Evaluation of protonation constants of glycylglycine in acetonitrile– and ethylene glycol–water mixtures

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ABSTRACT
Knowledge of the protonation constants of dipeptides is important and necessary for complete understanding of their physiochemical behavior. The effect of dielectric constant of medium on protonation equilibria has been studied by determining protonation constants of glycylglycine pH-metrically in various concentrations (0–60%v/v) of acetonitrile–water and ethylene glycol–water mixtures, at an ionic strength of 0.16 mol L−1 and at 303.0 ± 0.1 K. The protonation constants were calculated with the computer program MINIQUAD75 and selection of the best fit chemical models is based on the statistical parameters. Linear and non-linear variations of stepwise protonation constants with reciprocal of dielectric constant of the solvent mixtures have been attributed to the dominance of electrostatic and non-electrostatic forces, respectively. Distribution of species, protonation equilibria, and effect of influential parameters on the protonation constants have also been presented.

ARTICLE HISTORY
Received 22 July 2015
Accepted 13 August 2015

KEYWORDS
Glycylglycine; acetonitrile; ethylene glycol; protonation constants; MINIQUAD75; dielectric constant

Introduction
Peptides are an amazing class of compounds constructed from relatively simple building blocks, the amino acids. They are components of tissues [1] exhibiting a remarkable range of biological properties acting as antibiotics, hormones, food additives, poisons, or painkillers. [2] Investigations involving the peptides in acid–base reactions [3,4] particularly, small peptides have attracted great attention in relation to the bioinorganic chemistry because these compounds are usually considered as good model systems to attain a better insight into the characteristics of naturally occurring metalloproteins. [5,6] On the other hand, several metal complexes containing peptide groups have displayed diverse pharmacological activities. For instance, copper complexes with amino acids and peptides as ligands show anti-inflammatory and cytostatic activities.

Vast data are available on the protonation and stability constants of the amino acids and simple peptides in water and organic solvents. However, the protonation constants of amino acids and peptides in organic solvents are often different from those in water, as organic media tend to be lipophilic rather than hydrophilic.[7,8] Little is known about the chemistry of amino acids and simple peptides in mixed solvents, in regard to their protonation constants and experiments shown that one solvent alone is not an ideal model for in vivo reactions.

It has been suggested that mixture of solvents such as organic solvent–water mixtures provide a better model for in vivo reactions. Therefore, the influence which the solvent exerts on the protonation constant (K) values depends upon the extent and nature of the solute–solvent interaction, which involves species participating in the acid–base equilibrium.[9] Hence, studies in media made up of organic solvent–water mixtures should provide some understanding of the chemistry of peptides in living systems.

Glycylglycine (GG) is the simplest dipeptide made from the residues of two glycine molecules. Besides an important component of the tissue, glycylglycine is a potentially useful zwitterionic buffer in the physiological pH range (6.0–8.5). Ethylene glycol (EG) is a protophilic dipolar protic solvent and acts as a structure former. EG, having two hydroxyl groups, is distinctly different from monohydric alcohols. EG is more acidic (less basic) than water [10] due to the electron withdrawing effect [11] of the CH₂ group. EG offers several advantages as solvent in titration of weak bases.[12] EG plays an important role in protein conformation studies [13,14] because it is a weak protein denaturant compared to urea or other organic solvents such as ethanol, dioxan, etc. The protonation equilibria of glycylglycine have been studied in the presence of EG to understand the influence of solvent on the chemical speciation.
Acetonitrile (AN) is a weak base [15] and a much weaker acid [16] than water. Hence, cations and anions have lower solvation energies in AN than in water, except in those cases where there is specific interaction with the solvent.[17] It is a protophobic dipolar aprotic solvent, and it does not form any hydrogen bond with solute species. The protophobic character of AN may be due to the formation of dimers which are shown to exist from IR studies.

In this study, the protonation constants of glycylglycine have been determined pH-metrically in aqua-organic mixtures containing AN and EG frequently used as media in biochemistry and biology laboratories.

Materials and methods

Experimental

All the chemicals used in this investigation were of analytical reagent grade purity. Solution (0.05 mol L⁻¹) of glycylglycine (Merk, India) was prepared in triple-distilled water by maintaining 0.05 mol L⁻¹ hydrochloric acid concentration to increase the solubility. AN and EG (Merk, India) were used without further purification. Carbonate-free sodium hydroxide (Qualigens, India) pellets were used for the preparation of 0.4 mol L⁻¹ solution. Hydrochloric acid (Qualigens, India) of 0.2 mol L⁻¹ was prepared. Sodium chloride (Qualigens, India) of 2.0 mol L⁻¹ was prepared to maintain 0.16 mol L⁻¹ ionic strength in the titrant. Triple-distilled water was used throughout the experiment. The strengths of acid and alkali were determined using Gran plot method.[18] To assess the errors that might have crept into the determination of the concentrations, the data were subjected to analysis of variance of one-way classification (ANOVA).[19]

Alkalimetric titrations

Alkalimetric titrations were carried out with varying composition of AN and EG (0.0–60.0%v/v) maintaining an ionic strength of 0.16 mol L⁻¹ with sodium chloride at 303.0 ± 0.1 K. The purpose of this study was to mimic the equilibria taking place in biofluids, where the temperature is 37 °C. But at that temperature, the solvent may be evaporated. Hence, a temperature of 30 °C or 303 K has been chosen in this study. Amount of glycylglycine in the titrant ranged between 0.25 and 0.50 mmol. Elico Li-120 pH meter was used. Potassium hydrogen phthalate (0.05 mol L⁻¹) and borax (0.01 mol L⁻¹) solutions were used to calibrate the pH meter. The glass electrode was equilibrated in a well-stirred AN–water and EG–water mixture containing inert electrolyte for several days. At regular intervals, strong acid was titrated against alkali to check the complete equilibration of the glass electrode. The calomel electrode was refilled with AN (or EG)–water mixture of equivalent composition of the titrant. Experimental procedure and titration assembly have been detailed elsewhere.[20] The approximate protonation constants of glycylglycine were calculated with the computer program SCPHD.[21] The best fit chemical model for each system investigated was arrived at using non-linear least squares computer program, MINIQUAD75.[22]
emphasize the presence of two protonation–deprotonation equilibria in the pH range of this study.

The plots of $a$ vs. pH for glycglycine in both media were given in Figure 2. The negative values of $a$ correspond to the number of moles of free acid present in the titrant and the number of associable protons. The positive values of $a$ indicate the number of dissociable protons in the ligand molecules. The maximum value of $a$ in Figure 2 is +1, which indicates that glycglycine has one dissociable proton.

The best fit models containing the type of species and log values of overall formation constants ($\log \beta$) along with some of the important statistical parameters of this study are given in Table 1. A very low standard deviation (SD) in $\log \beta$ values and $U_{\text{corr}}$ (sum of the squares of deviations in concentrations of ligand and hydrogen ion at all experimental points corrected for degrees of freedom) indicates that the experimental data can be represented by the model. Small values of mean, SD, and mean deviation for the systems corroborate that the residuals are around a zero mean with little dispersion.

**Residual analysis [23]**

In data analysis with least squares methods, the residuals (the differences between the experimental data and the data simulated based on the model parameters) are assumed to follow Gaussian distribution. When the data are fit into the models, the residuals should be ideally equal to zero. Further, a model is considered adequate only if the residuals do not show any trend. Respecting the hypothesis of the least squares analysis, the residuals are tested for normal distribution. Such tests are $\chi^2$, skewness, kurtosis, and $R$-factor. These statistical parameters of the present data show that the best fit models portray the acido-basic equilibria of glycglycine in AN–water and EG–water mixtures, as discussed below.

**$\chi^2$ test**

$\chi^2$ is a special case of gamma distribution, whose probability density function is an asymmetrical function. This distribution measures the probability of residuals forming a part of standard normal distribution with zero...
Table 1. These data are compared with the experimental alkalimetric titration data, to verify the sufficiency of the models as shown in Figure 3. The overlap of the typical experimental and simulated titration data indicates that the proposed models represent the experimental data.

Effect of systematic errors in concentrations on best fit model
MINIQUAD75 does not have an inbuilt provision to study the effect of systematic errors in the concentration of ingredients like mineral acid, alkali, and ligand. In order to rely upon the best fit chemical model for critical evaluation and application, a brief investigation was made by introducing pessimistic errors in the ingredients. This type of investigation is useful because the data acquisition was done under varied experimental conditions with different accuracies. The results of a typical system given in Table 2 emphasize that the errors in the concentrations of alkali and mineral acid affect the protonation constants more than that of the ligand.

Effect of dielectric constant of medium
When the ionization of an acid gives a net increase of ions, a decrease in the dielectric constant of the solvent should be accompanied by an increase in the protonation constant of a weak acid dissolved in it. The variation of protonation constant or change in free energy with co-solvent content depends upon two factors, viz, electrostatic and non-electrostatic forces. Born’s classical treatment holds good in accounting for the electrostatic contribution to the free energy change. According to this treatment, the energy of electrostatic interaction or the logarithm of stepwise protonation constant ($\log K$) should vary linearly as a function of the reciprocal of the dielectric constant ($1/D$) of the medium.

The linear variation of $\log K$ values of glycylglycine in AN–water mixture shown in Figure 4 indicates the dominance of electrostatic forces over non-electrostatic forces.
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are shown in Figure 5. As the alkali is added to the titrant containing the ligands, the protonated forms of the ligand lose their protons. In the pH range of this study, glycylglycine loses carboxylic and amino protons successively.

The typical distribution plots shown in Figure 6 produced using the protonation constants from the best fit models Table 1 show the existence of $\text{LH}_2^+$, $\text{LH}^-$, and $\text{L}^-$ species. The $\text{LH}_2^+$ species is predominant at low pH, and its concentration decreases exponentially and becomes almost zero around pH 6.5. The most predominant species is LH (zwitterionic) form of glycylglycine present to an extent of 90% in the pH range 4.0–9.0. Around pH 5.0, the formation of the free ligand $\text{L}^-$ is observed while its concentration progressively increases and attains its maximum at higher pH in both media.
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**Conclusions**

Secondary formation functions indicated the existence of two protonation equilibria. The highest form of ligand is \( LH_2^+ \) which successively deprotonates to \( LH \) and \( L^- \) with increasing pH.

Increased standard deviations in the protonation constants with the introduction of errors in the concentrations of ingredients support the appropriateness of the experimental conditions. The change in the magnitude of protonation constant with co-solvent content indicates the influence of dielectric constant of the medium. The linear and non-linear variation of protonation constants in AN–water and EG–water mixtures, respectively, infers the dominance of electrostatic and non-electrostatic forces in the equilibria.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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