Involvement of Ser-451 and Ser-452 in the Catalysis of Human γ-Glutamyl Transpeptidase*

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The serine residue required for catalysis of γ-glutamyl transpeptidase was identified by site-specific mutagenesis of the conserved serine residues on the basis of sequence alignment of the light subunit of human, rat, pig and two bacterial enzymes. Recombinant human γ-glutamyl transpeptidases with replacements of these serine residues by Ala were expressed using a baculovirus-insect cell system. Substitutions of Ala at Ser-385, -413 or -425 yielded almost fully active enzymes. However, substitutions of Ala at Ser-451 or -452 yielded enzymes that were only about 1% as active as the wild-type enzyme. Further, their double mutant is only 0.002% as active as the wild type. Kinetic analysis of transpeptidation using glycylglycine as acceptor indicates that the Vmax values of Ser-451 and -452 mutants are substantially decreased (to about 3% of the wild type); however, their Km values for L-γ-glutamyl-p-nitroanilide as donor were only increased about 5 fold compared to that of the wild type. The double mutation of Ser-451 and -452 further decreased the Vmax value to only 0.005% of the wild type, while this mutation produced only a minor effect (2-fold increase) on the Km value for the donor. The kinetic values for the hydrolysis reaction of L-γ-glutamyl-p-nitroanilide in the mutants followed similar trends to those for transpeptidation. The rates of inactivation of Ser-451, -452 and their double mutant enzymes by acivicin, a potent inhibitor, were less than 1% that of the wild-type enzyme. The Km value of the double mutant for L-serine as a competitive inhibitor of the γ-glutamyl group is only 9 fold increased over that of the wild type, whereas the Kf for the serine-borate complex, which acts as an inhibitory transition-state analog, was more than 1,000 times higher than for the wild-type enzyme. These results suggest that both Ser-451 and -452 are located at the position able to interact with the γ-glutamyl group and participate in catalysis, probably as nucleophiles or through stabilization of the transition state.

γ-Glutamyl transpeptidase, a heterodimeric glycoprotein anchored to the extracellular surface of cell membrane, plays an important role in glutathione metabolism. It catalyzes the transfer reaction of a γ-glutamyl moiety from glutathione and related compounds to a variety of amino acids and dipeptides. The transfer of the γ-glutamyl moiety to water leads to hydrolysis (1, 2, 3). Both the large and the small subunit of γ-glutamyl transpeptidase are encoded by a common messenger RNA (4, 5, 6). The enzyme is translated as a single chain precursor, which yields subunits post-translationally by proteolytic processing (7, 8, 9).

A catalytic nucleophile, such as is found in thiol- or serine-class proteases, is assumed to form a covalent linkage with a γ-glutamyl group because reactions catalyzed by γ-glutamyl transpeptidase are thought to proceed via a γ-glutamyl-enzyme intermediate (10). Although γ-glutamyl transpeptidase from mammalian species possesses a unique thiol on the light subunit, which has the catalytic domain (11, 12), this cysteine residue is not required for catalysis (13). On the other hand, several studies suggest that a serine residue is involved in catalysis (14, 15). This is also supported by the recent observation that modification of γ-glutamyl transpeptidase with N-acetyl-l-methionyl led to a stabilized intermediate, and permitted the detection of a γ-glutamyl enzyme in which the γ-glutamyl moiety was bound on the light subunit (16). The nature of the linkage between enzyme and γ-glutamyl group was found to be consistent with an ester. Thus, the nucleophile in the active site was proposed to be a hydroxyl group, probably a serine residue on the light subunit. However, since the stabilized γ-glutamyl enzyme is hydrolyzed upon denaturation by guanidinium ions, the catalytic residue has not yet been identified. The detailed mechanism of the catalysis of γ-glutamyl transpeptidase is still unclear because its crystal structure is unknown. Several residues, however, have been identified to be at or near the active site (13, 16, 17, 19, 20).

Acivicin (L-αS,βS)-a-amino-3-chloro-4,5-dihydro-5-isoazole-2-carboxylic acid), a potent inhibitor of γ-glutamyl transpeptidase, inactivates the enzyme by its covalent attachment in or near the active site (2, 21, 22). By use of isotopically labeled acivicin, amino acid residues to which the acivicin moiety have been identified. These are Thr-523 in rat enzyme (17), Ser-405 in pig and Ser-406 (equivalent to Ser-405 in rat and pig) in human (20), all of which are on the light subunit. However, the human mutant γ-glutamyl transpeptidase in which these corresponding residues were replaced by Ala was found to be almost fully active, and the inactivation by acivicin was as rapidly for the mutant as for the wild-type enzyme; thus, these residues are not essential for catalysis (20). These findings suggested that another hydroxyl group reacts with acivicin initially, followed by transfer of acivicin moiety to the aforementioned residues. Such a primarily reactive hydroxyl group is likely to be identical to the serine residue involved in formation of γ-glutamyl enzyme intermediate because of structural similarity between the γ-glutamyl moiety and acivicin, and...
because of the requirement of a nucleophile for both the ω-glutamyl substrate and adicin to react with the enzyme.

In the current study, we selected seven conserved serine residues as candidates for mutagenesis by using amino acid sequence alignment of the light subunits of human, rat, pig and two bacterial ω-glutamyl transpeptidases. These candidates were examined by site-specific amino acid substitutions in order to identify the hydroxyl group(s) responsible for inactivation by adicin and for catalysis.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases and DNA modifying enzymes were obtained from New England Biolabs. Oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc. ω-Glutamyl-p-nitroanilide, glycyglycine and other common reagents were purchased from Sigma.

Site-directed Mutagenesis and Construction of the Transfer Plasmids—Site-directed mutagenesis was carried out according to Kunkel (23). The uracil-templates for the coding region of human ω-glutamyl transpeptidase, prepared from E. coli Cj 236, were subjected to mutagenesis as described in our previous study (13, 19). The synthetic oligonucleotide primers used to replace conserved serine residues with Ala are following: 5'-GCT CAC CGC GTC GTC GCA G-3'; S413GACGCTTCCCGGTGGTGGAGCTC-3'; S425A, 5'-ATGGACGACGCTTGGCCAGGACG-3'; S451A, 5'-AAGCAG CCT CTC ATG TGG CTC TCC GGC-3'; S452A, 5'-CAG CCT CTC AAC TGC TGC TCC GGC-3'; S451A/S452A (double mutant), 5'-AAG CAG CCT CTC GGC GTC ATG TGC CAG A-3'. After verification of mutant sequences by dye-excess sequencing (24), the BglII-EcoRI 0.8 kb fragments of these mutants were ligated together with the NotI-BglII 1.0 kb of the wild-type sequence into an NotI and an EcoRI sites of the transfer vector, pVL1392 (Invitrogen). The resultant transfer plasmids carrying whole coding regions of ω-glutamyl transpeptidases were purified with RPM kit (BIO-101).

Cell Culture—Spodoptera frugiperda (SF) 21 cells were maintained at 27°C under the insect media (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 3.33 g/l yeastolate, 3.33 g/l lactalbumin hydrolysate and 50 mg/l gentamicin.

Preparation of Recombinant Baculoviruses—The purified transfer plasmids and BaculoGold DNA (PharMingen) were subjected to co-transfection to obtain recombinant viruses, as described previously (13, 20, 25). Transfection was performed with Lipofectin (Life Technologies, Inc.) (26). The generated recombinant viruses were amplified to more than 5 × 10⁹ plaque forming units/ml prior to use for expression experiments. General manipulation of recombinant viruses was carried out as described (27).

Expression and Purification of Recombinant Human ω-Glutamyl Transpeptidases—10⁶ SF21 cells were harvested about 90 h after infection with recombinant viruses at a multiplicity of infection of 2. Recombinant enzymes were purified as described (13, 25). In brief, enzymes were extracted and solubilized from infected insect cells by Triton X-100, followed by papain treatment. Solubilized enzymes were further purified by column chromatography (hydroxyapatite (Bio-Rad), chromatofocusing (PBE94, Pharmacia) and gel filtration (Sephacryl S200HR, Pharmacia)).

Electrophoresis—The purified enzymes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 10% gels (28) with 20 μl glycyglycine as a donor in transpeptidation were obtained in the presence of 20 μl of glycyglycine as an acceptor. Alternatively,
parameters for the acceptor were determined with L-mML-light-subunits of L-glutamyltranspeptidases from a variety of species. Shaded boxes show the amino acid residues conserved among all species. The residue number for the human enzyme is given above the aligned sequences. The numbers on the right correspond to the residue number of each species. Arrow heads indicate the serine residues examined in the human enzyme.

**Fig. 1.** Sequence alignment of the light subunits of L-glutamyl transpeptidases from a variety of species. Shaded boxes show the amino acid residues conserved among all species. The residue number for the human enzyme is given above the aligned sequences. The numbers on the right correspond to the residue number of each species. Arrow heads indicate the serine residues examined in the human enzyme.

**Table I**

| Enzyme          | Specific activity (units/mg protein) |
|-----------------|-------------------------------------|
| Wild-type       | 580                                 |
| S385A           | 590                                 |
| S413A           | 330                                 |
| S425A           | 570                                 |
| S451A           | 4.4                                 |
| S452A           | 6.1                                 |
| S451A/S452A     | 0.011                               |

The decreased activity in the Ser-451 and -452 mutants is not likely to be due to an inability to use glycylglycine as an acceptor substrate because the presence of 20 mM glycylglycine substantially enhanced the $V_{\text{max}}$ values of both of these mutants (Table I and II). However, their $V_{\text{max}}$ values were 40 times lower (Table II). Furthermore, their double mutant showed saturation by the addition of only 0.5 mM of the acceptor. Hence, substitutions at Ser-451 -452 have decreased $K_m$ values for the acceptor.

**Inactivation of the Mutant Enzymes by Acivicin—**A nucleophilic residue on the enzyme is thought to be required for formation of the covalent adduct with acivicin (Fig. 2-B) that leads to inactivation of the enzyme. In order to evaluate which of the serine residues was necessary for interaction with acivicin, inactivation rates of the mutant enzymes were compared with that of the wild-type enzyme. Table IV shows inactivation rates of the mutants as compared to the rates for the wild-type enzyme. Replacements of Ser-385, -413 and -425 to L-serine complex to the L-glutamyl enzyme in a ping-pong mechanism proposed for the enzyme (10, 36).

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The inhibition constants of the wild-type and mutant enzymes by L-serine or serine-borate complex are given in Table V. All enzymes examined were inhibited in a competitive manner. The mutant enzymes in which Ser-385, 413 or 425 were replaced by Ala exhibited the same Ki values for L-serine and for serine-borate complex as for the wild-type enzyme. In the presence of saturating borate (10 mM), the Ki value of the wild type for L-serine decreased to 0.4% of that of L-serine alone. This decrease in Ki is attributed to the formation of a reversible linkage between the borate portion of the serine-borate complex and the hydroxyl group(s) of the enzyme. As expected from their Km values for L-γ-glutamyl-p-nitroanilide in the kinetic study, Ki values of Ser-431 and -452 mutants for L-serine in the absence of borate were only 5.5 and 3.8 fold higher than the wild-type, respectively.

Nevertheless, the usual decrease in Ki by addition of borate to the Ser-451, -452 and the double mutants was not as large as for the wild-type enzyme. Thus, substitution at Ser-451 and at Ser-452 greatly abolished (about 300 and 8 fold, respectively) inhibition by the serine-borate complex. Even at the concentration of L-serine higher than that of the borate, these inhibitions depended on the serine concentration. The borate appears to react with the complex of the enzyme and the serine because the borate needs the hydroxyl groups disposed appropriately to form the linkages. Therefore, Ser-451 and -452 residues appear to be necessary to form the linkage between the enzyme and borate. Moreover, inhibition of the double mutant appears to depend only on the serine portion of the serine-borate complex because the Ki values of the double mutant for serine-borate complex is much greater (>1,000 fold) than that of the wild type, while the Ki value of the double mutant for L-serine (without borate) is similar to the Ki value of the mutant for the serine-borate complex. This is in contrast to the wild-type and other active mutant enzymes. Additionally, the Ki of the double mutant for L-serine alone was relatively higher than expected from its Km values for the γ-glutamyl substrate, compared with the other mutants. This may be due to the lack of a hydrogen peroxide group located sufficiently near the position of the borate, corresponding to γ-carbonyl of the substrate, in the enzyme-inhibitor complex to form a reversible linkage between enzyme hydroxyl group and borate.

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The studies reported here indicate that Ser-451 or -452, or both, are essential for catalysis of γ-glutamyl transpeptidase, and that neither of these participate in the binding of the γ-glutamyl substrate. The results also suggest that these serine residues are positioned in the active site to allow interaction with the γ-carbonyl carbon of the γ-glutamyl moiety of the substrate. In addition to their spatial arrangement, the serine residues are nucleophilic so as to give rise to the nucleophilic substitution at the imide moiety of acivicin in the covalent-adduct formation. It also suggests that these residues would be primarily responsible for interaction with acivicin prior to the transfer to Ser-406 as proposed in previous studies (20). This seems analogous to the intramolecular transfer of the acyl group as found in fatty acid synthase (42). These findings are consistent with the suggestion that both Ser-451 and -452 are catalytic nucleophiles involved in formation of γ-glutamyl enzyme intermediate. However, it is also possible that these residues may stabilize the transition state in a manner similar to that of the oxanion hole found in serine proteases (37).

The rate of hydrolysis of L-γ-glutamyl-p-nitroanilide even by the double mutant, although virtually inactive, is still 2,000 times faster than without enzyme; the kcat of the double mutant was $1.7 \times 10^{-4}$ s⁻¹, while non-enzymatic hydrolysis was $9.5 \times 10^{-8}$ s⁻¹. This enhancement of the reaction rate may be achieved by a reaction pathway that is different from the normal mechanism. In such a pathway, a catalytic nucleophile may not be necessary. This possibility is similar to that for a subtilisin (a serine protease) mutant, where the catalytic serine was replaced by alanine. The mutant enzyme facilitated the hydrolysis of the acyl-p-nitroanilide substrate 3,000 times faster than in the absence of enzyme (38). This alternative mechanism of subtilisin for hydrolysis of the substrate without the catalytic serine is based on a detailed structure of the active site.

The motif, Pro-Leu-Ser⁴⁵₁-Ser⁴⁵₂-Met, is one of the most conservative regions in the complete sequences of γ-glutamyl transpeptidase from a variety of species. Further, the Ser-452 residue in the human enzyme is the only serine residue conserved among all γ-glutamyl transpeptidases for which the primary sequences are known and the two related enzymes, human γ-glutamyl transpeptidase-related enzyme (39) and Pseudomonas cephalosporin acylase (40). The later two are distinct from γ-glutamyl transpeptidase but have significant sequence homology. These comparisons suggest that these serine residues are of critical importance to enzyme function. Our data obtained using site-directed mutagenesis are in accord with this consideration in terms of sequence homology. Re-
Catalytic Role of Serine(s) in γ-Glutamyl Transpeptidase

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