Differentiation between α-Glutamyl Peptides, γ-Glutamyl Peptides, and α-Aminoacylglutamic Acids by PMR Spectroscopy

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Numerous γ-glutamyl derivatives of amino acids and amines occur naturally,[1] and methods for the unequivocal structure determination of these compounds are therefore of importance. Whereas standard methods can be used to establish the sequence of dipeptides containing glutamic acid the differentiation between α- and γ-glutamyl derivatives poses a specific problem. The only three reliable chemical solutions to this problem are synthesis, quantitative decarboxylation with ninhydrin,[2] and deamination with nitrous acid.[3] The latter two methods are destructive and demand considerable amounts of material. The higher pK_2-values for α-glutamyl α-amino acids than for γ-glutamyl α-amino acids determine the elution behaviour on strongly basic ion-exchange resins in the acetate form, γ-glutamyl peptides being eluted last with acetic acid. Differentiation between α- and γ-glutamyl peptides has been made on an amino acid analyser, where γ-glutamyl peptides are eluted before and α-glutamyl peptides after the second amino acid from a strongly acidic ion-exchange resin with citrate buffer.[4] Various methods depending on paper chromatography, electrophoresis, or acid lability for distinction between the two isomer possibilities require both isomers for reliable results.

The δ-value for the α-proton in the glutamic acid moiety is larger for α-glutamyl amino acids than for γ-glutamyl amino acids in aqueous solution, and this difference has been proposed as a means for differentiation between α- and γ-glutamyl derivatives.[4] However, the δ-value for the α-proton in γ-glutamylglutamic acid (3.92 ppm) is higher than the upper limit of 3.90 ppm given for the α-proton in γ-glutamyl derivatives,[4] and similar to the shift for the α-proton in glutamic acid in α-glutamylphenylalanine (3.93 ppm) and α-glutamyl-β-alanine (3.94 ppm).[4] Again the method will give reliable results only when both isomers are available.

The difference in δ-values for the α-proton is of course due to the different chemical surroundings of the α-protons. The surroundings of the α-protons vary with the ionization state of the dipeptides. Use of the changes in chemical shifts occurring on change in ionization[6] and analysis of the surroundings permits differentiation between the possible isomers in dipeptides containing glutamic acid and a second amino acid. Definite ionization states are obtained (i) in D_2O, (ii) in D_2O with excess trifluoroacetic acid, (iii) in D_2O with excess K_2HPO_4 (pH 7), and (iv) in D_2O with excess NaOH. In the first case, equal amounts of positively charged amino groups and negatively charged carboxyl groups are obtained, γ-glutamyl amino acids and aminoacylglutamic acids with negative charge on the α-carboxyl group in the glutamic acid moiety, and α-glutamyl α-amino acids with negative charge on the carboxyl group in the second amino acid. In case (ii), all carboxyl groups are free and all amino groups are positively charged, while in case (iii) all carboxyl groups are negatively charged and all amino groups are positively charged. Finally, in case (iv), all carboxyl groups are negatively charged and all amino groups are uncharged.

In Table 1 are listed the δ-values for the α-protons in various glutamic-acid-containing dipeptides and in related compounds. For elucidation the variable surroundings of the α-protons are listed. The changes in δ-values observed are in full agreement with those expected from the substitution patterns. Ionization of a carboxyl group positioned on the same carbon atom as the α-proton results in an upfield shift of from 0.12 to 0.37 ppm. Neutralization of an amino group positioned on the same carbon atom as the α-proton results in an upfield shift of from 0.43 to 0.77 ppm.

Only the signal for the α-proton in the γ-glutamyl peptides exhibits two shifts (between pH < 1 and pH 3 and between pH 7 and pH > 11) characteristic for a proton in a free α-aminocarboxylic acid setting. Therefore when a signal with two shifts is observed, it can be concluded that a γ-glutamyl peptide is present, and assignment of the signals for the two

Table 1. δ-Values and chemical surroundings for α-protons in glutamic acid containing dipeptides and related compounds.

	pH < 1[a]		pH ~ 3[b]		pH ~ 7[c]		pH > 11[d]	
	δ (ppm)	surroundings	δ (ppm)	surroundings	δ (ppm)	surroundings	δ (ppm)	surroundings
α-Proton in glutamic acid								
Glutamic acid	4.17		3.82		3.81		3.23	
Glutamine	4.20		–		3.83[b]		3.31	
γ-Glutamyl peptides								
γ-Glutamyl-alanine	4.15		3.82		3.77		3.25	
γ-Glutamyl-glutamic acid	4.14	$-COOH$ $-NH_3^+$	3.92	$-COO^-$ $-NH_3^+$	–	$-COO^-$ $-NH_3^+$	–	$-COO^-$ $-NH_2$
γ-Glutamyl-methionine sulfoxide	4.12		3.89		–		–	
γ-Glutamyl-phenylalanine	4.01		3.70		3.63		3.20[e]	
γ-Glutamyl-tyrosine	4.01		3.72		3.69		3.13	
Glutamic acid α-amide	4.17	$-CONH_2$ $-NH_3^+$	–	–	4.12[b]	$-CONH_2$ $-NH_3^+$	3.35	$-CONH_2$ $-NH_2$
α-Glutamyl peptides								
α-Glutamyl-alanine	4.15	$-CONHR$ $-NH_3^+$	4.10	$-CONHR$ $-NH_3^+$	4.05	$-CONHR$ $-NH_3^+$	3.37	$-CONHR$ $-NH_3$
α-Glutamyl-tyrosine	4.07		4.00		3.97		3.35	
α-Aminoacyl glutamic acids								
Alanylglutamic acid	4.53	$-COOH$ $-NHCOR$	4.27	$-COO^-$ $-NHCOR$	4.19	$-COO^-$ $-NHCOR$	4.15	$-COO^-$ $-NHCOR$
Tyrosyl-glutamic acid	4.45[e]		4.25[e]		4.23[e]		4.15	
α-Proton in second amino acid								
γ-Glutamyl peptides								
γ-Glutamyl-alanine	4.29		4.31		4.15		4.15	
γ-Glutamyl-glutamic acid	4.45		4.42		–		–	
γ-Glutamyl-methionine sulfoxide	4.54	$-COOH$ $-NHCOR$	4.54	$-COOH$ $-NHCOR$	–	$-COO^-$ $-NHCOR$	–	$-COO^-$ $-NHCOR$
γ-Glutamyl-phenylalanine	4.72		4.65		4.51		4.50	
γ-Glutamyl-tyrosine	4.65		4.60		4.48		4.40	

Table 1. Continued.

α-Glutamyl peptides								
α-Glutamyl-alanine	4.48	−COOH	4.28	−COO⁻	4.22	−COO⁻	4.17	−COO⁻
α-Glutamyl-tyrosine	4.70	−NHCOR	4.48	−NHCOR	4.46	−NHCOR	4.49	−NHCOR
α-Aminoacyl glutamic acids								
Alanyl-glutamic acid	4.20	−CONHR	4.15	−CONHR	4.17	−CONHR	3.52	−CONHR
Tyrosyl-glutamic acid	4.30[e]	$-NH_3^+$	4.20[e]	$-NH_3^+$	4.18[e]	$-NH_3^+$	3.60	$-NH_2$

[a] D_2O with trifluoroacetic acid. [b] D_2O. [c] $D_2O + K_2HPO_4$. [d] D_2O + NaOH. [e] The exact δ-values are difficult to obtain because of superposition of signals.

α-protons to the two constituent amino acids can be made. The signal for the proton in the second amino acid only shows a shift when pH is raised from 3 to 7. No shift is observed here for the two isomeric possibilities and thus further evidence for a γ-glutamyl structure can be obtained. Both α-glutamyl peptides and α-aminoacylglutamic acids show signals for one proton shifting between pH < 1 and pH 3 and for one proton shifting between pH 7 and pH > 11. Differentiation between these isomers can only be made when the two signals can be assigned to the two constituent amino acids.[6] In the present case this is easily done for the alanine derivatives since the α-proton in alanine gives a signal split into a quartet. For the tyrosine derivatives the coupling of the α-proton in tyrosine with the easily discernible benzylic protons permits assignment.

PMR-spectra of the compounds in different ionization states thus provide an unequivocal and nondestructive method for differentiation between on one side γ-glutamyl peptides and on the other side α-glutamyl peptides and aminoacylglutamic acids. Furthermore differentiation between α-glutamyl peptides and aminoacylglutamic acids can be made provided that the signals for the α-protons can be assigned to glutamic acid and the second amino acid. Not only the changes but also the actual δ-values found may be used for assignment of structure. However, especially when new amino acids are involved such assignments must be made with more caution than those made on the basis of changes in chemical shifts.

Methods and materials. PMR-spectra were measured on a JEOL C-6OHL instrument. δ-Values are in ppm relative to sodium 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionate. Spectra were measured on solutions containing between 1 and 4 % of peptide in D_2O containing excess trifluoroacetic acid (more than 6 %), in D_2O, in D_2O containing 1.5 mol of K_2HPO_4 per mol of glutamic acid derivative, and in 0.4 N NaOH in D_2O.

γ-L-Glutamyl-L-alanine, α-L-glutamyl-L-alanine, L-alanyl-L-glutamic acid, α-L-glutamyl-L-tyrosine, and L-tyrosyl-L-glutamic acid were obtained from Cyclo Chemical, U.S.A. γ-L-Glutamyl-L-phenylalanine was isolated from seeds of *Fagus silvatica*. L.[5] and unequivocally identified by comparison with previously verified material.[7] γ-L-Glutamyl-L-tyrosine was isolated from *Aubrietia deltoidea* DC.[7] γ-Glutamylglutamic acid and γ-glutamylmethionine sulfoxide were also isolated from seeds of *Fagus silvatica* L. The configurations of these compounds have not been established.[5] L-Glutamic acid α-amide was obtained from Calbiochem, U.S.A.

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