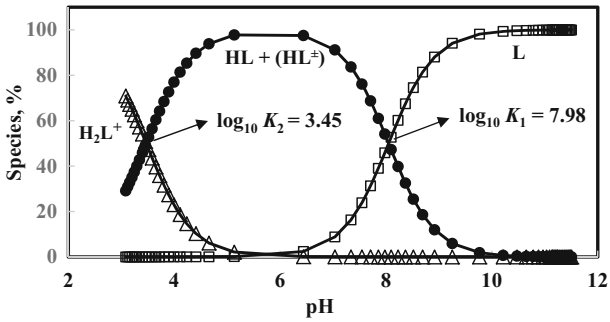


Fig. 4 Distribution diagrams of the different species of ala–val in 20 % v/v of DMSO–water mixture at 25 °C and an ionic strength of 0.1 mol·L^{-1} (NaCl)

HL + (HL$^{\pm}$) and L^{-}. These values are in good agreement with the experimental values of ala–val dipeptide given in Tables 4 and 5 for 20 % v/v of 1,4-dioxane–water (3.49 and 7.99) and DMSO–water mixtures (3.47 and 8.05).

Furthermore, the protonation constants of the alanyl dipeptides have different values in the two mixed solvent systems studied at the same percentage of organic solvent in the mixtures. This should be due to the differences in the dielectric constant values of the mixtures.

4 Conclusion

In this work the protonation constants of alanyl dipeptides (Ala–Tyr, Ala–Phe, Ala–Val, Ala–Leu, Ala–Ala and Ala–Met) were determined in binary mixtures of water with 1,4-dioxane and dimethyl sulfoxide containing (20, 40 and 60) % (v/v) using a potentiometric titration method at 25 ± 0.1 °C and constant ionic strength 0.10 mol·L^{-1} sodium chloride.

In the literature, there is no systematic study on the determination of the protonation constants of dipeptides in various aqueous organic solvent mixtures. Furthermore, dipeptides are becoming increasingly important in biological, pharmaceutical and other industrial applications. This study is therefore expected to provide an important addition to the literature. It is thought that the data obtained in 0.1 mol·L^{-1} sodium chloride, which simulates biological media, will be very useful for determining the effective mechanism of these compounds, which have significance in living organism. The results are discussed for the evaluation of the solute–solvent effect and predication of how the zwitterionic to neutral form ratio changes with the increase in the concentration of organic component in water–organic solvent mixtures. The effect of peptide formation on the $\log_{10}K_1$ and $\log_{10}K_2$ values of the corresponding amino acids was also discussed. In addition, the concentration distribution of the various dipeptide species in 1,4-dioxane–water and dimethyl sulfoxide–water mixtures was evaluated.

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