Active-Site Amino Acid Residues in γ -Glutamyltransferase and the Nature of the γ -Glutamyl-Enzyme Bond

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Active-site residues in rat kidney γ -glutamyltransferase (EC 2.3.2.2) were investigated by means of chemical modification. 1. In the presence of maleate, the activity was inhibited by phenylmethanesulphonyl fluoride, and the inhibition was not reversed by β -mercaptoethanol, suggesting that a serine residue is close to the active site, but is shielded except in the presence of maleate. 2. Treatment of the enzyme with *N*-acetylimidazole modified an amino group, exposed a previously inaccessible cysteine residue and inhibited hydrolysis of the γ -glutamyl-enzyme intermediate, but not its formation. 3. After reaction of the enzyme successively with *N*-acetylimidazole and with non-radioactive iodoacetamide/serine/borate, two active-site residues reacted with iodo[14 C]acetamide. One of these possessed a carboxy group, which formed a [14 C]glycollamide ester, and the other was cysteine, shown by isolation of *S*-[14 C]carboxymethylcysteine after acid hydrolysis. When *N*-acetylimidazole treatment was omitted, only the carboxy group reacted with iodo[14 C]acetamide. 4. Isolation of the γ -[14 C]glutamyl-enzyme intermediate was made easier by prior treatment of the enzyme with *N*-acetylimidazole. The γ -glutamyl-enzyme bond was stable to performic acid, and to hydroxylamine/urea at pH 10, but was hydrolysed slowly at pH 12, indicating attachment of the γ -[14 C]glutamyl group in amide linkage to an amino group on the enzyme. Proteolysis of the γ -[14 C]glutamyl-enzyme after performic acid oxidation gave rise to a small acidic radioactive peptide that was resistant to further proteolysis and was not identical with γ -glutamyl- ϵ -lysine. 5. A scheme for the catalytic mechanism is proposed.

The enzyme γ -glutamyltransferase (EC 2.3.2.2) (also known as γ -glutamyl transpeptidase) catalyses both hydrolysis of the γ -glutamyl group of many compounds and also transfer of the γ -glutamyl group to various acceptors, usually amino acids (Revel & Ball, 1959; Connell & Adamson, 1970; Orlowski & Meister, 1970; Thompson, 1970). The physiological function of the enzyme is not certain, but, being particularly abundant on the luminal surface of the kidney tubule, it has been suggested that the enzyme serves mainly for the hydrolysis of GSH and GSSG in the tubular filtrate (Young *et al.*, 1975; Schulman *et al.*, 1975; Elce & Broxmeyer, 1976; Pellefigue *et al.*, 1976; Inoue *et al.*, 1977a,b; Heinle *et al.*, 1977; Hahn *et al.*, 1978). Other work, however, suggested that the enzyme promotes amino acid transport into the tubular epithelial cells (Meister, 1973; Thompson & Meister, 1977; Griffith *et al.*, 1978; Karkowsky & Orlowski, 1978).

Abbreviations used: GSH, reduced glutathione; GSSG, oxidized glutathione.

Both hydrolysis and transfer reactions proceed through the formation of a covalent γ -glutamyl-enzyme intermediate, which has often been inferred from kinetic evidence, as reviewed by Thompson & Meister (1979), and the intermediate has been chemically demonstrated (Elce & Broxmeyer, 1976). The active site of the enzyme has not been further characterized, and the present work was aimed at identifying amino acid residues involved in the catalytic mechanism and in the covalent binding of the γ -glutamyl group. A preliminary account of some of this work has appeared (Elce, 1978).

Experimental

Materials and Methods

[U- 14 C]Glutamic acid-labelled glutathione ([14 C]-GSH) (16.6 mCi/mmol) was obtained and diluted to a specific radioactivity of 5 mCi/mmol as described by Elce & Broxmeyer (1976). Its purity was checked on a column of Dowex 1-X2 (Furano, 1971). Iodo-

[1-¹⁴C]acetamide (58 mCi/mmol) was obtained from the Amersham Corp. (Don Mills, Ont., Canada) and was diluted to a specific radioactivity of 5 mCi/mmol.

The enzyme was obtained from the kidneys of adult male Sprague-Dawley rats by established procedures. A microsomal pellet was treated with papain (Curthoys & Kuhlenschmidt, 1975), and the solubilized enzyme was purified by passage through columns of Sephadex G-200 and concanavalin A-Sepharose 4B (Takahashi *et al.*, 1974; Hughey & Curthoys, 1976). The activity of the enzyme was assayed in a total volume of 1 ml containing 2.5 mM- γ -glutamic acid *p*-nitroanilide, 20 mM-glycylglycine and enzyme sample in 50 mM-2-amino-2-methylpropane-1,3-diol, pH 8.0, at 20°C, by observing the rate of increase of absorption at 410 nm (Orlowski & Meister, 1965; Szasz, 1969). One unit of enzyme activity is defined as the amount of enzyme that catalyses the formation of 1 μ mol of *p*-nitroaniline/min. The peak tubes from the concanavalin A-Sepharose 4B columns contained enzyme with a specific activity of 50–55 units/nmol, assuming a mol.wt. of 68 000 (Hughey & Curthoys, 1976). From the report of Tate & Ross (1977), for comparison, it can be calculated that the human kidney γ -glutamyltransferase possesses a specific activity of 69 units/nmol. Hydrolysis of [¹⁴C]GSH by γ -glutamyltransferase was measured as described by Elce & Broxmeyer (1976).

Formic acid was prepared by mixing 9 ml of 90% (w/v) formic acid with 1 ml of 30% (w/w) H₂O₂ solution and leaving the mixture at 0°C for 1–2 h before use (Hirs, 1967). High-voltage electrophoresis was carried out on Whatman 3MM paper at 60 V/cm for 45 min, in acetic acid/formic acid/water (4:1:45, by vol.), pH 1.9, and in acetic acid/pyridine/water (10:3:500, by vol.), pH 3.9. Descending paper chromatography was performed in butan-1-ol/acetic acid/water (12:3:5, by vol.) on Whatman no. 1 paper for 24 h. Amino acids and peptides were detected on paper by their reaction with ninhydrin, and radioactivity was detected by scanning with a Berthold Aerograph radiochromatogram scanner.

Chemical modification experiments were carried out at 20°C with 3–10 units of enzyme in a total volume of 0.5–1.0 ml of 5 mM-Tris/HCl buffer, pH 7.5, unless otherwise stated, small samples being removed for assay at appropriate times. The combined presence of serine and sodium borate, each at a final concentration of 25 mM, acts as a competitive inhibitor, and was used to protect the active site of the enzyme (Szewczuk & Connell, 1965; Tate & Meister, 1978). The assay with γ -glutamic acid *p*-nitroanilide requires 0.03–0.05 unit of enzyme, so that the resultant dilution in most cases prevented interference in the assay by the modifying reagents

or by serine/borate without the need for prior dialysis. Control incubations were performed without the modifying reagents and the effects of these reagents on the assay system were checked.

S-Carboxymethylcysteine was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other carboxymethyl derivatives were obtained by treatment of methionine, *N*-acetyltyrosine, α -*N*-acetylhistidine and polylysine with iodoacetic acid (Gurd, 1972; Gundlach *et al.*, 1959*a,b*), followed by acid hydrolysis and purification on a small DEAE-Sephadex column (Green & Elce, 1978).

Results

Preliminary work showed that di-isopropyl phosphorofluoridate, L-1-tosylamido-2-phenylethyl chloromethyl ketone ('TPCK'), 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK') and phenylmethanesulphonyl fluoride had no effect on enzyme activity. As in the report by Inoue *et al.* (1978), it was observed that the enzyme was inhibited by 1 mM-phenylmethanesulphonyl fluoride in the presence of maleate; the inhibition was further shown not to be reversed by excess β -mercaptoethanol. [Maleate is known to promote hydrolysis of most substrates by γ -glutamyltransferase at the expense of transpeptidation (Thompson & Meister, 1979).]

The enzyme was 80% inhibited in 100 min by incubation with 0.2 M-1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide at 20°C in 0.1 M-sodium phosphate buffer, pH 6.0 (Huc *et al.*, 1975), and was not protected by serine/borate. The inhibition was not reversed by subsequent incubation of the inhibited enzyme at 20°C for 24 h with 1 M-NH₂OH at pH 7.

Treatment with 6.7 mM-pyridoxal 5'-phosphate at pH 7.0 did not inhibit the enzyme, with or without subsequent treatment with NaBH₄ (Klein & Sager, 1967; Carne & Flynn, 1977). However, treatment with 1 mM-trinitrobenzenesulphonic acid at pH 8.0 (Carne & Flynn, 1977) did inhibit the enzyme, but the reagent also interfered with the enzyme assay and reacted with serine/borate, so that this method of modification was not further studied.

N-Acetylimidazole and iodoacetamide

The most useful results were obtained as a result of treatment of the enzyme with *N*-acetylimidazole, iodoacetamide and serine/borate, in various combinations and sequences.

Enzyme activity was lost on incubation with *N*-acetylimidazole (Means & Feeney, 1971) at pH 7.5 (Fig. 1). At this pH the reagent was rapidly hydrolysed, whereas at pH 6 it was much more stable (Jencks & Carriuolo, 1959; Bruce, 1963), but did not inhibit the enzyme. The inhibition at pH 7.5 was not prevented by the presence of serine/borate,

Fig. 1. Inhibition of γ -glutamyltransferase by *N*-acetylimidazole

Samples of the enzyme (2.0 units) were incubated with *N*-acetylimidazole in a total volume of 0.4 ml of 5 mM-Tris/HCl buffer, pH 7.5, at 20°C, and samples were withdrawn for assay at the indicated times. The concentrations of *N*-acetylimidazole at zero time and after 80 min were determined from the absorbance at 245 nm (ϵ_{245} 2600 M⁻¹·cm⁻¹; Bruice, 1963), and were respectively: 3.1 mM, 0.23 mM (O); 12.6 mM, 3.0 mM (●); 31 mM, 7.4 mM (Δ); 56 mM (▲).

20 mM-glycylglycine (a good acceptor substrate for the transpeptidation reaction) or 1 mM-GSH. Samples of enzyme 80% inhibited by *N*-acetylimidazole treatment were incubated at pH 4.7 and at pH 9.3 for 24 h, with 1 M-NH₂OH at pH 7.5 for 3 h, and with 20 mM-dithiothreitol for 15 min, all at 20°C, and the degree of inhibition did not change. Control samples of unmodified enzyme retained their full activity under these conditions.

Further, it was observed that *N*-acetylimidazole treatment of the enzyme, which inhibited to the same extent hydrolysis both of γ -glutamic acid *p*-nitro-anilide and of [¹⁴C]GSH, did not however diminish the binding of the γ -[¹⁴C]glutamyl group to the enzyme. As shown in Table 1, the protein-bound radioactivity derived from [¹⁴C]GSH was the same in the control and in the *N*-acetylimidazole-treated enzyme samples.

Incubation of γ -glutamyltransferase at 20°C with 10 mM-iodoacetamide in 5 mM-Tris/HCl buffer, pH 8.0, caused 80% inhibition of enzyme activity in 1 h, and the inhibition was completely prevented by the presence of 25 mM-serine/borate. Both the hydrolysis of [¹⁴C]GSH and the binding of the γ -[¹⁴C]glutamyl group were also abolished by treatment of the enzyme with iodoacetamide.

To confine attention to active-site residues that react with iodoacetamide, the enzyme was first treated with a high concentration of non-radioactive iodoacetamide in the presence of serine/borate, thereby modifying all reactive groups remote from the active site, and then dialysed. The enzyme retained its full activity during this treatment. On sub-

sequent exposure of the enzyme to 10 mM-iodo[1-¹⁴C]acetamide, enzyme activity was completely lost and an amount of radioactivity became protein-bound that was consistent (after dialysis and allowing for manipulative losses) with one [¹⁴C]carboxamidomethyl group per molecule of enzyme (Table 2). If, however, the enzyme was treated after the non-radioactive iodoacetamide/serine/borate step with *N*-acetylimidazole, dialysed and then exposed to iodo[¹⁴C]acetamide, the amount of radioactivity incorporated was consistent with two [¹⁴C]carboxamidomethyl groups per molecule of enzyme (Table 2). When the enzyme was treated first with *N*-acetylimidazole, causing 90–95% inhibition, then with non-radioactive iodoacetamide/serine/borate, dialysed and finally exposed to iodo[¹⁴C]acetamide, two [¹⁴C]carboxamidomethyl groups were again bound per molecule of enzyme.

Radioactivity (d.p.m. per fraction)

Fraction no.	<i>N</i> -Acetylimidazole-	
	treated	Control
1	17	27
2	164	3
3	2111	832
4	410	1786
5	32680	400
6	226000	57500

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Table 2. *Iodo[¹⁴C]acetamide labelling of the active site of γ -glutamyltransferase*

γ -Glutamyltransferase (650 units; 12 nmol) in 5 mM-Tris/HCl buffer, pH 7.5, was preincubated with 25 mM-serine/borate in a total volume of 6.6 ml for 25 min. Solid non-radioactive iodoacetamide was added to a final concentration of 20 mM, and the mixture left for 3 h at 20°C. Excess β -mercaptoethanol was then added and the mixture was dialysed against 3 $\times$ 500 ml of 1 mM-Tris/HCl buffer, pH 8.0, at 4°C for 24 h. The total activity observed at this point was 550 units. The dialysed solution was divided into two equal portions, I and II. To portion II was added solid *N*-acetylimidazole to a final concentration of 100 mM, and both mixtures were left for 24 h at 4°C before being precipitated with 5 vol. of acetone. The precipitates were redissolved and transferred in 0.25 ml of 10 mM-Tris/HCl buffer, pH 8.7, to small vials, and both received 0.25 ml of 20 mM-iodo[¹⁴C]acetamide (5 mCi/mmol). The mixtures were left in the dark at 4°C overnight and then separately applied to Sephadex G-50 columns (45 cm $\times$ 1 cm) equilibrated and eluted with 5 mM-sodium phosphate buffer, pH 7.3; 2.2 ml fractions were collected. The results are given as units of enzyme activity per fraction and as ¹⁴C radioactivity (d.p.m.) per fraction.

Fraction no.	Sample I		Sample II	
	Enzyme activity (units/fraction)	Radioactivity (d.p.m./fraction)	Enzyme activity (units/fraction)	Radioactivity (d.p.m./fraction)
1-5	—	—	—	—
6	3.88*	55 731†	0.27	110 100†
7	0.45	9000	0.12	47 184
8	—	—	—	6400
9-11	—	—	—	—
12-16	—	42 $\times$ 10 ⁶	—	50 $\times$ 10 ⁶

* The residual enzyme activity acts as a marker for the 4-5 nmol of enzyme protein that is present.

† The radioactivity in fractions 6 and 7 was decreased by about 30% by dialysis (see Table 3).

Characterization of the [¹⁴C]carboxamidomethyl groups

In sample I (Table 2) it appeared that one [¹⁴C]-carboxamidomethyl group had been bound per molecule of enzyme, and it had been suggested that this might be located on a carboxy group of the enzyme (Connell & Adamson, 1970). Three other examples of this type of binding have been described (Takahashi *et al.*, 1967; Bonner & Bloch, 1972; Nigen *et al.*, 1973), and the tests described in those reports were therefore employed (Table 3), and gave the expected confirmation. In addition, portions of fractions 6 and 7 from sample I (Table 2) were mixed with 50 μ g of carrier glycollic acid and incubated in 0.1 M-NaOH overnight at 20°C. The samples were then passed through columns of Dowex 50 (5 cm $\times$ 0.5 cm) in water, and all the radioactivity was recovered in the first 4 ml of eluate. This was evaporated to dryness and the residue was re-dissolved and subjected to high-voltage electrophoresis at pH 2 for 1 h. The only radioactive peak detected by scanning coincided with the glycollic acid standard (approx. 5 cm from the origin to wards the cathode), and on elution from the paper the peak area contained about 60% of the initial radioactivity.

When portions of fractions 6 or 7 from sample II (Table 2) were treated with 0.1 M-NaOH overnight and then dialysed, the loss of radioactivity was 46% compared with the control, showing that one of the two [¹⁴C]carboxamidomethyl groups was not alkali-labile. The dialysed material was freeze-dried and

Table 3. *Chemical stability of the single [¹⁴C]carboxamidomethyl-enzyme bond*

Portions of γ -glutamyltransferase labelled with 1 equiv. of [¹⁴C]carboxamidomethyl group (fraction 6, sample I, Table 2) were treated as follows: (a) radioactivity determined without further treatment; (b) mixed with 1 ml of 6 M-urea in 0.1 M-sodium acetate buffer, pH 5.5, left at 20°C for 5 h and dialysed overnight against 500 ml of 6 M-urea in 0.1 M-sodium acetate buffer, pH 5.5; (c) mixed with 1 ml of 0.1 M-NaOH, left at 20°C for 5 h, neutralized with 0.2 ml of 1 M-HCl and dialysed as in (b); (d) mixed with 1 ml of 1 M-NH₂OH in 6 M-urea, pH 9.0, left at 20°C for 5 h and dialysed as in (b). After dialysis the total radioactivity in each mixture was determined.

Treatment	Total radioactivity (d.p.m.)	Percentage loss relative to (b)
(a)	4344	—
(b)	3088	0
(c)	655	79
(d)	1379	55

hydrolysed in 1.5 ml of 6 M-HCl for 8 h. The sample was then evaporated to dryness and applied to a column of DEAE-Sephadex with a gradient of 0-1 M-acetic acid (Green & Elce, 1978). The radioactivity was eluted in fractions 8-10, and portions of the radioactive material was subjected to high-voltage electrophoresis at pH 2 and to descending paper chromatography, together with standards of mono-

and di-*N*-carboxymethyl-lysine, *O*-carboxymethyl-tyrosine, *S*-carboxymethylhomocysteine and *S*-carboxymethylcysteine. On high-voltage electrophoresis the cysteine and tyrosine derivatives were not separated, but were clearly distinguished from the lysine, homocysteine and histidine derivatives; on paper chromatography the cysteine and tyrosine derivatives were well resolved. The only observed radioactive peak corresponded precisely to *S*-carboxymethylcysteine in both systems.

As stated above, when the enzyme was treated in the order *N*-acetylimidazole, iodoacetamide/serine/borate and iodo[^{14}C]acetamide, two [^{14}C]carboxamidomethyl groups were bound per molecule. Half of this protein-bound radioactivity was alkali-labile, and the other half, after hydrolysis in 6M-HCl at 110°C for 16h, was again shown to correspond to *S*-carboxymethylcysteine.

γ -[^{14}C]Glutamyl-enzyme intermediate

For amounts of enzyme up to 1–2nmol, the γ -[^{14}C]glutamyl-enzyme was prepared as described in the legend to Table 1. As a control, it was shown that free [^3H]glutamate did not become bound to the enzyme.

The radioactive enzyme intermediate was treated in various ways to determine the nature of the binding to the enzyme (Table 4). The bound radioactivity was stable to treatment with performic acid and was stable up to pH 10 in the presence of 1M-NH₂OH in 6M-urea. It was approx. 65% hydrolysed in 20h in 0.1M-sodium phosphate buffer, pH 12.

In an attempt to isolate γ -[^{14}C]glutamyl- ϵ -lysine from the intermediate, the enzyme (22nmol, 1200 units) was treated first with *N*-acetylimidazole and then permitted to react for 4s with 2 μ mol of [^{14}C]-GSH (5mCi/mmol) in a total volume of 2.8ml, followed by addition of 3ml of performic acid and chromatography on a Sephadex G-50 column (50cm $\times$ 1cm). The yield of protein-bound radioactivity was 102000 d.p.m. (9.2nmol). After treat-

ment with Pronase (Sigma), leucine aminopeptidase and carboxypeptidase A, and purification on a column (40cm $\times$ 1cm) of Bio-Gel P-2 in 1% (v/v) acetic acid, a small radioactive peptide was obtained. This was subsequently treated further with Prolidase (Sigma) and Dispase (Boehringer) without altering its electrophoretic mobility (Hill & Schmidt, 1962). The radioactive material could pass through a dialysis membrane and could be hydrolysed by either 1M- or 6M-HCl at 110°C for 16h to [^{14}C]glutamate. The peptide had a mobility midway between those of glutamate and aspartate on high-voltage electrophoresis at pH 3.9, and was clearly distinct from pyrrolid-5-one-2-carboxylic acid and from standard γ -glutamyl- ϵ -lysine.

Discussion

The earliest preparations of γ -glutamyltransferase employed detergent extracts of microsomal fractions and yielded a very-high-molecular-weight form still associated with membrane components and soluble with difficulty in the absence of detergents (Revel & Ball, 1959; Connell & Adamson, 1970; Orłowski & Meister, 1970; Takahashi *et al.*, 1974). The enzyme obtained by papain treatment of microsomal fractions, in contrast, is freely soluble in water and more readily purified. It has been shown that the papain-treated enzyme from rat kidney is a heterodimer containing subunits of approx. 46000 and 22000 mol.wt. and that the smaller subunit contains the active site (Hughes & Curthoys, 1976; Tate & Meister, 1976; Tate & Ross, 1977; Horiuchi *et al.*, 1978).

Although much kinetic work on γ -glutamyltransferase has been reported, few reports have appeared of work attempting to identify specific active-site residues. Szewczuk & Connell (1965) described the reaction of kidney γ -glutamyltransferase with iodoacetamide, but were unable to identify the modified residues. Goore & Thompson (1967) obtained evidence from variation of the activity of a plant γ -glu-

Table 4. Chemical stability of the γ -[^{14}C]glutamyl-enzyme bond

Portions of γ -[^{14}C]glutamyl-enzyme solution were incubated in final concentrations as described below for 20h at 20°C, in final volumes of 0.75ml, and then dialysed for 24h against 1 litre of 10mM-sodium acetate buffer (pH 4.0)/0.1% (w/v) sodium dodecyl sulphate. The total radioactivity remaining within the dialysis bags was determined. The radioactivity in the diffusate was negligible.

Treatment	Non-diffusible radioactivity (d.p.m.)
1. 0.14 M-Sodium phosphate (pH 7.2)/1 M-NH ₂ OH	221
2. 0.14 M-Tris/HCl (pH 10.0)/1 M-NH ₂ OH	250
3. 0.14 M-Tris/HCl (pH 10.0)/1 M-NH ₂ OH/6 M-urea	253
4. 0.14 M-Sodium phosphate (pH 12.0)	90
5. 0.14 M-Tris/HCl (pH 10.0)/0.5 M-NH ₃	222
6. 3.3 mM-Sodium acetate (pH 4.0)	244

tamyltransferase with pH for involvement of tyrosine and imidazole residues, but there is no evidence for an important role of these residues in the rat kidney enzyme. In addition, Katunuma *et al.* (1973) briefly described some inhibition experiments on a kidney phosphate-independent glutaminase, an enzyme later shown to be identical with γ -glutamyltransferase (Curthoys & Kuhlenschmidt, 1975; Tate & Meister, 1975).

The enzyme was not inhibited by several reagents commonly used to inhibit the serine proteinases, and inhibition by phenylmethanesulphonyl fluoride occurred only in the presence of maleate (Inoue *et al.*, 1978). The inhibition could not be reversed by β -mercaptoethanol, which tends to exclude the possibility that the phenylmethanesulphonyl fluoride had reacted with a cysteine residue (Whitaker & Perez-Villasenor, 1968). The inhibition caused by 6-diazo-5-oxonorleucine has also been shown to occur very much more rapidly in the presence of maleate, and was prevented by the presence of GSH (Tate & Meister, 1977; Inoue *et al.*, 1977a,b). The site of covalent attachment of the diazo compound has proved difficult to identify, which suggests that it is not a cysteine residue, and it has been argued that both 6-diazo-5-oxonorleucine and phenylmethanesulphonyl fluoride react with the same serine residue (Inoue *et al.*, 1978). These results suggest that a serine residue close to the active site is normally inaccessible, but is exposed as a result of a conformational change induced by maleate.

Reaction of the enzyme with carbodi-imide caused an inhibition that was not reversible by 1 M-NH₂OH at pH 7, indicating that tyrosine and cysteine residues were not involved (Huc *et al.*, 1975), and that reaction of a carboxy group was responsible for the observed inhibition. Since serine/borate did not in this case protect the enzyme, it is likely that the carboxy group that reacts with carbodi-imide is not the same as that which reacts, as discussed below, with iodoacetamide only in the absence of serine/borate.

N-Acetylimidazole

N-Acetylimidazole is capable of acetylating several types of amino acid side chain (Means & Feeney, 1971), but the persistence of the inhibition of treated enzyme on further incubation at pH 4.7 and 9.3, with NH₂OH at pH 7.5, and with dithiothreitol, and the fact that these treatments did not affect the control enzyme activity, excludes acetylation of histidine (Bruce, 1963), tyrosine (Riordan *et al.*, 1965) and cysteine residues (Roskoski, 1973; Knappe *et al.*, 1974). In the case of γ -glutamyltransferase, therefore, it appears likely that acetylation of an amino group was responsible for the observed inhibition. Since acetylation prevented hydrolysis of the γ -glutamyl-enzyme, but not its formation (Table

1), it is probable that this amino group is catalytically active in the hydrolytic step, even though it is not protected from acetylation by serine/borate or by substrates.

From Table 1 it may also be calculated that 12.5 units of enzyme bound 0.24 nmol of γ -glutamyl group, which corresponds to 52 units/nmol. Since the enzyme specific activity is known to be 50–55 units/nmol, these values show that the stoichiometry of γ -glutamyl binding to the enzyme was 1 : 1.

Iodoacetamide

In confirmation of the suggestion made by Connell & Adamson (1970), it was shown that a carboxy group at the active site could react with iodoacetamide (Tables 2 and 3), giving rise to a carboxamidomethyl group attached by an alkali-labile ester bond between an enzyme carboxy group and the primary alcohol group of glycollamide. Reaction with a carboxy group is also consistent with the fact that iodoacetic acid does not inhibit the enzyme (Szewczuk & Connell, 1965), presumably as a result of charge repulsion.

Treatment of the enzyme with *N*-acetylimidazole exposed a cysteine residue to reaction with iodo-[¹⁴C]acetamide, as shown by isolation of *S*-[¹⁴C]-carboxymethylcysteine after acid hydrolysis. The reaction could be prevented by serine/borate, so that the cysteine residue must be close to the active site. This result was surprising in that γ -glutamyltransferase is not inhibited by mercurial reagents (Szewczuk & Connell, 1965; Katunuma *et al.*, 1973), and it seems unlikely that the cysteine is directly involved in catalysis.

γ -Glutamyl-enzyme bond

The normal rate of hydrolysis of the γ -glutamyl-enzyme intermediate is extremely high, and even after *N*-acetylimidazole treatment the residual hydrolytic activity removed [¹⁴C]GSH very rapidly and made it difficult to trap the covalent enzyme intermediate in large amounts. Performic acid was used to stop the reaction, since the oxidation of all thiol and disulphide groups to sulphonic acids gave rise to a cleaner separation on the Sephadex G-50 column of the enzyme-bound radioactivity from the great excess of free radioactivity ([¹⁴C]glutamate and [¹⁴C]glutathionesulphonate). It had been shown previously (Elce & Broxmeyer, 1976) that the glutamate moiety from GSH was preferentially bound, rather than the whole GSH molecule, and as a further control it was shown that free [³H]glutamate did not become bound to the enzyme. The stability of the bound radioactivity towards performic acid, apart from eliminating any possible binding of the whole GSH molecule by means of a disulphide bond, excluded also the presence of a γ -glutamyl thioester (Perham & Harris, 1963; Riordan *et al.*, 1965;

Roskoski, 1973; Knappe *et al.*, 1974). A γ -glutamylhistidine also would not survive exposure to acid (Bruice, 1963). Phenolic esters are rapidly hydrolysed above pH 8 (Riordan *et al.*, 1965), and like thioesters are not stable above pH 7 in the presence of NH_2OH (Riordan *et al.*, 1965; Roskoski, 1973). The same arguments apply to carboxylic acid anhydrides. With respect to a possible γ -glutamyl-serine bond, aliphatic esters are less stable in alkali than are amides (Bonner & Bloch, 1972) and, as shown in Table 4, the bound radioactivity in the γ -glutamyl-enzyme was stable up to pH 10 with or without the addition of NH_2OH or NH_3 , being released rather slowly at pH 12. There is in addition a close similarity in the nature of the ester bond formed by iodoacetamide treatment of γ -glutamyl-transferase:

and the ester bond that would exist in a γ -glutamyl-serine intermediate:

The former ester was shown to be 55% decomposed by exposure to 1M- NH_2OH in 6M-urea at pH 9 for 5 h (Table 3), whereas the γ -glutamyl-enzyme bond was unaffected in 1M- NH_2OH in 6M-urea at pH 10 for 20 h (Table 4).

Attachment of the γ -glutamyl group to cysteine, histidine, serine, and tyrosine residues, and to aspartic acid and glutamic acid residues, has therefore been excluded, and involvement of arginine, methionine and tryptophan residues is not feasible. The slow hydrolysis of the γ -glutamyl group at pH 12 and its stability in 1M- NH_2OH seem therefore to be compatible only with an amide bond between the γ -carboxy group and an enzyme amino group. This is presumed to consist of a γ -glutamyl- ϵ -lysine bond, although an α -amino group is also possible. The peptide γ -glutamyl- ϵ -lysine has been isolated and identified as a cross-link in fibrin by means of exhaustive proteolysis (Lorand, 1972; Pisano *et al.*, 1972), but in the present case this has not been achieved, since the product of exhaustive proteolysis was a small acidic peptide that was not identical with standard γ -glutamyl- ϵ -lysine. It is possible that the peptide contained a cysteic acid residue, having been exposed to performic acid, which could account for both its acidic behaviour and its resistance to further proteolysis, but insufficient material remained to investigate this.

The possibility cannot be excluded, however, that the initial γ -glutamyl bond is to a serine residue, followed by an *O*- to *N*-acyl shift, although it has been argued that the serine residue at the active site may normally be inaccessible. A γ -glutamyl-serine bond would be stable in performic acid and below

pH 7 (Narita, 1959), but an *O*- to *N*-acyl shift could occur above pH 8 (Flavin & Slaughter, 1965; Nagai & Flavin, 1967), i.e. in most of the conditions described in Table 4, provided that an amino group were suitably placed. (I am indebted to Dr. S. S. Tate for this suggestion.)

Possible catalytic mechanism

The chemical evidence of this and previous work makes it clear that one amino group, as the γ -glutamyl binding site, a second amino group whose acetylation inhibits hydrolysis of the γ -glutamyl-enzyme intermediate, one carboxy group, one cysteine residue and one serine residue are all close to the active site, since, apart from the acetylated amino group, all these residues are protected by serine/borate or by GSH. It has been argued, however, that the serine residue is inaccessible in the absence of maleate, and the cysteine residue is certainly inaccessible without *N*-acetylimidazole treatment, so that neither of these two residues is likely to be directly involved in catalysis.

It therefore appears that the catalytic mechanism depends on two amino groups and a carboxy group. Precise measurements of kinetically important pK values have not yet been made for a mammalian γ -glutamyltransferase, and might provide evidence for the ionic states of these two amino groups during reaction. It is known that the transfer reaction prevails over hydrolysis at higher pH values (Connell & Adamson, 1970; Orłowski & Meister, 1970), but this probably reflects only the increased proportion of the acceptor substrate amino group in the unprotonated form. However, Goore & Thompson (1967), working with the γ -glutamyltransferase of kidney bean fruit, observed pK values for the hydrolytic reaction of 7 and 9.5, and attributed these values to histidine and tyrosine residues, without supporting evidence. If these values can be carried over to the rat kidney enzyme, where there is no evidence in support of important histidine and tyrosine residues, it is possible that their correct attribution is to the two amino groups at the active site. Acetylation of one of these amino groups by *N*-acetylimidazole, which then inhibited hydrolysis of the γ -glutamyl-enzyme intermediate, occurred at pH 7.5 but not at pH 6, implying that the amino group has a relatively low pK value, and it is therefore possible to suggest that this amino group, in the unprotonated form, could act as a general base in assisting attack by a water molecule on the γ -glutamyl-enzyme bond. It might also be expected that the second amino group should be in the unprotonated form to act as a good nucleophile for attack on the substrate, GSH. However, the carboxy group on the enzyme must be very close to the amino group, since its carboxamidomethylation abolishes enzyme activity, and since both are protected by serine/borate. In such

Scheme 1. *Proposed catalytic mechanism for γ -glutamyltransferase*

In the case of transfer to an acceptor amino acid, rather than hydrolysis by water, the acceptor amino group competes with the molecule of water for attack on the γ -glutamyl carbonyl group.

proximity, the amino group is likely to be stabilized in the protonated form by a salt link to the carboxy group, and will therefore have a higher pK value. If it is protonated, the amino group would then need some assistance to act as a nucleophile, conceivably from the carboxy group. These speculations lead to a mechanism with the general features indicated in Scheme 1, but further work is required to extend, confirm or revise this, and to correlate it with other topological studies of the active site of γ -glutamyltransferase (Thompson & Meister, 1977, 1979).

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References

- Bonner, W. M. & Bloch, K. (1972) *J. Biol. Chem.* **247**, 3123–3133
- Bruice, T. C. (1963) *Methods Enzymol.* **6**, 606–610
- Carne, T. J. & Flynn, T. G. (1977) *Can. J. Biochem.* **55**, 856–864
- Connell, G. E. & Adamson, E. D. (1970) *Methods Enzymol.* **19**, 782–789
- Curthoys, N. P. & Kuhlenschmidt, T. (1975) *J. Biol. Chem.* **250**, 2099–2105
- Elce, J. S. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* **359**, 1034–1035
- Elce, J. S. & Broxmeyer, B. (1976) *Biochem. J.* **153**, 223–232
- Flavin, M. & Slaughter, C. (1965) *Biochemistry* **4**, 1370–1375
- Furano, A. V. (1971) *Methods Enzymol.* **17B**, 509–510
- Goore, M. Y. & Thompson, J. F. (1967) *Biochim. Biophys. Acta* **132**, 15–26
- Green, R. M. & Elce, J. S. (1978) *Can. J. Biochem.* **56**, 1069–1074
- Griffith, O. W., Bridges, R. J. & Meister, A. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5405–5408
- Gundlach, H. G., Stein, W. H. & Moore, S. (1959a) *J. Biol. Chem.* **234**, 1754–1760
- Gundlach, H. G., Moore, S. & Stein, W. H. (1959b) *J. Biol. Chem.* **234**, 1761–1764
- Gurd, F. R. N. (1972) *Methods Enzymol.* **25**, 424–438
- Hahn, R., Wendel, A. & Flohé, L. (1978) *Biochim. Biophys. Acta* **539**, 324–337
- Heinle, H., Wendel, A. & Schmidt, V. (1977) *FEBS Lett.* **73**, 220–224
- Hill, R. L. & Schmidt, W. R. (1962) *J. Biol. Chem.* **237**, 389–396
- Hirs, C. H. W. (1967) *Methods Enzymol.* **11**, 197–199
- Horiuchi, S., Inoue, M. & Morino, Y. (1978) *Eur. J. Biochem.* **87**, 429–437
- Huc, C., Olomucki, A. & Thomé-Beau, F. (1975) *FEBS Lett.* **60**, 414–418
- Hughey, R. P. & Curthoys, N. P. (1976) *J. Biol. Chem.* **251**, 7863–7870
- Inoue, M., Horiuchi, S. & Morino, Y. (1977a) *Biochem. Biophys. Res. Commun.* **79**, 1104–1110
- Inoue, M., Horiuchi, S. & Morino, Y. (1977b) *Eur. J. Biochem.* **73**, 335–342
- Inoue, M., Horiuchi, S. & Morino, Y. (1978) *Biochem. Biophys. Res. Commun.* **82**, 1183–1188
- Jencks, W. P. & Carriuolo, J. (1959) *J. Biol. Chem.* **234**, 1272–1279

- Karkowsky, A. M. & Orlowski, M. (1978) *J. Biol. Chem.* **253**, 1574–1581
- Katunuma, N., Katsunuma, T., Towatari, T. & Tomino, I. (1973) in *The Enzymes of Glutamine Metabolism* (Prusiner, S. & Stadtman, E. R., eds.), pp. 227–254, Academic Press, New York
- Klein, S. M. & Sager, R. D. (1967) *J. Biol. Chem.* **242**, 301–305
- Knappe, J., Blaschkowski, H. P., Gröbner, P. & Schmitt, T. (1974) *Eur. J. Biochem.* **50**, 253–263
- Lorand, L. (1972) *Ann. N.Y. Acad. Sci.* **202**, 6–30
- Means, G. E. & Feeney, R. E. (1971) *Chemical Modification of Proteins*, pp. 72–74, Holden-Day, San Francisco, London and Amsterdam
- Meister, A. (1973) *Science* **180**, 33–39
- Nagai, S. & Flavin, M. (1967) *J. Biol. Chem.* **242**, 3884–3895
- Narita, K. (1959) *J. Am. Chem. Soc.* **81**, 1751–1756
- Nigen, A. M., Keim, P., Marshall, R. C., Morrow, J. S., Vigna, R. A. & Gurd, F. R. N. (1973) *J. Biol. Chem.* **248**, 3724–3732
- Orlowski, M. & Meister, A. (1965) *J. Biol. Chem.* **240**, 338–347
- Orlowski, M. & Meister, A. (1970) *Methods Enzymol.* **17A**, 883–889
- Pellefigue, F., Butler, J. D., Spielberg, S. P., Hollenberg, M. D., Goodman, S. I. & Schulman, J. D. (1976) *Biochem. Biophys. Res. Commun.* **73**, 997–1002
- Perham, R. N. & Harris, J. I. (1963) *J. Mol. Biol.* **7**, 316–320
- Pisano, J. J., Bronzert, T. J., Peyton, M. P. & Finlayson, J. S. (1972) *Ann. N.Y. Acad. Sci.* **202**, 99–113
- Revel, J. P. & Ball, E. G. (1959) *J. Biol. Chem.* **234**, 577–582
- Riordan, J. F., Wacker, W. E. C. & Vallee, B. L. (1965) *Biochemistry* **4**, 1758–1765
- Roskoski, R. (1973) *Biochemistry* **12**, 3709–3714
- Schulman, J. D., Goodman, S. I., Mace, J. W., Patrick, A. D., Tietz, F. & Butler, E. J. (1975) *Biochem. Biophys. Res. Commun.* **65**, 68–74
- Szasz, G. (1969) *Clin. Chem.* **15**, 124–136
- Szewczuk, A. & Connell, G. E. (1965) *Biochem. Biophys. Acta* **105**, 352–367
- Takahashi, K., Stein, W. H. & Moore, S. (1967) *J. Biol. Chem.* **242**, 4682–4690
- Takahashi, S., Pollack, J. & Seifter, S. (1974) *Biochim. Biophys. Acta* **371**, 71–75
- Tate, S. S. & Meister, A. (1975) *J. Biol. Chem.* **250**, 4619–4627
- Tate, S. S. & Meister, A. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2599–2603
- Tate, S. S. & Meister, A. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 931–935
- Tate, S. S. & Meister, A. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4806–4809
- Tate, S. S. & Ross, M. E. (1977) *J. Biol. Chem.* **252**, 6042–6045
- Thompson, G. A. & Meister, A. (1977) *J. Biol. Chem.* **252**, 6792–6798
- Thompson, G. A. & Meister, A. (1979) *J. Biol. Chem.* **254**, 2956–2960
- Thompson, J. F. (1970) *Methods Enzymol.* **17A**, 894–900
- Whitaker, J. R. & Perez-Villasenor, J. (1968) *Arch. Biochem. Biophys.* **124**, 70–78
- Young, J. D., Ellory, J. C. & Wright, P. C. (1975) *Biochem. J.* **152**, 713–715