New role for leucyl aminopeptidase in glutathione turnover

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A manganese-dependent cysteinyl-glycine hydrolysing activity has been purified to electrophoretic homogeneity from bovine lens. The characterization of the purified enzyme (molecular mass of the native protein, molecular mass of the subunit and extensive primary structure analysis) allowed the unequivocal attribution of the cysteinyl-glycine hydrolysing activity, which is usually associated with alanyl aminopeptidase (EC 3.4.11.2) or membrane-bound dipeptidase (EC 3.4.13.19), to LAP (leucyl aminopeptidase; EC 3.4.11.1). Analysis of the pH dependence of Cys-Gly hydrolysis catalysed by LAP, supported by a molecular modelling approach to the enzyme–substrate conformation, gave insights into the ability of the enzyme to recognize Cys-Gly as a substrate. Due to the effectiveness of LAP in hydrolysing Cys-Gly ($K_m = 0.57$ mM, $k_{cat} = 6.0 \times 10^3$ min^{-1} at pH 7.4 and 25 °C) with respect to other dipeptide substrates, a new role for this enzyme in glutathione turnover is proposed.

Key words: cysteinyl-glycine, cysteinyl-glycine hydrolase, glutathione metabolism, leucyl aminopeptidase, leucyl-glycine.

INTRODUCTION

Glutathione, the main cellular defence against oxidative stress, is catabolized by the ecto-enzyme GGT (γ-glutamyltranspeptidase), which transfers the glutamate moiety to an α-amino acid, to give γ-glutamyl amino acid and Cys-Gly. The role of GGT in allowing the recovery of extracellular GSH through the generation of membrane-permeable precursors, useful for intracellular resynthesis of GSH, is widely accepted [1–3]. Moreover, the body of experimental evidence is growing, suggesting the pro-oxidant capability of GGT or GGT-related enzymes, especially in the presence of transition metal ions, on different targets and in several cell models [4–10]. Indeed, it appears to be clear now that the pro-oxidant action of the GGT/GSH/Me^{n+}/$Me^{(n-1)+}$ system is related to the special redox features of Cys-Gly, which is formed in the GGT-catalysed reaction [4,5,11–14]. Thus, whereas Cys-Gly acts as an effective source of reactive oxygen species in the presence of Fe^{3+} or Cu^{2+}, its oxidation product, Cys-Gly disulphide, is able to intervene as an active protein thiolating agent *in vivo* [14–16] and *in vitro* [17,18]. Because of its cell damaging ability [5–13], as well as its physiologically relevant signalling potential [13,19,20], the level of Cys-Gly needs to be controlled. Thus enzymes capable of catabolizing Cys-Gly can play a role in the homeostasis of the reduced status of the cell.

In the 1950s, an enzyme activity capable of hydrolysing Cys-Gly was isolated from pig kidney [21–23]. Later on, in the late 1970s, such a Cys-Gly hydrolase activity was associated with the brush-border aminopeptidase M (now called alanyl aminopeptidase, EC 3.4.11.2) [24,25] and the properties of the former attributed to the latter [26]. Another enzyme capable of hydrolysing Cys-Gly is the membrane-bound dipeptidase (EC 3.4.13.19), first purified from pig kidney [27], which is considered to be the major route of degradation of the oxidized dipeptide [28]. In a preliminary communication, we proposed that most of the high Cys-Gly hydrolase activity, measurable in bovine lens, is associated with cytosol LAP (leucyl aminopeptidase; EC 3.4.11.1) [29]. More recently, on the basis of kinetic and immunoprecipitation studies performed on crude cytosolic fraction of rat liver, it was reported that leucyl aminopeptidase may be responsible for the hydrolysis of Cys-Gly in rat liver [30].

In the present study, we describe the purification of lens cysteinyl-glycine hydrolase activity to electrophoretic homogeneity and show unequivocal evidence that the lens Cys-Gly hydrolase activity is actually associated with the soluble enzyme LAP; we also give the rationale for Cys-Gly hydrolase activity of LAP, which then becomes a new potential tool for the control of the cell redox status.

EXPERIMENTAL

Materials

Cys-Gly, DTT (dithiothreitol), iodoacetamide, trypsin, BSA, SDS, sequencing grade β-lactoglobulin and molecular-mass markers were purchased from Sigma (St. Louis, MO, U.S.A.). Ninhydrin was from Merck (Darmstadt, Germany). DEAE-cellulose (DE-52) was obtained from Whatman. Sephacryl S-300, PhastGel Gradient 10–15 electrophoresis gels and PD10 columns were from Amersham Biosciences (Uppsala, Sweden). Centricon 30 microconcentrators were from Amicon. All inorganic chemicals were of reagent grade from BDH (Poole, U.K.).

Calf eyes were obtained from freshly slaughtered animals at the local slaughterhouse and the lenses were frozen until use.

Cys-Gly hydrolase assay

A colorimetric method and a capillary electrophoresis method were developed to measure Cys-Gly hydrolase activity. In both

Abbreviations used: DTT, dithiothreitol; ESI-MS, electrospray ionization MS; GGT, γ-glutamyltranspeptidase; LAP, leucyl aminopeptidase; LC-ESI-MS/MS, liquid chromatography 'on line' coupled with electrospray tandem MS.

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cases, the standard incubation mixture (final volume 250 μl) contained 0.2 mM $MnCl_2$, 5 mM DTT and 2 mM Cys-Gly in 32 mM Tris borate buffer (pH 8.5). The reaction was started by the addition of the extract.

For the colorimetric assay, essentially based on the specific reaction between cysteine and ninhydrin [31], the reaction was stopped at appropriate times by the addition of 5 % trichloroacetic acid and the incubation mixture was centrifuged at 12 000 ***g*** for 1 min in a Beckman Microfuge E. Supernatant (200 μl) was added to 200 μl of glacial acetic acid and 200 μl of a reagent prepared by dissolving 250 mg of ninhydrin in 10 ml of acetic acid/4 M HCl (3 : 2) [31]. The mixture was placed in a boiling bath for 10 min and then cooled on ice. After cooling, 300 μl of the mixture was diluted with 1 ml of 95 % ethanol and used for spectrophotometric measurements at 560 nm.

For the capillary electrophoresis assay, the reaction was stopped at the appropriate time by placing the incubation mixture in a boiling bath for 3 min and the incubation mixture was centrifuged for 1 min at 12 000 ***g***. The supernatant was subjected to ultrafiltration through Amicon Centricon 3 and the ultrafiltrate used for the determination of cysteine and Cys-Gly residues. Capillary electrophoresis was performed at a constant voltage of 30 kV with the cathode positioned at the outlet, using 100 mM Tris/borate buffer (pH 8.5) as running buffer. The temperature of the capillary during electrophoresis was kept constant at 25 °C. Between runs, the capillary was washed with 0.1 M sodium hydroxide and water, followed by reconditioning with running buffer. The samples were injected by pressurization with nitrogen at 5×10^5 Pa for 8 s and electropherograms were monitored at 214 nm.

Cysteine concentration was calculated for both methods after subtraction of a blank obtained by terminating the reaction at time 0.

One unit of Cys-Gly hydrolase activity is defined as the amount of enzyme that catalyses the hydrolysis of 1 μmol/min of Cys-Gly at 25 °C under the above assay conditions.

Equipment

Capillary electrophoresis analysis was performed with a Beckman P/ACE system 2000 equipped with an IBM PS/2 (Windows™-based control including System Gold™ software from Beckman). Capillaries were of fused silica, 50 cm long, 75 μM inner diameter, supplied by Polymicro Technologies (Phoenix, AZ, U.S.A.) and assembled in the Beckman cartridge format. The spectrophotometric analyses were performed with a Beckman DU-7 spectrophotometer.

Purification of bovine lens Cys-Gly hydrolase

Bovine lens Cys-Gly hydrolase activity was purified by the procedure first described by Binkley et al. [22] for pig kidney with some modifications. All procedures were carried out at 4 °C unless otherwise stated. Bovine lenses were suspended in about 5 vol. (w/v) of 10 mM sodium phosphate buffer (pH 7.0), supplemented with 5 mM DTT and stirred in an ice-cold bath for 1 h. The suspension was then centrifuged at 20 000 ***g*** for 20 min at 4 °C. The supernatant, which is referred to as 'lens extract', was added to 0.25 vol. of DE-52 equilibrated with 10 mM sodium phosphate buffer (pH 7.0), supplemented with 2 mM DTT (standard buffer) and stirred in an ice-cold bath. After 45 min, the resin was filtered through a Buchner funnel and the filtrate containing the Cys-Gly hydrolase activity was used for further purification. Ice-cold ethanol (1.5 vol) was slowly added to the DE-52 filtrate. After 30 min stirring, the suspension was centrifuged at 17 500 ***g*** for 20 min. After centrifugation, the precipitate was discarded and an additional volume of ethanol was added to the ensuing supernatant. After 30 min stirring, the suspension was again centrifuged. The precipitate containing the Cys-Gly hydrolase activity was collected and dissolved in standard buffer. The solution was made to 0.1 M with magnesium acetate and brought, under stirring, to pH 9.3, by dropwise addition of ammonium hydroxide. After 15 min, the precipitate was removed by centrifugation and discarded. An equal volume of a mixture of chloroform/octanol (19 : 1) was added to the supernatant and the mixture was vigorously shaken at room temperature (20 °C) for 10 min. The emulsion was then centrifuged at 250 ***g*** for 3 min and the supernatant, which contained the enzyme activity, was collected by suction. Chloroform/octanol treatment was repeated until little emulsion was formed. The supernatant resulting from the last step was allowed to stand overnight at 4 °C and then centrifuged at 15 000 ***g*** for 20 min. Aliquots of 3.5 ml of the resulting supernatant were applied to a Sephacryl S-300 column (1.5 × 90 cm), equilibrated with standard buffer supplemented with 0.2 mM manganese chloride. The flow rate was 30 ml/h and 2 ml fractions were collected. The active fractions were pooled, analysed by SDS/PAGE, concentrated by ultrafiltration on Amicon Centricon 30 to approx. 0.2 units/ml and stored at −80 °C until use.

In-gel alkylation and digestion

Bands from SDS/PAGE analysis were excised, washed with water, reduced with 10 mM DTT in 50 mM NH_4HCO_3 (45 min at 55 °C) and S-alkylated with 55 mM iodoacetamide in 50 mM NH_4HCO_3 (30 min at 25 °C). Gel particles were washed with 50 mM NH_4HCO_3 and acetonitrile (twice), dried and rehydrated with the digestion solution (25 ng/μl of trypsin in 50 mM NH_4HCO_3/5 mM $CaCl_2$). After incubation at 5 °C for 1 h, the supernatant was replaced by 50 mM NH_4HCO_3, 5 mM $CaCl_2$, and gel particles were incubated overnight at 37 °C. Peptides were extracted with 25 mM NH_4HCO_3/acetonitrile (1 : 1, v/v) by sonication and concentrated.

LC-ESI-MS/MS (liquid chromatography 'on line' coupled with electrospray tandem MS) analysis

Protein digests were analysed using an LCQ Deca Xp Plus mass spectrometer (ThermoFinnigan, San Josè, CA, U.S.A.) equipped with an electrospray source connected to a Phoenix 40 pump (ThermoFinnigan). Peptide mixtures were separated on a capillary Phenomenex Jupiter C_{18} column (250 × 0.32 mm, 5 μm), using a linear gradient from 10 to 50 % acetonitrile in 0.2 % formic acid, over a period of 65 min, at a flow rate of 10 μl/min. Spectra were acquired 'on-line' in the range m/z 200–2000. The instrument was calibrated using a mixture of caffeine, MRFA peptide and Ultramark 1621. Data were analysed using BioWorks software provided by the manufacturer. TurboSequest software package was used to identify protein bands from independent non-redundant protein sequence databases.

Protein sequence analysis

Automated N-terminal degradation of the purified peptides was performed by using a Milligen 6600 protein sequencer. The N-terminal sequence obtained was compared with those stored in the SwissProt database using a PC gene program.

Alkylation under non-reducing conditions

To block cysteine residues in the native reduced state, protein samples were alkylated with 1.1 M iodoacetamide, 0.25 M Tris/HCl, 1.25 mM EDTA and 6 M guanidinium chloride (pH 7.0), at room temperature for 1 min. Protein samples were freed from salt and reagent excess by passing the reaction mixture through a PD10 column. Samples were manually collected, dried and further analysed by SDS/PAGE or ESI-MS (electrospray ionization-MS).

ESI-MS analysis

Native or alkylated protein samples (50 μg) were purified on a narrow bore Vydac 214TP52 column (250 mm × 2.1 mm, 5 μm), eluted with a linear gradient from 10 to 60 % acetonitrile in 0.1 % trifluoroacetic acid, over a period of 60 min, at a flow rate of 0.2 ml/min. Protein samples were manually collected, dried and dissolved in 10 % acetic acid. ESI mass spectra were recorded using an API-100 single quadrupole mass spectrometer (PerkinElmer-SCIEX, Thornhill, Canada) equipped with an atmospheric pressure ionization source, as previously reported [18]. Data were analysed using the BioMultiView 3.1 software provided by the manufacturer. Mass calibration was performed by means of the multiply charged ions from a separate injection of horse heart myoglobin (molecular mass 16.9515 kDa). Masses are reported as average values.

Other methods

SDS/PAGE of the purified enzyme was performed according to the method of Laemmli [32], and gels were stained with silver nitrate according to the method of Wray et al. [33]. The proteins used as molecular-mass standards for the determination of the apparent molecular mass of the subunit by SDS/PAGE were BSA (molecular mass, 66 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa) and carbonic anhydrase (29 kDa).

The proteins used as molecular mass standards for the determination of the apparent molecular mass of the native enzyme by gel filtration on S-300 were apoferritin (443 kDa), β-amylase (200 kDa), ovalbumin (43 kDa) and ribonuclease A (13.7 kDa).

Protein concentration was estimated by the Coomassie Blue binding assay [34] with BSA as the standard. The activity of the enzyme towards dipeptides other than Cys-Gly was measured by following the decrease in A_{225} and A_{230} associated with the hydrolysis of the dipeptide.

Kinetic constants of the enzyme were calculated by non-linear regression analysis of at least five different rate measurements.

Molecular modelling

Modelling of complexes in which proteins bind to bi- or tervalent metal ions presents a rather problematic task, because of the difficulty in predicting correct complex geometries using the current simplified biomolecular force fields [35]. The problem is even more severe when: (i) more than one ion is involved in a single complexation site; (ii) no direct experimental information is available to derive geometrical constraints on the site; and (iii) investigations of either dynamic features of different complexation states or compared analysis of different ligand behaviours, prevent application of a totally restrained approach, in which the geometrical parameters of each metal binding site are kept fixed during all simulations. Unfortunately, in our system, these three unfavourable conditions occur. Thus, as an alternative to a sophisticated and time-consuming approach, based on an extensive reparametrization of the force-field (even potentially including modification in the functional forms of several terms), in combination with expensive quantum-mechanical calculations, which is currently under development in our laboratory, we decided to apply a very simple and apparently reductive approach in which only the substrates (and optionally few LAP side chains) were allowed to relax.

Modelled complexes of LAP with dipeptides were based on the highest resolution structure so far available for LAP complexes, the 1.65 Å (1 Å = 0.1 nm) structure of the complex of LAP(Zn_2) with an inhibitor (L-leucine phosphonic acid) [36] and 1LCP entry in the Protein Data Bank database [37]. The LAP(MnZn) form of the enzyme was obtained by replacement of the Zn ion in the activation site (I) with a Mn bivalent ion. In the adopted model, all the intra-molecular and the inter-molecular LAP–metal ion interactions were model-invariant. In particular, the two metal ions form a network of fixed interactions: site (I) with $Asp^{255}O_{\delta 2}$ (2.12 Å), $Asp^{332}O$ (2.19 Å) and $O_{\delta 1}$ (2.02 Å), and $Glu^{334}O_{\varepsilon 1}$ (2.01 Å), and site (II) with Lys^{250} (2.12 Å), $Asp^{273}O_{\delta 2}$ (2.12 Å), $Glu^{334}O_{\varepsilon 2}$ (2.07 Å) and substrate peptide N-terminus (2.28 Å). Thus only the structure of the peptide (except for the fixed metal-complexing N-terminal atom) was energy-minimized, while keeping fixed the structure of LAP and metal ions.

Besides Cys-Gly, Leu-Gly, a representative substrate of LAP, and Asp-Gly, a dipeptide which is not hydrolysed by LAP (see Table 3), were built by a best fit of their backbone atoms with the corresponding atoms of the LAP inhibitor. The different side-chain arrangements sampled for each peptide were based on the criteria of maximum superposition with the template inhibitor side chain, minimal bump with LAP atoms and (for Cys-Gly and Asp-Gly) maximum direct interaction of side chains with metal ions in the active site.

In vacuo energy minimization calculations were performed with the AMBER 5 package [38], using the all-atom 1986 force field of Weiner et al. [39], a distance-dependent dielectric and a convergence criterion based on the energy gradient (r.m.s. of the Cartesian elements of the gradient < 0.001 kcal $\cdot$ mol^{-1} $\cdot$ Å^{-1}). The non-bonded terms used for the Zn ion were those already present in the Weiner et al. [39] force field, whereas for the Mn ion, we used the non-bonded terms of Bradbrook et al. [40].

The molecular graphics program MOLMOL was used to analyse and represent the structures of the different LAP complexes [41].

RESULTS

Measurement of Cys-Gly hydrolase activity

Two methods have been developed for the measurement of Cys-Gly hydrolase activity and both of them were used to follow the purification and to perform the characterization of the enzyme.

The first method is based on the separation of Cys-Gly and cysteine by capillary electrophoresis. Thus both the residual substrate and reaction product can be detected with this method. The inset of Figure 1 shows the electropherogram obtained when a mixture of cysteine, Cys-Gly and DTT was subjected to capillary electrophoresis separation. DTT was added to the mixture to avoid the oxidation of the two thiol compounds. Cysteine and Cys-Gly showed a migration time of 5.2 and 6.9 min respectively. A linear relationship between peak area and concentration was observed in the range of concentrations tested (Figure 1). The limits of detection were 0.1 mM and 0.2 mM for cysteine and Cys-Gly respectively.

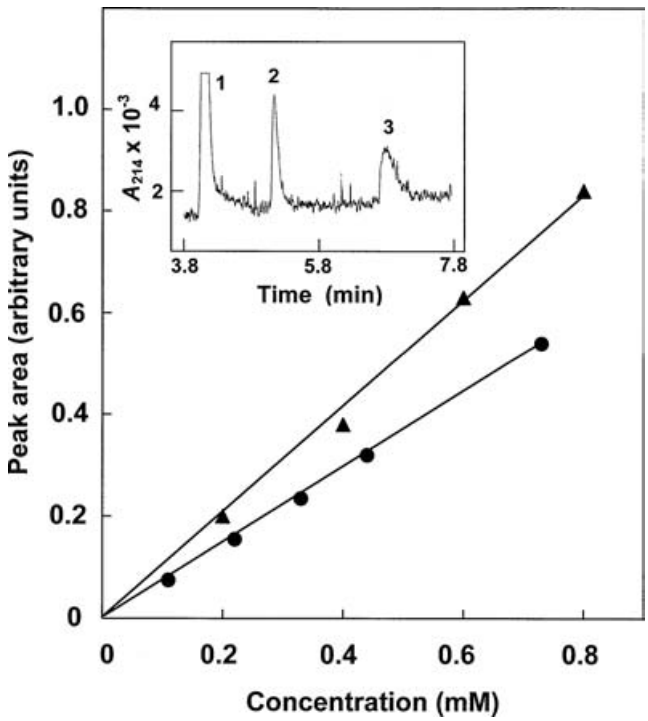


Figure 1 Determination of cysteine and Cys-Gly concentrations by capillary electrophoresis method

Peak area versus concentration is shown for cysteine (●) and Cys-Gly (▲). Inset: separation of 0.4 mM cysteine (peak 2), 0.4 mM Cys-Gly (peak 3) and 4 mM DTT (peak 1) by capillary electrophoresis.

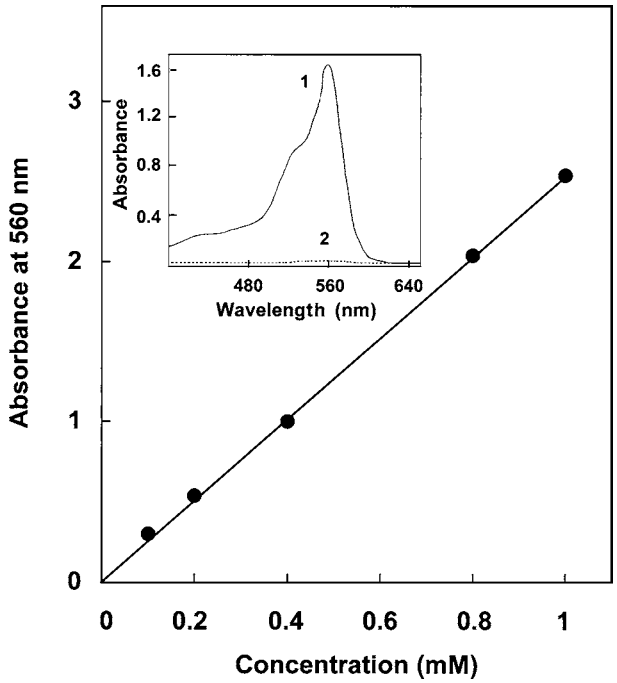


Figure 2 Determination of cysteine concentration by colorimetric method

The A_{560} versus cysteine concentration is shown. Inset: spectra obtained after treatment of 1 mM cysteine (spectrum 1) or 1 mM Cys-Gly (spectrum 2) with ninhydrin reagent as described in the Experimental section.

The second method adopted is based on the determination of the cysteine produced by spectrophotometric analysis of the product of the reaction between cysteine and ninhydrin under acidic conditions [31]. Cysteine specifically reacts with ninhydrin at 100 °C in the presence of acetic acid/HCl mixture to give a pink coloured complex that can be estimated spectrophotometrically. Inset to Figure 2 shows the absorption spectra obtained after boiling a mixture of 1 mM cysteine (solid line) or 1 mM Cys-Gly (dotted line) with ninhydrin. The spectrum of the treated cysteine shows a characteristic peak in the 500–600 nm zone with a maximum at 560 nm, which is absent in the spectrum of treated Cys-Gly. From the spectrum, an absorption coefficient of 34.8 $mM^{-1} \cdot cm^{-1}$ was calculated for cysteine at 560 nm. A calibration curve was drawn by plotting the absorbance measured at 560 nm versus cysteine concentration: a linear relationship was obtained up to 1 mM (Figure 2). The detection limit for cysteine was 0.025 mM. Besides Cys-Gly, neither DTT, GSH nor other physiological thiols interfered in the reaction between cysteine and ninhydrin [31].

Calibration curves drawn for both methods were used for the measurement of Cys-Gly hydrolase activity in lens extracts prepared as described in the Experimental section. Time courses of the reaction showed a linear relationship between the amount of cysteine produced and time for at least 25 min with both methods (results not shown). The rate of cysteine formation as a function of increasing protein concentrations was linear at least up to 8 mg/ml (results not shown).

Enzyme purification

Cys-Gly hydrolase activity of bovine lens was purified approx. 1000-fold with an overall yield of 12 % with a procedure including DE-52 chromatography, ethanol precipitation, treatment with chloroform/octanol and S-300 chromatography. Table 1 summarizes the purification procedure of the enzyme to apparent electrophoretic homogeneity.

Cys-Gly hydrolase activity was stable for at least 6 months at 4 °C in the preparation derived from the DE-52 batch step and for at least 2 weeks in the supernatant from chloroform/octanol treatment maintained at 4 °C. Further purification of the enzyme resulted in a loss of stability. The omission of $MnCl_2$ from the buffer utilized for S-300 column elution resulted in the complete inactivation of the enzyme within 48 h. Pure enzyme was stable for at least 2 weeks if stored at − 80 °C in the presence of 0.2 mM $MnCl_2$.

Characterization of the purified enzyme

Purified Cys-Gly hydrolase activity showed a single band on SDS/PAGE with an apparent molecular mass of 53.7 kDa (Figure 3, inset). Purity of the enzyme preparation was also confirmed by sequence analysis data; in fact, the Edman degradation of the protein gave a single residue in each of the first ten cycles with yields comparable with those measured with sequencing grade β-lactoglobulin used as control. A molecular mass of approx. 320 kDa was evaluated for the native enzyme obtained by gel filtration on Sephacryl S-300 (Figure 3), thus suggesting an homohexameric structure for the enzyme.

To identify the protein species reported in Figure 3, the gel band was reduced, alkylated, digested *in situ* with trypsin and the extracted peptides were directly subjected to LC-ESI-MS/MS analysis. Figure 4 shows two collision-induced dissociation spectra, among those obtained for 23 different peptides, generated following chromatographic separation. All tandem MS data were used to search non-redundant protein databases with an efficient program. This analysis clearly identified the species as bovine

Table 1 Purification of bovine lens Cys-Gly hydrolase

Fraction	Total volume (ml)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Lens extract	40	12	0.010		
DE-52 filtrate	40	11.6	0.019	97	1.9
Ethanol precipitation	24	9.8	0.013	82	1.3
Magnesium acetate precipitation	14	2.5	0.045	21	4.5
Chloroform precipitation	10.8	2.8	0.081	23	8.1
Sephacryl S-300	8	1.44	10.9	12	1090

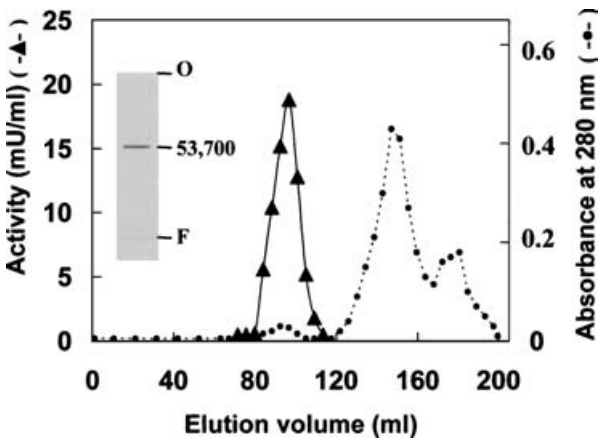


Figure 3 Gel chromatography on Sephacryl S-300 column

See the Experimental section for details. Inset: SDS/PAGE analysis of Cys-Gly hydrolase activity purified from bovine lens; O: origin, F: front.

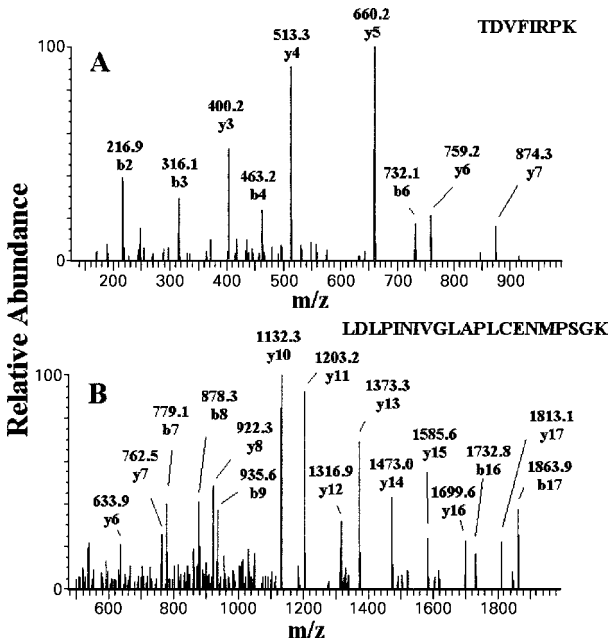


Figure 4 Tandem mass spectra resulting from tryptic digest peptides of the purified Cys-Gly hydrolase

Spectra were generated from the doubly charged parent ions at *m/z* 488.4 (**A**) and 1126.5 (**B**). In both panels, the observed masses of some fragment ions are shown together with the identified sequence.

Table 2 LC-ESI-MS-MS analysis of a LAP sample alkylated under denaturing and non-reducing condition, following digestion with trypsin

The assignment of each signal to the corresponding peptide was obtained on the basis of the mass value, protease specificity and mass fragmentation data. For simplicity, only the peptides containing cysteine residues are reported. CAM, carboxamidomethylated.

Mass (kDa)	Peptide
3.8908 ± 0.0011	(99–134) CAM
3.3019 ± 0.0008	(397–423) CAM
2.2501 ± 0.0008	(290–310) CAM
1.8457 ± 0.0004	(337–352) CAM
1.6926 ± 0.0007	(272–289) CAM
1.6859 ± 0.0005	(409–423) CAM
1.1667 ± 0.0002	(426–437) CAM
0.7746 ± 0.0003	(91–98) CAM

cytosolic LAP (SwissProt entry P00727). The peptide map obtained covered almost 41 % of the entire sequence and suggested the absence of the amino acid substitutions previously reported for this enzyme [42,43]. Moreover, an independent ESI-MS analysis of the intact purified protein showed a molecular mass of 52.8742 ± 0.0069 kDa. This value was not in agreement with the theoretical one (52.9124 kDa), calculated on the basis of the LAP primary structure and the presence of an acetyl group at the N-terminus (SwissProt P00727). The observed mass difference ($\Delta m \approx -0.042$ kDa) was associated with a probable absence of this blocking group. This hypothesis was confirmed by automated Edman degradation that allowed the determination of the sequence of the first ten residues as TKGLVLGIYS, thus demonstrating that the enzyme in lens tissues is not acetylated. Furthermore, an independent ESI-MS analysis of the intact enzyme following alkylation under denaturing and non-reducing conditions (measured molecular mass, 53.2739 ± 0.0054 kDa) and mapping experiments on its tryptic digest (Table 2) confirmed that all seven cysteine residues present in LAP occur in a reduced state.

A relevant feature of the Cys-Gly hydrolase activity of the purified bovine lens LAP was its dependence on divalent cations. The purified enzyme was inactive if assays were carried out in the presence of 5 mM EDTA. Dialysis of the purified enzyme, which was stored in the presence of $MnCl_2$, against a buffer lacking the metal ion resulted in the complete loss of enzyme activity (results not shown). The dialysed enzyme was reactivated after the addition of $MnCl_2$, while $ZnCl_2$ had no effect.

Effect of pH on Leu-Gly and Cys-Gly hydrolase activity of LAP

The kinetic parameters of the enzyme towards Cys-Gly and other dipeptides were determined at three different pH values and are reported in Table 3. The dipeptides tested were chosen on the basis of known features of LAP [44], which is reported to prefer a bulky hydrophobic amino acid at the N-terminus of dipeptides. Surprisingly, the enzyme displays the lowest K_m values for

Table 3 Kinetic parameters of bovine lens Cys-Gly hydrolase

Kinetic parameters are expressed as estimated value ± S.E.M.

	K_m (mM)			k_{cat} (min^{-1})		
Substrate	pH 6.9	pH 7.4	pH 8.3	pH 6.9	pH 7.4	pH 8.3
Cys-Gly	0.42 ± 0.048	0.57 ± 0.057	0.59 ± 0.012	3400 ± 350	6000 ± 150	7100 ± 150
Leu-Gly	2.3 ± 0.50	2.5 ± 0.20	1.5 ± 0.15	11500 ± 2000	24000 ± 650	40500 ± 6000
Met-Gly	2.8 ± 0.58	1.5 ± 0.24	1.3 ± 0.10	8150 ± 950	28100 ± 1650	59300 ± 10000
Ser-Gly	N.D.*	5.2 ± 0.29	5.0 ± 0.61	N.D.	1000 ± 25	2500 ± 150
Asp-Gly	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

*N.D., not detectable.

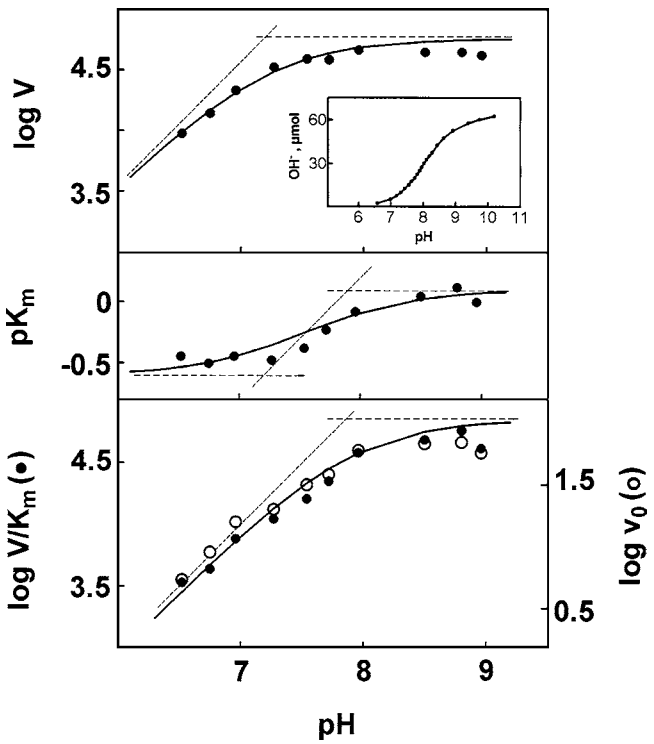


Figure 5 Effect of pH on the kinetic parameters of Leu-Gly hydrolysis by bovine lens LAP

log V, pK_m and log V/K_m are shown as a function of pH with Leu-Gly as substrate. Bottom panel: log of the reaction rate v_0 at a low Leu-Gly concentration as a function of pH is also reported. K_m and V values were obtained between pH 6.52 and 8.96 by interpolation of at least six rate measurements. Solid curves are computer-drawn plots of the theoretical pH curves obtained by fixing a pK_a for the enzyme complex at 7.2 and a pK_a for the free substrate at 7.9. To highlight the pK_a values, dashed lines, drawn according to the classical approach to analyse pH curves [45], were superimposed. Inset: 15 ml of 5 mM Leu-Gly was titrated by the addition of 0.5 M NaOH.

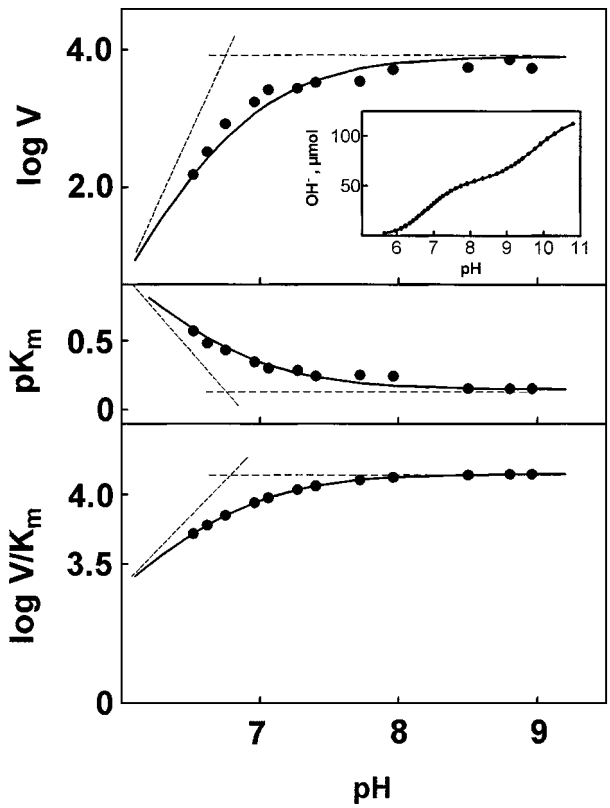


Figure 6 Effect of pH on the kinetic parameters of Cys-Gly hydrolysis by bovine lens LAP

log V, pK_m and log V/K_m are plotted as a function of pH with Cys-Gly as substrate. K_m and V values were obtained between pH 6.52 and 8.96 by interpolation of at least six rate measurements. Solid curves are computer-drawn plots of the theoretical pH curves obtained by fixing two concurrent pK_a for the enzyme complex at 6.75 and a pK_a for the free substrate at 6.75. To highlight the pK_a values, dashed lines, drawn according to the classical approach to analyse pH curves [45], were superimposed. Inset: 15 ml of 5 mM Cys-Gly was titrated by the addition of 0.5 M NaOH.

Cys-Gly and its catalytic efficiency is comparable with, if not higher than, that observed for the hydrophobic peptides Leu-Gly and Met-Gly, especially near physiological pH values. To identify the special features enabling Cys-Gly to be recognized as a substrate for LAP, a comparative analysis of the pH effect on the Cys-Gly and Leu-Gly enzymic hydrolysis was performed. V and K_m values for Cys-Gly and Leu-Gly were determined at different pH values with concentrations of Leu-Gly ranging from 0.5 to 5 mM (Figure 5) or concentrations of Cys-Gly ranging from 0.2 to 1.2 mM (Figure 6). For Leu-Gly used as substrate for LAP, analysis of the plot of log V versus pH revealed a dissociation process on the enzyme–substrate complex affecting the catalytic activity with an apparent pK_a of 7.2 (Figure 5, top panel). A similar pK_a value, associated with the enzyme–substrate complex (upward concavity) was evaluated from the pK_m versus pH curve (Figure 5, middle panel), which also revealed an additional dissociation process (downward concavity, pK_a of 7.9) either at the active site of the free enzyme or on the free substrate. Indeed, a dissociation process with an apparent pK_a

of 7.9 either on the free enzyme or on the free substrate was also shown by the data on the effect of pH on the reaction rates (v_0) measured in far sub-saturating conditions (i.e. $[S] \ll K_m$) (Figure 5, bottom panel). Analysis of the plot of log V versus pH for Cys-Gly as substrate (Figure 6, top panel), indicated an increase in the catalytic activity of the enzyme linked to two events of proton dissociation on the enzyme–substrate complex with an overall apparent pK_a of 6.75. A dissociative event with the same apparent pK_a of 6.75 was also observed in the curve representing the pH dependence of K_m (Figure 6, middle panel). In this case, the upward concavity of the curve (from slope -1 to 0), while confirming the relevance of catalysis of a dissociative phenomenon on the enzyme–substrate complex, is indicative of a relevant dissociation process occurring either on the free enzyme or, quite probably, on the free substrate. In this regard, because of the low values of K_m of LAP for Cys-Gly, a direct measurement of v_0 in highly sub-saturating conditions at different pH values was not feasible.

To define possible association between the dissociation processes as evidenced in the kinetic study and the substrate dissociation equilibria, both Leu-Gly and Cys-Gly were subjected to acid/base titration. A pK_a of 7.9 for the dissociation of the NH_2 group of Leu-Gly and two apparent pK_as of approx. 6.8 and 9.5 for the dissociation processes of the -SH and -NH_3^+ groups of Cys-Gly were found (see the Discussion section for the attribution of the two pK_a values to the dissociable groups). The titration curves for Leu-Gly and Cys-Gly are shown in the insets of Figures 5 and 6 respectively.

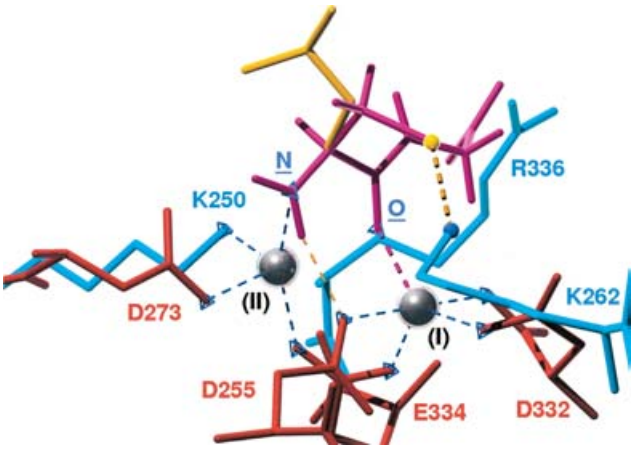


Figure 7 Molecular interactions of LAP with Leu-Gly and Cys-Gly

Superimposed models of LAP(Mn_2) complexes with Leu-Gly (yellow sticks) and Cys-Gly (magenta sticks) dipeptides are shown. The residues involved in metal complexation and proton transfer in the enzyme active site are labelled and shown as red (acid) or cyan (basic) sticks. Metal complexation is shown by representing the involved atoms as blue tetrahedrons and the corresponding interactions by blue (for the invariant part of the model, see the Experimental section) or magenta dashed lines. Orange dashed lines show intermolecular substrate–enzyme H bonds. The predicted H bond involving the side-chain sulphur atom (yellow sphere) of Cys-Gly and the side-chain nitrogen atom of enzyme K^{262} (blue sphere) is represented by a bold orange dashed line. Metal ions are labelled according to their usual numbering scheme in LAP literature. The substrate N and O atoms involved in metal complexation are in blue and underlined.

Molecular modelling

The modelling approach showed that, independently of the initial side-chain conformation of the tested dipeptides, a single stable conformation was obtained for the dipeptide–LAP complexes, depending on both the specific dipeptide sampled and the LAP form considered. This provided a putative interpretation at the atomic level for the different behaviours exhibited by these substrates in hydrolysis experiments.

The Leu-Gly dipeptide formed very similar complexes with both LAP(Zn_2) and LAP(MnZn). In addition to model-invariant interactions, the metal ion in site (I) strongly interacted with the Leu O atom of the Leu-Gly [2.44 Å in LAP(Zn_2) and 2.15 Å in LAP(MnZn)]. Similarly, the metal ion in site (II) formed, in addition to model-invariant contacts, strong interaction with the Leu O atom of Leu-Gly [2.46 Å in LAP(Zn_2) and 2.56 Å in LAP(MnZn)].

Apart from a shorter distance between the Leu O atom and metal ion (I) predicted for the MnZn complex [which could be related to the higher activity exhibited by LAP(MnZn)] [44], the same distance pattern was obtained in both cases, fully compatible with all suggested reaction paths of this enzyme [46].

Cys-Gly exhibited a completely different behaviour, with a peptide complexation of the two metal ions resembling that found for Leu-Gly in MnZn complex alone [Cys O distances from ions are 2.20 and 2.45 Å for site (I) and (II), respectively], whereas in the Zn_2 complex, the Cys O atom was widely separated (3.77 Å) from site (I), having been replaced in the Zn(I) co-ordination sphere by the Cys side chain, which in the pH range corresponding to maximum activity of the enzyme is presumably in its anionic S^- form [Zn(I)–S distance = 2.58 Å].

This model easily explained the observed lack of reactivity of LAP(Zn_2) towards Cys-Gly, and it fully agreed with the observed relative propensity of ligands containing NH_2, CO_2H and SH groups to bind Zn^{2+} rather than Mn^{2+} ions [47]. In particular, whereas thiols represent good ligands for a Zn^{2+} ion, no similar stable complex is known for Mn^{2+}, which preferably binds O or N donor groups.

The complexation of the Mn^{2+} ion by a carboxylic ligand was observed in the Asp-Gly complexation, where a strong interaction (2.20 Å) between Asp $O_{\delta 2}$ and Mn(I) is responsible for a substantial distortion of the co-ordination around the bimetallic centre. A different, but equally destructive, arrangement occurred in the LAP(Zn_2) complex with Asp-Gly, where an intramolecular H bond between the Asp side chain and NH_2 group of Gly brought the Asp O atom away from Zn(I) (2.51 Å), thus preventing an optimal arrangement of relevant groups for peptide activation.

Therefore, in spite of its crudeness, the adopted model seemed to be capable of predicting both qualitative and semi-quantitative features of the interaction among LAP, metal ions and potential substrate dipeptides in the enzyme active site. This gives support to the 'local' nature of the activating/deactivating interactions involving a Cys side chain against more complex models involving other regions of the site, or even the channel that provides access to the active site of each monomer in the LAP hexamer.

On this basis, the occurrence of interactions of Cys-Gly in the LAP(MnZn) complex, offering an explanation for the experimental pH dependence exhibited by this system, was explored. As shown in Figure 7, a strong interaction of Lys^{262} with the Cys side chain of Cys-Gly (S–N distance = 2.68 Å) was observed in the LAP(MnZn) : Cys-Gly complex.

DISCUSSION

A major problem when dealing with enzymes that hydrolyse peptide bonds is the fact that often such activities are assayed and studied by using synthetic substrates that resemble possible physiological ones. This fact, together with the overlapping

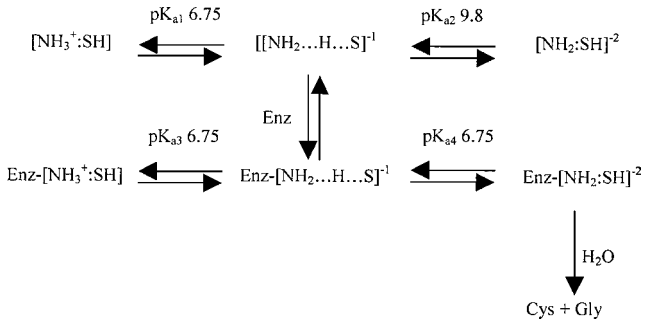
substrate specificity shared by many peptidases, makes it difficult to associate a physiological substrate with the enzyme responsible for its hydrolysis. Such a difficulty is reflected in the literature that often appears somewhat confusing. Some of the peptidases have changed name over the years, and some others, initially considered to be linked to different proteins, have been finally attributed to the same enzyme [48]. Cys-Gly is an important physiological dipeptide, resulting as a product of glutathione catabolism by GGT. It has been suggested that Cys-Gly may act as a pro-oxidant, thus intervening in the processes responsible for the redox status of cysteine residues of proteins [4,14]. Moreover, it has been also proposed that Cys-Gly might have signalling function [13]. Thus the identification of the enzyme(s) responsible for the hydrolysis of Cys-Gly can improve the overall picture of processes involving this relevant dipeptide. The activity responsible for the hydrolysis of Cys-Gly is at present attributed to alanyl aminopeptidase (also called aminopeptidase N; EC 3.4.11.2) [49], mainly on the basis of a paper by Rankin et al. [25], which demonstrated that a peptidase activity towards S-benzyl-cysteine-*p*-nitroanilide isolated from rat renal brush border was due to the activity of this enzyme.

Available methods for the determination of Cys-Gly hydrolase require complex and/or time consuming steps [25,50]. Alternatively, Cys-Gly hydrolase activity can be monitored by using the chromogenic synthetic substrate S-benzoylcysteinyl-*p*-nitroanilide and measuring spectrophotometrically the amount of *p*-nitroaniline produced [51]. However, this method, although simple and rapid, does not utilize a physiological substrate, and it is obviously inappropriate to determine kinetic parameters for Cys-Gly. To circumvent this major problem and with the aim to isolate from bovine lens an activity capable of hydrolysing Cys-Gly, we optimized two simple and sensitive assay methods (i.e. the spectrophotometric and the capillary electrophoresis methods), which were found to be suitable for routine measurements both in crude extracts and purified enzyme preparations. Making use of both methods, we developed a purification procedure for Cys-Gly hydrolase activity mainly based on the original work of Binkley et al. [22], whose initial purification steps take advantage of the unusual stability of the enzyme to chloroform treatment. After chloroform treatment, a single chromatographic step on a Sephacryl S-300 column was sufficient to accomplish the complete purification of the enzyme. MS analysis, together with the analysis of the molecular mass (320 kDa) of the native enzyme, subunit composition and the metal-ion requirement, allowed unequivocal identification of the purified Cys-Gly hydrolysing enzyme with LAP (EC 3.4.11.1) (for a review of this enzyme, see [46]). Analysis of kinetic parameters of the enzyme towards Cys-Gly (Table 3) showed that Cys-Gly is a better substrate than most synthetic substrates and as good as the peptides usually considered as good substrates for the enzyme [44]. The catalytic ability of LAP towards Cys-Gly hydrolysis was especially evident at near physiological pH values, where a specificity constant of about $10\,000\ \mathrm{min^{-1} \cdot mM^{-1}}$ was observed. It is clear from the data of Table 3 that the catalytic effectiveness of LAP is associated with a remarkable apparent affinity for Cys-Gly with respect to other substrates rather than a special ability of the enzyme at the level of the substrate hydrolysis step. The finding that LAP may efficiently hydrolyse Cys-Gly, while disclosing a potential specific role for LAP, raises a question regarding the features required for molecules to be a substrate for the enzyme, which was reported to prefer a bulky hydrophobic amino acid at the N-terminal end of dipeptides [44]. Indeed, the nature of the side chain of a cysteine residue, both for sterical and charge reasons, would hardly allow its allocation into the Leu-sized hydrophobic pocket, making it difficult to predict for Cys-Gly any possible role as substrate for LAP. Nevertheless, both a detailed kinetic analysis at different pH values and a molecular modelling approach indicated a possible mechanism of interaction that can explain the ability of LAP to hydrolyse Cys-Gly in the presence of Mn^{2+}.

The pH dependence of the kinetic parameters V_{max}, K_m and v_0 (i.e. V_{max}/K_m), obtained for Leu-Gly, was clearly in line with the postulated mechanism of action of LAP that, as generally accepted [44], can catalyse peptide hydrolysis only if the α-NH_2 group of the substrate is unprotonated. A dissociation event with a pK_a of 7.9, which can be associated to the deprotonation of the α-NH_2 group of the dipeptide (Figure 5, inset) was observed on the pK_m and log v_0 versus pH curves (Figure 5, middle and bottom panels respectively). Moreover, dissociation with a pK_a of approx. 7.25, probably ascribed to the deprotonation of the substrate when engaged in the enzyme–substrate complex, was evident both in the log V_{max} and pK_m versus pH curves (Figure 5, top and middle panels respectively). When Cys-Gly was used as substrate, the pH dependence of V_{max} indicated the occurrence of two events of proton dissociation on the enzyme–substrate complex as a relevant process for the hydrolytic breakdown of the dipeptide, with an overall apparent pK_a of 6.75 (Figure 6, top panel). The peculiar effect of pH on V_{max} was clearly evident when compared with the results related to the effect of pH on K_m. In fact, the upward concavity present on the pK_m versus pH curve (Figure 6, middle panel) at an apparent pK_a of 6.75 indicated that a dissociation process, either at the level of the free enzyme or free substrate was affecting the enzyme activity. The apparent lack of dissociation events characterized by a pK_a of 6.75 in the hydrolysis of Leu-Gly would suggest no involvement of the enzyme; thus, the dissociation event observed with Cys-Gly as substrate (pK_a 6.75) is likely to be ascribed to a free substrate dissociation. Indeed, when subjected to acid/base titration, Cys-Gly displayed a first dissociation with an apparent pK_a of 6.8 (Figure 6, inset). Even though $[NH_2 : SH]^{-1}$ is considered to be the predominant ionic form derived from the first dissociation of the $[NH_3^+ : SH]$ form of Cys-Gly [52], it is difficult to ascribe unequivocally this dissociation to one of the ionizable groups present (NH_3^+ and SH). Indeed, the generated monoanionic species $[NH_2 : SH]^{-1}$ is more appropriately described by a status in which the remaining proton is shared between the sulphide and the NH_2 groups ($[NH_2...H...S]^{-1}$). This intermediate undergoes further dissociation with an apparent pK_a of 9.8 (Figure 6, inset). Despite its high pK_a value, the dissociation may still take part in the pH dependence mechanism of Cys-Gly hydrolysis catalysed by LAP.

In fact, the molecular modelling approach to LAP(MnZn) : Cys-Gly complex revealed that, although unable to enter into the Leu hydrophobic pocket, the sulphur atom of the Cys side chain of Cys-Gly can optimally interact with Lys^{262} (Figure 7). This residue, located at the active site and postulated to take part in the catalytic action of LAP [46], has the potential to exert a strong inductive effect on the substrate at the level of the enzyme–substrate complex, with a significant decrease in the final dissociation pK_a. Thus, the last dissociation step of Cys-Gly may become indistinguishable from the first one in terms of dissociation constant. The occurrence of such an event will contribute to the steepness at the acidic side of the log V_{max} versus pH curve of the LAP-dependent hydrolysis of Cys-Gly, justifying the observed slope of 2 (Figure 6, top panel).

A detailed, quantitative explanation of the differences detected in pH dependence of LAP activity between Leu-Gly and Cys-Gly substrates is a challenging task. This is because of the lack of a well-stated mechanism for the enzyme activity [36,46,53], in turn influenced by the lack of precise knowledge of both the protonation state of several LAP/substrate residues and water molecules, and a bicarbonate ion, whose presence has been



Scheme 1 Model of the breakdown of Cys-Gly by LAP

Dissociation equilibria of Cys-Gly and enzyme–Cys-Gly complexes involved in Cys-Gly hydrolysis are reported. See text for details.

detected in some LAP crystal structures [54]. Nevertheless, the rather simple modelling approach adopted in the present study, together with kinetic data, allowed us to propose a mechanism explaining the pH dependence of the enzymic breakdown of Cys-Gly (Scheme 1). Release of the last proton from Cys-Gly, deprives the α-NH_2 group of the dipeptide of positive charge and then co-ordinates the Zn^{2+} ion at the active site. It also induces the generation of an anionic side chain that, interacting with the NH_2 group of the Lys^{262} side-chain, a functional group probably involved in the catalytic event of dipeptide hydrolysis [46], gives the rationale for the rather modest catalytic effectiveness (k_{cat}) of the enzyme towards Cys-Gly and, at the same time, for the unpredictable relevant affinity (i.e. a reduced k_{off}) for this substrate.

In conclusion, our results cannot rule out the possibility that different enzymes (i.e. alanyl aminopeptidase and/or membrane-bound dipeptidase) may intervene in the lens during Cys-Gly degradation. Indeed, less than 5–10 % of the total Cys-Gly hydrolase activity could be measured in membrane preparations (results not shown). However, this study clearly states that most of the remarkable Cys-Gly hydrolase activity of the lens has to be attributed to LAP unpredictably. Therefore, an unexpected role for this lens-abundant enzyme in glutathione turnover and in the processes responsible for the redox status control of the lens is disclosed. Indeed, the recent report suggesting that, in rat liver, Cys-Gly hydrolysis may be associated with LAP [30] supports the idea of a more general involvement of LAP in glutathione metabolism.

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