Identification of a highly reactive threonine residue at the active site of γ -glutamyl transpeptidase

(glutathione/ L -(α S, α S)- α -amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid/glycoprotein/ β -hydroxyglutamic acid)

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ABSTRACT γ -Glutamyl transpeptidase [(5-glutamyl)-peptide:amino-acid 5-glutamyltransferase, EC 2.3.2.2], an enzyme of major importance in glutathione metabolism, was inactivated by treating it with L -(α S, α S)- α -amino-3-chloro-4,5-dihydro-5-[3- 14 C]isoxazoleacetic acid. This selective reagent binds stoichiometrically to the enzyme; more than 90% of the label was bound to its light subunit. Enzymatic digestion of the light subunit gave a 14 C-labeled peptide that corresponds to amino acid residues 517–527 of the enzyme and two incomplete digestion products that contain this labeled peptide moiety. The radioactivity associated with this peptide was released with threonine-523 during sequencing by the automated gas-phase Edman method. The light subunit contains 14 other threonine residues and a total of 19 serine residues; these were not labeled. Threonine-523 is situated in the enzyme in an environment that greatly increases its reactivity, indicating that other amino acid residues of the enzyme must also participate in the active-site chemistry of the enzyme.

γ -Glutamyl transpeptidase [(5-glutamyl)-peptide:amino-acid 5-glutamyltransferase, EC 2.3.2.2], the enzyme that catalyzes the cleavage of the γ -glutamyl bond of glutathione and related γ -glutamyl compounds, plays a key role in metabolism by virtue of its function in the cleavage and formation of γ -glutamyl bonds (1–4). Such reactions are involved in the processing of various S-conjugates of glutathione (formed from endogenous and exogenous compounds), in the transport of amino acids as γ -glutamyl amino acids, and in the cellular recovery of cysteine moieties as γ -glutamylcystine. The reactions catalyzed by γ -glutamyl transpeptidase are thought to involve formation of a γ -glutamyl enzyme and transfer of the γ -glutamyl moiety to acceptors such as amino acids to form the corresponding γ -glutamyl compounds. Hydrolysis of the γ -glutamyl donor occurs when water is the acceptor. γ -Glutamyl transpeptidase is widely distributed and has been found, for example, in kidney, pancreas, epididymis, jejunal mucosa, biliary epithelium, ciliary body, and choroid plexus (5). The enzyme is typically membrane-bound and is extensively glycosylated.

Rat kidney γ -glutamyl transpeptidase consists of two subunits (heavy subunit of $M_r \approx 51,000$ and light subunit of $M_r \approx 22,000$). The heavy subunit is linked to the cell membrane through its N-terminal segment, whereas the light subunit is attached to the heavy subunit by noncovalent interactions (1). The two subunits of rat kidney γ -glutamyl transpeptidase are synthesized as a single peptide chain, and glycosylation and membrane insertion take place as cotranslational events (1, 6–8). The enzyme subunits, which are formed by cleavage of the proenzyme, are encoded by a common mRNA. The complete amino acid sequence of the two subunits has been deduced from the nucleotide sequence of the corresponding

cDNA (9–11). These sequence studies support the conclusion that a 21-amino acid peptide moiety at the N terminus of the heavy subunit is closely associated with the cell membrane (12, 13). There are as yet no studies that identify specific amino acid residues of the enzyme that function in catalysis at the active site. Previously, evidence was obtained suggesting that the γ -glutamyl moiety of the substrate and various inhibitors bind to a hydroxyl group on the enzyme (14). Findings that suggest involvement of an enzyme amino group (15), a histidine moiety (16), a carboxyl group (15, 17), a cysteine residue (15, 17), and an arginine residue (18, 19) also have been reported.

In the present work, the enzyme was inactivated by incubation with L -(α S, α S)- α -amino-3-chloro-4,5-dihydro-5-[3- 14 C]isoxazoleacetic acid, a selective irreversible inhibitor (20–22). Inactivation was associated with binding of 1 mol of inhibitor per mol of enzyme and the inhibitor was found to be attached to a specific threonine residue (Thr-523) of the light subunit.

EXPERIMENTAL PROCEDURES

Materials. γ -Glutamyl transpeptidase, isolated from frozen rat kidneys (Pel-Freez Biologicals) (1), had a specific activity of 1100 μ mol/min per mg when assayed at 37°C with 1 mM L - γ -glutamyl- p -nitroanilide (23) and 20 mM glycylglycine in 60 mM Tris-HCl buffer at pH 8.0. L -(α S, α S)- α -Amino-3-chloro-4,5-dihydro-5-[3- 14 C]isoxazoleacetic acid (acivicin, AT-125; specific activity, 67,800 cpm/nmol as determined by PICO-TAG amino acid analysis and comparison with an authentic sample of acivicin standard) was supplied by Richard S. P. Hsi (Upjohn). Endoproteinase Lys-C purified from *Lysobacter enzymogenes* (specific activity, 30 units/mg) (24), pyroglutamate aminopeptidase purified from calf liver, and endoproteinase Glu-C purified from *Staphylococcus aureus* V8 were purchased from Boehringer Mannheim. Trifluoroacetic acid, trifluoromethanesulfonic acid, Tris, glycylglycine, Sephadex G-25 fine, L - γ -glutamyl- p -nitroanilide, L -1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin, and peptide: N -glycosidase F (PNGase F) isolated from *Flavobacterium meningosepticum* were obtained from Sigma. Acetonitrile (ChromAR HPLC) was purchased from Mallinckrodt. Guanidinium hydrochloride and urea (ultra pure; recrystallized before use) were products of Schwarz/Mann. Methylamine was obtained from Eastman Kodak, and triethylamine, constant-boiling HCl, and phenyl isothiocyanate were obtained from Pierce. Compounds used in peptide sequencing were obtained from Applied Biosystems. Mono-fluor scintillation liquid was purchased from National Diagnostics (Somerville, NJ).

Methods. γ -Glutamyl transpeptidase (6 mg; 91 nmol) was incubated with [14 C]acivicin (910 nmol) in a final 0.3-ml volume of 50 mM NaH₂PO₄ (pH 7.5) at 37°C. Portions (1 μ l)

were withdrawn at various times and assayed (1) to monitor the degree of inactivation. The inactivated enzyme was recovered by gel filtration on Sephadex G-25 (0.7 cm × 40 cm) (0.1 M NH₄HCO₃ at pH 7.5).

The subunits of the denatured enzyme were separated by reverse-phase HPLC on a protein C₄ column (5 μm; 0.46 cm × 25 cm) (Vydac, Hesperia, CA) with a Waters system and 0.1% trifluoroacetic acid as solvent A and 95% (vol/vol) acetonitrile/0.1% trifluoroacetic acid as solvent B. After the enzyme was denatured by treatment at 37°C with 8 M guanidinium hydrochloride in 1 M acetic acid for 15 hr, the sample was injected into the column and eluted with a linear gradient run from 20–60% (vol/vol) solvent B over 40 min at a flow rate of 1.5 ml/min. The effluent was monitored at 214 nm. Portions of the eluted peptides were taken for liquid scintillation counting on an LKB-1218 Rackbeta scintillation counter. The separated peptides were examined by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) (25). Samples (1.5 mg) of isolated light subunit were treated with PNGase F (6 units) in 0.3 ml of 0.25 M NH₄HCO₃ (pH 8.6) at 37°C for 24 hr. The neutral carbohydrate content of the treated enzyme was determined by the phenol/sulfuric acid method using D-galactose as a standard (26); 60% of the carbohydrate was removed by treatment with PNGase F.

The labeled light subunit (5 mg/ml) was digested at 37°C with 10% of its weight of endoproteinase Lys-C in 100 mM Tris-HCl, pH 8.5/2 M urea/20 mM methylamine for 24 hr. Peptides generated from endoproteinase Lys-C digestion of the light subunit were fractionated on a Waters μBondapak C₁₈ ODS column (10 μm; 3.9 mm × 30 mm) with solvents A and B as described above. The flow rate was 0.7 ml/min. The peptides were eluted with a linear gradient between solvent A and 60% (vol/vol) solvent B in 60 min and 60%–100% (vol/vol) solvent B in 10 min. Portions of the eluate were taken for liquid scintillation counting.

Endoproteinase Glu-C digestions were conducted in 50 mM NaH₂PO₄ (pH 7.8) at 37°C for 24 hr with 10% endoproteinase Glu-C. The peptides generated were separated by HPLC as described above.

Residual N-linked oligosaccharides, and O-linked carbohydrate were removed from the light subunit by treatment with trifluoromethanesulfonic acid at 0°C for 3 hr as described (27); the reagent was removed by ether extraction. The protein was precipitated by adding 20% (wt/vol) trichloroacetic acid. The pellet was washed with ice-cold acetone and then digested with 10% of its weight of trypsin at 37°C in 2 M urea/100 mM Tris-HCl, pH 8.5/20 mM methylamine for 24 hr. The tryptic peptides were separated by C₁₈ reverse-phase HPLC as described above.

Amino acid analyses were performed by the PICO-TAG system essentially as described (28). Peptides were hydrolyzed with 6 M HCl at 110°C under N₂ for 24 hr. The hydrolysates were neutralized, and the amino acids present were converted to the corresponding phenylthiocarbamyl-conjugated amino acids. Automated Edman degradations were performed on an Applied Biosystems model 470A gas-phase protein sequencer (29). Amino acids were detected as the corresponding phenylthiohydantoin derivatives by HPLC. Portions of each phenylthiohydantoin-conjugated amino acid fraction were examined for radioactivity.

RESULTS

Incubation of the enzyme with [¹⁴C]acivicin led to 98% loss of activity. Addition of 5 mM L-serine and 5 mM sodium borate, which selectively complex with the active-site region of the enzyme (14, 30), protected about 90% against inactivation and the associated incorporation of label. The inactivated enzyme, recovered by gel filtration, contained 1.05 ± 0.18 (SD; *n* = 5) mol of [¹⁴C]acivicin per mol of enzyme. The

inactivated labeled enzyme was separated by HPLC into light and heavy subunits, which were examined by SDS/PAGE; 92% of the radioactivity bound to the recovered protein was associated with the light subunit.

Digestion of the PNGase F-treated labeled light subunit with endoproteinase Lys-C, followed by fractionation on a C₁₈ column, gave three peptide fractions (I, II, and III), each containing about one-third of the total peptide-bound radioactivity recovered. Amino acid analysis indicated that the composition of fraction I agrees closely with the expected composition of a specific peptide corresponding to amino acid residues 517–527. Automated Edman degradation of fraction I confirmed this and gave the sequence Asn-Ile-Asp-Gln-Val-Val-Thr-Ala-Gly-Leu-Lys (Fig. 1). Radioactivity was eluted from the sequencer with residue 7 of fraction I, which corresponds to Thr-523; small amounts were carried over into cycles 8 and 9. That radioactivity carried over into later cycles suggests that either the Edman cleavage reaction or the extraction of the cyclized acivicin-derivatized threonine complex is less efficient than for threonine. The peptide was estimated in several separate sequencing runs to be 90–100% pure. No evidence was obtained for the presence of other peptides derived from γ-glutamyl transpeptidase. There is a gradual loss of label from the protein during the several procedures applied, reflecting the lability of the acivicin–enzyme linkage under the conditions used. The acivicin–enzyme bond is presumably formed through reaction at C-3 of acivicin. The resulting adduct is labile at both acid and alkaline pH but is less stable at alkaline pH, consistent with an ester-type linkage. Amino acid analysis of fraction I, isolated in several runs, indicated that the specific radioactivity of this fraction was between 35% and 40% of the original. Fraction II had a specific radioactivity that was 54–71% of the original.

Sequencing of fraction II, revealed that the first 10 residues corresponded to residues 407–416 of the enzyme; the radioactivity remained attached to the unsequenced portion of the peptide. After additional treatment of fraction II with endoproteinase Lys-C, about 15% of the radioactivity of this fraction moved with fraction I, and no other labeled species were formed. After further treatment of fraction III (which may contain unsplit light subunit and/or a peptide equivalent to 380–561) with endoproteinase Lys-C, about 40% of the radioactivity chromatographed with fraction II and 15% of it with fraction I. When endoproteinase Lys-C-treated fractions II and III were incubated with trypsin, only a small amount of radioactivity moved in the elution position of fraction I, probably because the presence of peptide-linked

FIG. 1. Elution of phenylthiohydantoin (>PhNCS)-conjugated amino acids during sequencing of the labeled peptide in fraction I. The numbers at the top refer to amino acid residues of γ-glutamyl transpeptidase; the single letter code for amino acid residues is used. ■, >PhNCS-amino acids; Δ, cpm.

carbohydrate inhibited proteolysis. Therefore, fractions II and III were treated with trifluoromethanesulfonic acid to remove carbohydrate (27); subsequent tryptic digestion converted 85% of the label to a product that moved with fraction I. Sequencing of the trypsin-treated mixture was complicated by the presence of many peptides; 15 cycles were performed and the only radioactivity released was found in cycle 7, a finding consistent with the data given in Fig. 1.

Digestion of fraction II with endoproteinase Glu-C gave a labeled peptide in 90–95% yield whose composition agreed with the sequence 520–534. Although the N terminus was mainly blocked by cyclization of Gln-520, sequencing through 10 cycles of the small amount of unblocked peptide showed elution of ^{14}C in cycle 4, which is consistent with labeling of Thr-523. (Treatment of the peptide with pyroglutamate aminopeptidase did not yield an unblocked N terminus.)

No evidence was obtained for binding of acivicin to other amino acid residues. Labeled light subunit, when treated with cyanogen bromide, gave a peptide corresponding to residues 467–568 that contains all of the ^{14}C . Thus, Cys-453, Ser-384, -387, -391, -397, -405, -409, -412, -424, -425, -437, -450, -451, and -455 and Thr-380, -396, -398, and -429 are not labeled. Sequencing of several other peptides isolated in the course of this work excluded labeling of Ser-471, -479, and -487, and Thr-474, -477, -478, -511, -512, -513, -528, -533, and -536. The digestion with endoproteinase Glu-C indirectly excludes labeling of Ser-550, -557, and -559 and Thr-549. This accounts for all of the serine, threonine, and cysteine residues of the light subunit.

Hydrolysis of [^{14}C]acivicin and of the [^{14}C]acivicin-treated holoenzyme with 6 M HCl at 110°C for 24 hr led to a labeled amino acid product that was eluted from the PICO-TAG system in the position of β -hydroxyglutamate. Mass spectrometry verified the structure of the product obtained from acivicin hydrolysis as β -hydroxyglutamate.

DISCUSSION

These findings show that Thr-523 is located at the active site of γ -glutamyl transpeptidase and that it participates in binding of acivicin presumably in a manner analogous to that in which the γ -glutamyl moieties of glutathione and related compounds bind. Thus, Thr-523 seems to supply the previously postulated (14) active-site hydroxyl moiety. However, one cannot unequivocally exclude the possibility that acivicin binds to a site close to but different from that at which γ -glutamylation occurs, and thus conceivably serine borate could block both sites.

The present studies identify the first of the amino acid side chains that comprise the catalytic center of γ -glutamyl transpeptidase. At this time one may only speculate about the nature of the other amino acid side chains present. Thr-523 might be activated by a mechanism similar to the activation of serine by the catalytic triad of the serine proteases (31). For example, carboxylate and histidine residues may be involved; indeed, there is indirect evidence for involvement of carboxylate (15, 17) and imidazole (16) moieties in the reaction catalyzed by γ -glutamyl transpeptidase. However, there are other possibilities.

The threonine hydroxyl group, in contrast to that of serine, is oriented in a specific position on an asymmetric carbon atom of the side chain. This may be of special significance in relation to the reaction mechanism of γ -glutamyl transpeptidase. Whereas the deacylation step of the reaction catalyzed by a serine protease involves reaction of water, that of γ -glutamyl transpeptidase involves attack of the amino group of an acceptor molecule, which is bound to the enzyme at a specific site. The use of the threonine hydroxyl group by γ -glutamyl transpeptidase may have other structural or

mechanistic significance or may be related to the evolutionary development of an active site that interacts with the γ -glutamyl moiety.

Earlier studies on the interaction of 6-diazo-5-oxo-norleucine with bacterial glutaminase-asparaginases (32) showed that this inhibitor binds to an enzyme threonine residue, which occurs in a sequence that is completely different from that found about the labeled threonine residue of γ -glutamyl transpeptidase. The labeled threonine residues of the bacterial glutaminase-asparaginases occur in a sequence (-Ala-Thr-Gly-Gly-Thr-) that is similar to that of residues 470–474 (-Ala-Ser-Gly-Gly-Thr-) of rat kidney γ -glutamyl transpeptidase. This region of the labeled transpeptidase was sequenced in the present studies and found to be devoid of radioactivity. Another amidase (*Escherichia coli* asparaginase) has a sequence (-Ala-Thr-Gly-Gly-Thr-) that is identical to that found in bacterial glutaminase-asparaginase; but an inhibitor (5-diazo-4-oxo-norvaline) is thought to bind to a serine residue that is located elsewhere in this enzyme (33). Acivicin (34), like azaserine, 6-diazo-5-oxo-L-norleucine, and L-2-amino-4-oxo-5-chloropentanoate (see, for example refs. 35–40) is also known to inactivate glutamine amidotransferases. Such inactivation is associated with alkylation of a cysteine residue at or close to the glutamine binding site of these enzymes. The light subunit of γ -glutamyl transpeptidase has a single cysteine residue (Cys-453); this residue was found to be unlabeled in the present studies. Further studies of γ -glutamyl transpeptidase are now needed to extend our knowledge of the structure of the catalytic center and to achieve understanding of the active site chemistry of this enzyme.

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