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Research paper

Protection of the peptide glutathione by complex formation with α-cyclodextrin: NMR spectroscopic analysis and stability study

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Abstract

The main objective of this work was to investigate the complexation mechanism of the tripeptide glutathione with α-cyclodextrin (α-CyD). The final purpose was to explore the possibility of using this complexation approach for preserving the stability of this peptide in all biological environments relevant for oral drug delivery. The complexes between the peptide and α-CyD were formed in aqueous solution and the complexation mechanism was investigated using different ^{1}H NMR experimental approaches. The resulting complexes were also studied with respect to their ability to protect the peptide against proteolytic degradation by the exopeptidase, γ-glutamyltranspeptidase. The NMR experiment, 1D-saturation transfer NOE difference (STD), evidenced the interaction between α-CyD and glutathione. The binding constants, calculated by a titration method, were in the range of 55–70 M^{-1} at 25 °C and in the range 68–72 M^{-1} at 37 °C. Moreover, from the 1D-pulse field gradient spin echo-transverse-rotating frame nuclear Overhauser (PFGSE-T ROESY) spectra it was concluded that α-CyD binds preferably to the L-glutamate (side chain) moiety of glutathione, leaving the glycine residue exposed to the external medium. This result was consistent with those of the in vitro stability study, which indicated that the degradation of glutathione was markedly reduced to the half in 2 h upon inclusion in α-CyD. Overall, these results show the possibility of protecting specific peptide groups by their inclusion in CyDs as well as the utility of NMR experiments for the understanding of this stabilization strategy.

Keywords: Cyclodextrins; Glutathione; Inclusion complex; Nuclear magnetic resonance; Peptide stability

1. Introduction

Glutathione (γ-glutamylcysteinylglycine, GSH, Fig. 1) is the major thiolated small peptide present in living cells. Due to its reducing and nucleophilic properties, GSH acts as a redox buffer, thus preventing oxidative damage. In addition, it has been found that, in HIV-positive patients, systemic GSH deficiency is associated to an increase in virus replication [1]. Similarly, GSH depletion has been observed in lung- and neurological-diseases such as the acute respiratory-, and Parkinson's-disease, respectively [2]. With regard to its clinical use, GSH is indicated in the treatment of alcohol and drug poisoning, as well as for protection against toxicity induced by cytotoxic chemotherapy and radiation trauma and also in the treatment of AIDS-associated cachexia [3]. Unfortunately, due to its low and variable bioavailability, [4] GSH needs to be administered intravenously. This limited and variable absorption has mainly been attributed to the chemical and enzymatic degradation of the peptide in the jejunum. More specifically, it is known that the thiol group of the cysteine moiety in GSH is susceptible to enzymatic (γ-glutamyl-transpeptidase) and non-enzymatic pH-dependent oxidation, [5] leading to the formation of non-active products. Therefore, the development of a technological approach that helps to

Abbreviations: STD, saturation transfer NOE difference; PFGSE-T ROESY, pulse field gradient spin echo-transverse rotating-frame Overhauser effect spectroscopy.

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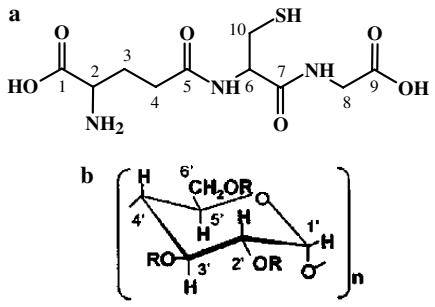

Fig. 1. Structures of (a) glutathione and (b) a monomer of α-CyD ($n = 6$) with the corresponding proton numbering.

protect the peptide from its degradation in the gastrointestinal tract has been conceived as a way to increase the use and clinical value of GSH.

Among the possible approaches for the protection of delicate molecules against degradation, the use of cyclodextrins (CyDs) as stabilizers [6] or as artificial chaperones [7,8] has recently attracted significant attention. Indeed, several reports have shown that the non-covalent interactions between CyDs and the hydrophobic peptide residues, forming host–guest inclusion complexes, led to an enhancement of the chemical stability and, hence, the intestinal absorption of these sensitive drugs. Examples of macromolecules which have been protected against degradation by their partial inclusion in the cyclodextrin cavity are insulin, [9] busereline, [10] and growth hormone [11]. In general, the protection has been achieved due to incorporation of the aromatic side chains of the peptides, mainly L-tryptophan and L-tyrosine residues, into the hydrophobic environment of the CyD cavity. In addition to this protective effect, it has been reported that hydrophilic CyDs are able to inhibit the P-glycoprotein function, thus leading to an enhancement of the oral bioavailability of some drugs [12–14]. Finally, the low toxicity of CyDs and their easy and economic availability make these host compounds particularly amenable for the design of drug carriers.

Taking this information into account, the aim of this work was, first, to study the possible mechanism of interaction between GSH and α-CyD and, second, to investigate whether or not this interaction enhances the stability of the peptide upon contact with degrading enzymes. From most analytic methods for investigation of complexes (circular dichroism, differential scanning calorimetry, solubility measurements), the spatial geometry of the complex cannot be drawn because these techniques disturb the drug–CyD complex equilibrium. For this purpose, we have performed ^{1}H nuclear magnetic resonance (NMR) spectroscopy experiments to resemble the real dynamic conformation of the GSH–α-CyD complex in solution and analyzed the stability of the associated peptide in simulated intestinal fluid in presence of γ-glutamyltranspeptidase.

2. Materials and methods

2.1. Materials

α-Cyclodextrin (α-CyD), glutathione (GSH), 2,2-dimethyl-silapentane-5-sulfonate sodium salt (DSS) and equine kidney γ-glutamyltranspeptidase were purchased from Sigma Co. (Spain) while deuterium oxide (D_2O, deuteration degree $\geqslant$99.9%) was purchased from Merck Farma y Quimica S.A. (Spain). Other chemicals and solvents were of analytical reagent grade or better. Ultrapure water (MilliQ Plus, Millipore Iberica, Spain) was used throughout the study.

2.2. ^{1}H NMR spectroscopy

All ^{1}H NMR spectra were acquired on an Inova Varian spectrometer operating at 750 MHz and processed with the NMR processing software Mestre-C (v.4.6.3.0) (Mestrelab Research Inc.) [15]. Samples were dissolved in D_2O and placed in standard 5-mm sample tubes. The ^{1}H NMR signals of GSH and α-CD were assigned by standard 1D and 2D techniques. Assignment of proton resonances of cyclodextrin was in agreement with those previously found [16].

A proton-NMR titration study was performed at 25 and 37 °C using DSS as external chemical shift reference standard, that was dissolved in D_2O and placed coaxially in a capillary tube inside the NMR tube. All the chemical shifts throughout this paper are given in reference to the most upfield signal of DSS (0 ppm).

The stoichiometry of the complexes of GSH with α-CyD in D_2O at 25 °C was determined by the continuous variation method [17,18]. For this purpose, the total concentration of GSH and α-CyD was kept constant at 0.048 M in D_2O. The stability constants (K_a) for the complexes in D_2O at 25 °C and at 37 °C were determined according to Hanna's [19] and Scatchard's [20] equations. For these samples, the concentration of GSH was set at 2.1 mM and the concentrations of α-CyD were in the range between 92 and 4 mM in D_2O.

1D-saturation transfer NOE difference (STD) [21] was performed at the beginning of the study to confirm the occurrence of the complexation process. For these experiments all well-isolated signals of GSH at $\delta = 2.0$, 2.5, 2.9 and 4.6 ppm were selectively saturated during 3 s by a train of Gaussian pulses separated by a short delay (1 ms), at $\gamma B_1/2\pi = 100$ Hz. 1D-pulse field gradient spin echo-transverse-rotating frame nuclear Overhauser spectroscopy (PFGSE-T ROESY) [22] experiments were performed to provide information about the chemical groups of GSH that might be included within the CyD cavity. For each PFGSE-T ROESY experiment a 180° selective shaped pulse was calibrated to affect a single resonance of GSH within the PFGSE scheme. The PFGSE-T ROESY experiments were also performed with all the well-isolated signals of GSH (2.0, 2.5, 2.9, and 4.6 ppm). Both the STD and the PFGSE-T ROESY experiments were acquired at

25 °C for a NMR sample of α-CyD/GSH 16:1 dissolved in D_2O.

2.3. Stability studies of GSH

The study of the degradation of GSH, either free or complexed with α-CyD, was performed at 37 °C in simulated intestinal fluid (USP XXVI, pH 6.8, without pancreatin), containing γ-glutamyltranspeptidase, under mechanical agitation. The selection of these experimental conditions was based on the known fact that γ-glutamyltranspeptidase catalyzes the breakdown of GSH to produce L-glutamate and L-cysteinylglycine [23]. Before starting the degradation experiment, the enzymatic medium was pre-warmed for 15 min. Free GSH (1.9 mM), 8:1 and 32:1 molar ratio stoichiometry α-CyD/GSH complexes were incubated in this enzymatic medium. The concentration of γ-glutamyltranspeptidase was kept constant at 0.8 I.U./ml of the incubation medium in each experiment. At appropriate time intervals, an aliquot (0.8 ml) was withdrawn and mixed with 500 μl of $HClO_4$, in order to stop the enzymatic reaction. These samples were then centrifuged (16,000*g*, 15 °C; Beckmann Avanti30, Beckmann, USA) for 15 min. The supernatant was analyzed for their content in non graded degraded GSH by high performance liquid chromatography (HPLC).

2.4. HPLC analysis

Quantitative determinations of GSH were performed by HPLC, using an Agilent 1100 system (Agilent Technologies, S.L., Spain) equipped with a UV-visible wavelength detector, and a reversed phase Hydro Synergy C_{18} (25 cm × 4.6 mm; 4 μm particles Phenomenex (Torrance, CA)) column in conjunction with a pre-column C_{18} insert. The eluting phase consisted of a mixture of acetonitrile and 0.025 M phosphate buffer, pH 2.7 (1:99 v:v). The flow rate of 0.7 ml/min was maintained and the absorption was monitored continuously at 220 nm. The standard calibration curves of GSH were linear ($r^2 > 0.999$) in the range of concentrations between 10 and 1000 μg/ml.

3. Results and discussion

As indicated in the introduction, a major goal of this work was to investigate the potential mechanism of complexation of GSH in α-CyD using different ^1H NMR experimental approaches. This specific CyD was selected taking into account previous studies which showed the efficient inclusion of the –SH group in its cavity [24]. In addition, α-CyD is known by its low toxicity, being its LD_{50}, after oral administration to rats, higher than 10,000 mg/kg [25].

The numbers corresponding to the NMR signals of GSH and α-CyD are shown in Tables 1 and 2. Proton labels are the same as those in the drawing of their molecular structures (Fig. 1).

Table 1
Proton chemical shift assignment (ppm) of GSH referred to external reference standard DSS at 25 and 37 °C

Proton	Chemical shifts (ppm)[a]	Chemical shifts (ppm)[b]	Multiplicity
H3	2.148–2.180	2.150–2.179	Multiplet
H4	2.511–2.588	2.513–2.578	Multiplet
H10	2.911–2.970	2.904–2.970	Multiplet
H2	3.808–3.816–3.825	3.800–3.808–3.817	Triplet
H8	3.947–3.971–3.976–3.999	3.965–3.968	Multiplet
H6	4.555–4.563–4.571	4.556–4.564–4.571	Triplet

[a] Chemical shifts observed at 25 °C.
[b] Chemical shifts observed at 37 °C.

Table 2
Proton chemical shift assignment (ppm) of α-CyD referred to external reference standard DSS at 25 °C

Proton	Chemical shifts (ppm)	Multiplicity
H4′	3.568–3.58–3.920	Multiplet
H2′	3.620–3.633	Multiplet
H5′	3.842–3.847–3.865	Multiplet
H6′	3.898–3.914	Multiplet
H3′	3.965–3.977–3.990	Triplet
H1′	5.056	Doublet

3.1. Nuclear Overhauser effect (NOE)-saturation transfer difference (STD) experiments

Among the different NMR experimental approaches, the NOE experiments are particularly useful for the determination of structural arrangements of molecular complexes. NOE experiments are based on the observation that selective excitation of some nuclei may affect the transitions of other nuclei of the sample. Such changes are considered to be driven by dipolar interactions between each pain of nuclei, typically at distances between 1.8 and 4.5 Å. The NOE-STD experiment is particularly suited for the identification of molecular association processes and the evaluation of the intermolecular binding affinity [26]. Moreover, specific advantages of this experimental approach are that (i) the STD can be detected even in the case that one of the interacting molecules is not detectable by liquid-state NMR (such as very high molecular weight macromolecules) and (ii) the STD is detected independently of the rate of chemical exchange. In summary, very low intermolecular affinity is evidenced by the absence of response in a STD spectrum. Although previous works on NOE measurements of the interacting atoms of α-CyD and drugs provided insight into the structure of these complexes, [27,28] to our knowledge, there is no report where NOE-STD coupled experiment was applied for screening the molecular association between a drug and a CyD.

The spectrum in Fig. 2 shows the result of a STD experiment performed after selective excitation of the GSH proton at $\delta = 4.6$ ppm. It can be seen that the excitation of these protons was transferred to the CyD nuclei (signals between 3.4 and 4.1 ppm). Similar results were obtained for the other well-identified GSH signals (2.0, 2.5 and

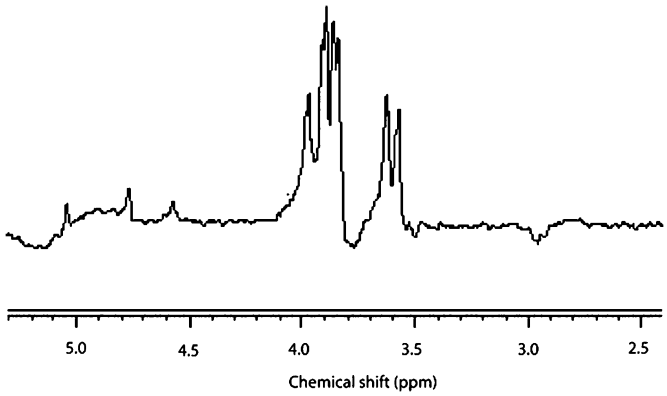

Fig. 2. STD spectrum of the complex GSH/α-CyD (2.1:33.6 mM) obtained at 25 °C in D_2O. The signal of GSH at 4.6 ppm was selectively saturated.

2.9 ppm, data not shown). Overall, these results indicated the occurrence of inter molecular interactions between the CyD and the peptide. Consequently, the next step of the work was aimed at identifying the interacting groups and characterizing the association dynamics.

3.2. NMR titration experiments

We performed these NMR titration experiments taking into account previous information on their utility for the determination of the association constants and the geometry of CyD/proteins complexes [10,29,30]. Table 3A and 3B show the maximum ^{1}H-chemical shift displacements detected for the H3, H4, H6, H10 signals of the tripeptide after the addition of α-CyD at different concentrations. Unfortunately, H8 protons of the GSH molecule could not be quantitatively analyzed because of the overlap of their signals with those of α-CyD. In the case of H2 protons, signal overlap occurred only at low tripeptide to α-CyD ratios. Therefore, the values shown in Table 3 represent the maximum chemical shift displacement observed for this peak in conditions where it could be unequivocally identified. From these results it can be deduced that the effect of α-CyD on the chemical shifts characteristics of GSH was particularly relevant in the case of the γ-glutamate side chain protons (H2, H3 and H4), whereas it was slightly noticeable for the proton of the chiral center of L-cysteine, H6. Taking into account that the most affected protons of the guest should be the ones included inside the cavity of the CyD, these results suggest that the γ-glutamate residue of GSH is preferentially included in the α-CyD cavity, exhibiting the *C*-terminus of the peptide outside the cavity.

Table 3
^{1}H NMR chemical shift displacements, Δδ (ppm) of: H3, H4, H6 and H10 of GSH as a function of concentrations of α-CyD at 25 °C (A) and 37 °C (B)

Proton	α-CyD (M)	Δδ (ppm)
(*A*)		
H3′	0.0336	0.0155 (±0.0015)
H4′	0.0336	0.010 (±0.000)
H6′	0.0336	0.0040 (±0.000)
H10′	0.0924	−0.0055 (±0.0005)
H2′[a]	0.0084	0.0225 (±0.0005)
(*B*)		
H3′	0.0336	0.0170 (±0.0)
H4′	0.0336	0.0135 (±0.0005)
H6′	0.0336	0.0123 (±0.0017)
H10′	0.0924	−0.0060 (±0.002)

Chemical shift displacements were expressed as $\Delta\delta = \delta_{\text{free GSH}} - \delta_{\text{complexed GSH}}$. The concentration of GSH was 2.0 mM.

[a] This chemical shift displacement was observed at the concentration of α-CyD where no overlapping occurred between the H2′ of GSH and the H5′ of α-CyD.

The molecular interactions taking place between host and guest were further investigated by monitoring the chemical shifts of the H2 protons of GSH. These continuous variation plots were intended for determining the stoichiometry of the complexation reaction. These plots (Fig. 3) showed a maximum at a 0.5 host/guest molar ratio, thus indicating that in aqueous solution the 1:1 tripeptide/α-CyD complex is formed.

Once known the stoichiometry of the complex, we applied the Hanna's equation (Eq. (1)) derived on the basis of the 1:1 complex formation, in order to obtain the association binding constant (K_a) of the complexes:

$$1/\Delta\delta_{obs} = 1/(K_a \cdot \Delta\delta[H_0]) + 1/\Delta\delta_{max} \quad (1)$$

where $\Delta\delta_{obs}$ is the chemical shift difference of GSH in the absence and presence of different concentrations of CyD, $\Delta\delta_{max}$ is the chemical shift difference between a nucleus in the guest molecule and the same nucleus in the host–guest complex, and $[H_0]$ is the concentration of CyD.

As shown in Fig. 4, the linear plots of Eq. (1) made feasible the calculation of the K_a values corresponding to H3 and H4. These values did not change significantly depending on the temperature, being in the range of 55–70 M^{-1} at 25 °C and in the range 68–72 M^{-1} at 37 °C.

On the other hand, given the low K_a values (less than 100 M^{-1}) we found it important to validate these data by calculating the stability constants according to the alternative Scatchard plot (Fig. 5), Eq. (2) [17]. The values calculated for H3 by this linearization procedure (75.2 M^{-1} at

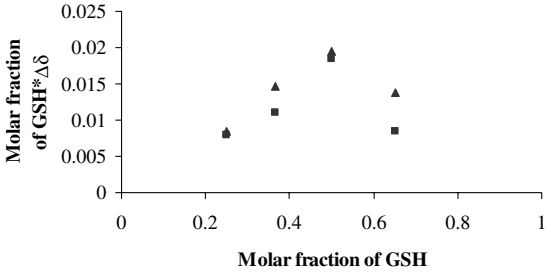

Fig. 3. ^{1}H NMR chemical shift displacement, Δδ (ppm), determined at different molar fractions GSH/α-CyD in D_2O at 25 °C. Chemical shifts of H2 protons of GSH were monitored. The total concentration of GSH and α-CyD was 0.048 M. H2 (▲), H2′ (■).

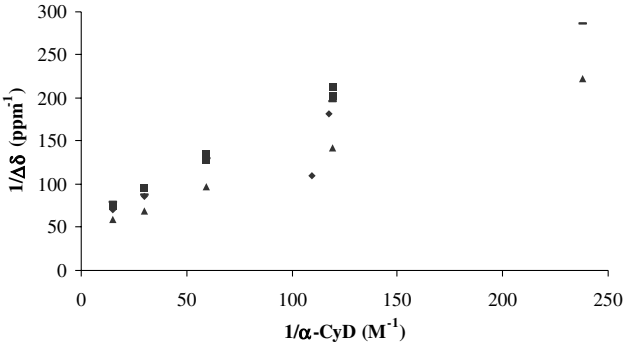

Fig. 4. Plot of the Hanna data treatment according to Eq. (1) for the ^{1}H NMR chemical shift displacements, $\Delta\delta$ (ppm), of different protons of GSH. H3, 25 °C (♦); H4, 25 °C (■); H3, 37 °C (▲); H4, 37 °C (–). Fit results were the following: H3 25 °C, $y = 0.7805x + 58.645$, $r = 0.838$; H4 25 °C, $y = 1.2732x + 56.05$, $r = 0.997$; H3 37 °C, $y = 0.7338x + 50.388$, $r = 0.998$; H4 37 °C, $y = 0.944x + 68.148$, $r = 0.994$.

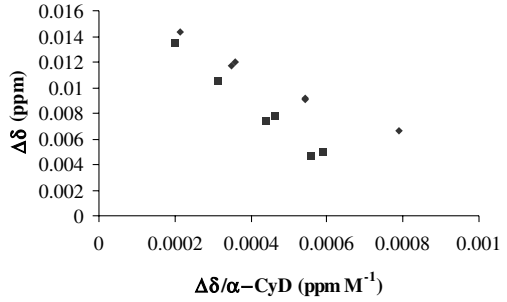

Fig. 5. Plot of the Scatchard data treatment according to Eq. (2) for the ^{1}H NMR chemical shift displacements, $\Delta\delta$ (ppm), of different protons of GSH. H3, 25 °C (♦); H4, 25 °C (■); H3, 37 °C (▲); H4, 37 °C (–). Fit results were the following: H3 25 °C, $y = -13.29x + 0.0167$, $r = 0.989$; H4 25 °C, $y = -22.651x + 0.0178$, $r = 0.992$; H3 37 °C, $y = -15.82x + 0.0208$, $r = 0.991$; H4 37 °C, $y = -15.513x + 0.0156$, $r = 0.992$.

25 °C and 63.3 M^{-1} at 37 °C) were in close agreement with those previously obtained by the Hanna's equation.

$$\Delta\delta/[H_0] = -(1/K_a) \cdot \Delta\delta + K_a \cdot \Delta\delta_{max} \quad (2)$$

3.3. 1D PFGSE-T ROESY experiments

As previously mentioned, STD experiments are particularly suited for the identification of molecular interactions between different molecular species. However, the relative long saturation time used for the STD experiments (typically 1–3 s) could be a limit of this experimental approach because it may cause some excitation to diffuse through the spin-system. Thus, the resulting STD will show the signals of not only the interacting groups, but also those of other groups affected by spin-diffusion (e.g. Fig. 2). A possible alternative to overcome this problem is to use the classical transient NOE experiments (NOESY), where the shorter mixing time (typically 100–500 ms) may reduce spin-diffusion enough so that only the interacting groups will be seen [31]. Unfortunately, the typical molecular weight range of CyD complexes (between 1000 and 2000 Da) have usually tumbling rates in which the competence between the positive and negative zero quantum relaxation transitions leads to a net NOE zero effect that is independent of how close the two dipolar coupled protons are in space. For these cases, experiments based on the Rotatory Overhauser Effect (ROE) become a suitable alternative [32]. Despite the fact that ROE experiments are less sensitive than those based on the NOE, ROE does not have a zero-effect zone for molecules of certain molecular weights. Consequently, ROE studies are mainly indicated for systems in the zero-NOE range (i.e. those between 1000 and 2000 Da), as is the case of the of GSH–α-CyD complexes.

Fig 6 shows the result of selective 1D-PFGSE-ROESY experiments performed on the well-isolated (vide infra) GSH signals (from top to bottom: 4.6, 2.9, 2.5 and 2.0 ppm). No ROE effect was observed for the first three groups. On the other hand, a clear intramolecular ROE could be detected at 2.0 ppm. Indeed, excitation of the H3 of GSH led to the detection of a triplet at 3.99 ppm ($J = 9.4$ Hz), that has been assigned to the H3 proton of α-CyD. The triplet structure of the observed ROE signal allows us to rule out any confusion with the H8 proton of GSH as this chemical group shows a signal in this region in the form of a doublet with two "satellite" peaks that integrate each one for less than 5% of the whole multiplet (Fig. 7). Furthermore, the observed coupling constant of the triplet was quite similar to that observed for the CyD ($J = 8.8$ Hz) and the chemical shifts in best agreement with those of the H3 proton of α-CyD. These protons are considered to be in the inner CyD cavity, close to the wider rim of the toroid that the CyD forms in water. On the contrary, the other observed ROEs (signals at 3.8 and 2.5 ppm) were assigned to signals arising from the GSH molecule and therefore, are considered indicative of intramolecular interactions. ROE results were in good agreement with

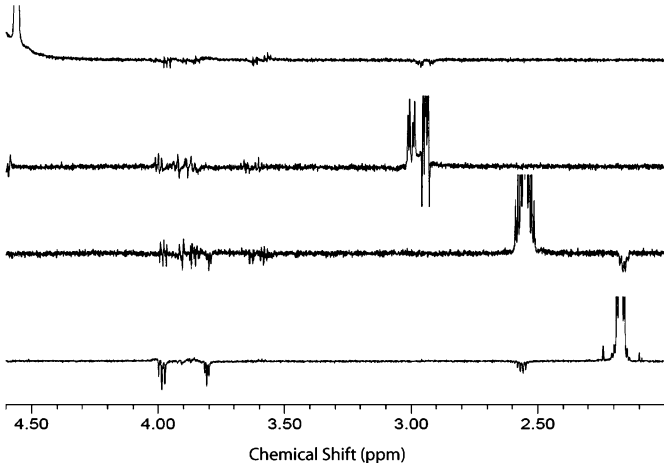

Fig. 6. Selective 1D-PFGSE-T ROESY experiments performed on GSH signals (from top to bottom: 4.6, 2.9, 2.5 and 2.1 ppm). The concentrations for α-CyD and GSH were 2.1 and 33.6 mM, respectively at 25 °C in D_2O.

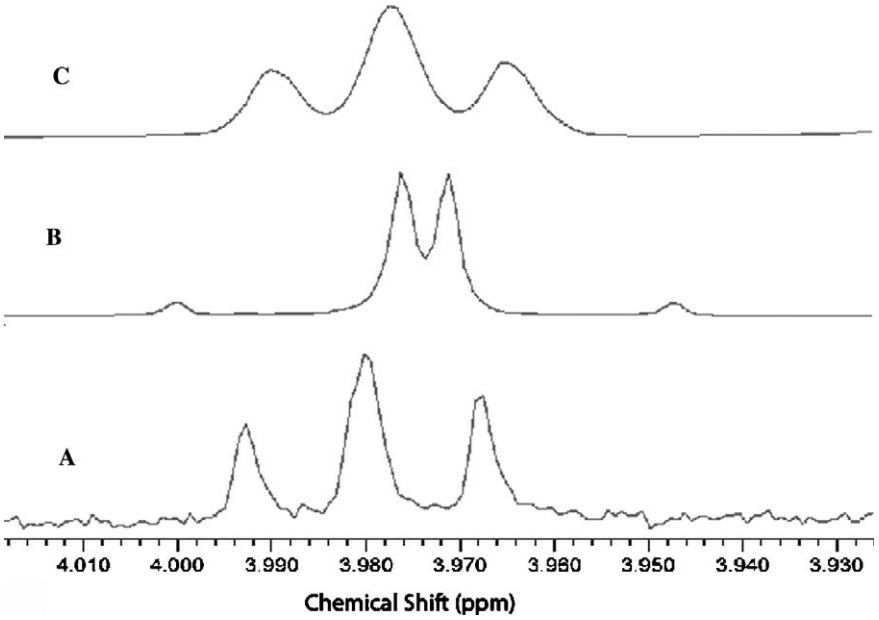

Fig. 7. Analysis of the peak shapes relevant for ROE identification: (A) ROE spectrum after selective irradiation of H3′ of GSH; (B) H8′ of GSH; (C) H3′ of α-CyD.

the data from the NMR titration experiments. In these last experiments, H3 and H4 were the two protons most affected by CyD complexation ($\Delta\delta_{max}$ for H3 = 0.0356 ppm and $\Delta\delta_{max}$ for H4 = 0.0165). The H2 group of GSH is a triplet at 3.8 ppm, that is overlapped at low GSH/CyD ratios. Due to this fact, we have excluded H2 group from any titration or ROE experiment. Nevertheless, it is noteworthy that at low GSH/CyD ratios, H2 was indeed the most affected signal (above 0.02 ppm already at 1:4 GSH/CyD ratio), and therefore, it is suggested that it may be a group deeply immersed in the CyD cavity. A graphical representation of the suggested inclusion mode is displayed in Fig. 8. With regard to the experimental procedures, some works have been previously reported where two-dimensional ROESY experiments gained insight into the inclusion mechanism of peptide–CyD complexes [24,33]. The enhanced sensibility of 1D-PFGSE-ROESY experiments due to the selective pulses led us to confirm that it is a convenient NMR tool that facilitates the interpretation of dynamic interactions such as those originating from drug–CyD complexation.

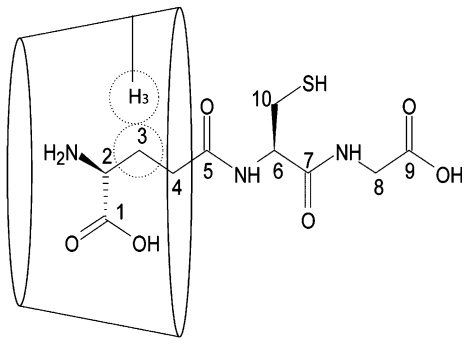

Fig. 8. Model of the inclusion of GSH in α-CyD consistent with the NMR titration and 1D-DPFGSE-T ROESY results.

3.4. Effect of α-CyD on the degradation of GSH

Studies performed in rats have shown that the administration of GSH could be beneficial in cases when peptide depletion occurs due to pathological conditions [34]. Unfortunately, this peptide cannot be administered by the most acceptable route of administration (i.e. orally) because it undergoes enzymatic degradation by the enzyme γ-glutamyltranspeptidase, a process characterized by the cleavage of the peptide bond between L-glutamate and L-cysteinylglycine by γ-glutamyltranspeptidase [35,36]. In the present work, our hypothesis was that the complexation of the peptide with CyD could help in reducing this degradation process. With this idea in mind, we analyzed the degradation of GSH, either in the free form or complexed with α-CyD, upon incubation with γ-glutamyltranspeptidase. The complexes selected for this study were those consisting of 8:1 and 32:1, α-CyD/GSH molar ratios. In order to study the peptide degradation process we have quantified by RP-HPLC analysis the area of the peak corresponding to non-degraded GSH that eluted at 7.4 min, rather than the peaks corresponding to the degradation products, L-glutamate and cysteinylglycine-dipeptide, because these latter molecules have a very short retention time and their peaks may lead to misinterpretations.

Fig. 9 shows the effects of α-CyD complexes on the degradation rate of GSH. The results show a 50% GSH degradation of the free peptide after two hours of incubation. Interestingly, this degradation rate could be reduced down to 40% in the case of the 8:1 GSH/α-CyD complex and to 27% for the 32:1 GSH/α-CyD complex. This marked

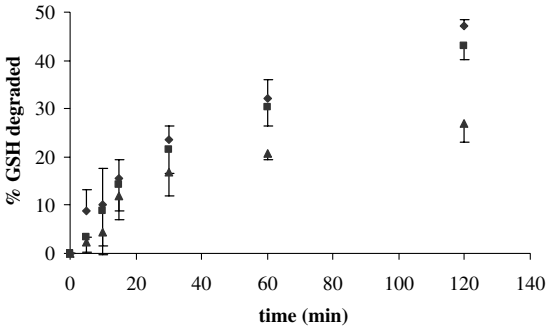

Fig. 9. Degradation of GSH (1.63 mM) with or without α-CyD in phosphate buffer (pH 6.8) with γ-glutamyltranspeptidase (0.8 U.I./ml) at 37 °C. Free GSH (♦), α-CyD/GSH 8:1 (■), α-CyD/GSH 32:1 (▲).

reduction of the enzymatic hydrolysis in a α-CyD concentration-dependent manner is attributed to the formation of a peptide–CyD complex that shields the molecule from enzymatic attack. Moreover, these data together with those from the NMR studies clearly evidence that the immersion of the L-glutamate (side chain) of GSH in the cavity of α-CyD protected this chemical moiety from degradation. On a wider perspective, this work highlights the stabilization strategy for peptides and proteins via the use of CyDs, a beneficial role that has been mainly evidenced for protein therapeutics including γ-globulin [37], lactate dehydrogenase [38], the Alzheimer amyloid β-A4 peptide [39].

4. Conclusion

In this work, we propose the formation of inclusion complexes between α-CyD and GSH for its protection in the gastrointestinal tract. The results of the NMR spectroscopic analysis indicate that the L-glutamate side chain of GSH, which is the one susceptible to enzymatic degradation, is included in the α-CyD cavity. The *in vitro* stability studies of GSH have confirmed the enhanced stability of the peptide thanks to its complexation with α-CyD.

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