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Different sites of acivicin binding and inactivation of γ-glutamyl transpeptidases

(glutathione/enzyme inhibition/active site)

TERRY K. SMITH*, YOSHITAKA IKEDA*, JUNICHI FUJII†, NAOYUKI TANIGUCHI†, AND ALTON MEISTER*

*Department of Biochemistry, Cornell University Medical College, 1300 York Avenue, New York, NY 10021; and †Department of Biochemistry, Osaka University Medical School, 2-2 Yamadaoka, Suita 565, Osaka, Japan

Contributed by Alton Meister, December 19, 1994

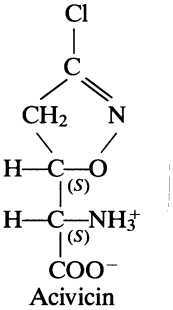
ABSTRACT **Acivicin is a potent inhibitor of γ-glutamyl transpeptidase (EC 2.3.2.2), an enzyme of importance in glutathione metabolism. Acivicin inhibition and binding are prevented by γ-glutamyl substrates and analogs (e.g., serine plus borate), consistent with the previous postulate that acivicin and substrates bind to the same enzyme site. Inactivation of rat kidney transpeptidase by acivicin leads to its binding as an ester to Thr-523. The pig enzyme, which has Ala-523 in place of Thr-523, is inhibited by acivicin with esterification at Ser-405. The human enzyme has Thr-524 (corresponding to Thr-523 in rat); its inactivation leads to esterification of Ser-406 (corresponding to Ser-405 in rat and pig). Hydroxylamine treatment of the acivicin-inactivated enzymes restores activity and releases the acivicin-derived *threo*-β-hydroxyglutamate moiety. The findings indicate that there are significant structural differences between the active site region of the rat enzyme and the active site regions of the human and pig. Human mutant enzymes in which Thr-524 and Ser-406 were replaced by Ala, separately and together, are enzymatically active, indicating that these amino acid residues are not required for catalysis. However, esterification of these residues (and of another near the active site) effectively blocks the active site or hinders its function. Acivicin can bind at enzyme sites that are close to that at which γ-glutamylation occurs; it may bind at the latter site and then be transesterified to another enzyme site.**

γ-Glutamyl transpeptidase (EC 2.3.2.2) plays a key role in glutathione metabolism by catalyzing the cleavage and formation of γ-glutamyl bonds (1–5). These reactions are thought to proceed through a γ-glutamyl-enzyme intermediate (reaction **1**),

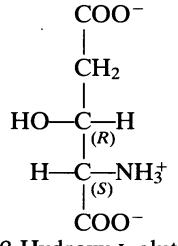
$$\gamma\text{-glutamylcysteinylglycine} + \text{enzyme} \rightleftharpoons \gamma\text{-glutamyl-enzyme} + \text{cysteinylglycine} \quad [1]$$

whose γ-glutamyl moiety can be transferred to amino acid acceptors to form γ-glutamyl amino acids, or to water, leading to hydrolysis. The enzyme is typically a highly glycosylated heterodimeric species in which the heavy subunit is anchored to the cell membrane through its N-terminal segment (residues 1–21) and the light subunit is noncovalently attached to the heavy subunit. The subunits are formed by cleavage of a single peptide chain proenzyme encoded by a common mRNA (6–8). The amino acid sequences of the enzymes from several species have been deduced from the cDNAs (9–12).

Acivicin is a highly active inhibitor of the enzyme, and its use has been of major importance in elucidating several aspects of glutathione metabolism (1, 4). There is evidence that both the heavy and the light subunits of the enzyme are involved in its action on glutathione (13, 14), but incubation of the enzyme with labeled acivicin leads to labeling that is predominantly on the light subunit (15). In previous studies it was found that when the enzyme was incubated with [^{14}C]acivicin, >90% of the label was bound to Thr-523 of the rat kidney enzyme (15). Since binding was prevented by adding γ-glutamyl substrate or substrate analogs such as serine + borate (16), it appears that acivicin interacts with the enzyme at or close to the active site. Previous work also showed that inhibition of the enzyme by acivicin involves conversion of acivicin on the enzyme to a form which is attached, apparently as an ester, to an enzyme hydroxyl group (17). The very slow rate of release of this species (to form *threo*-β-hydroxyglutamic acid) appears to account for the inhibition. An ester linkage is consistent with the finding that when the [^{14}C]acivicin-inhibited enzyme is incubated with hydroxylamine, enzymatic activity is restored, and there is associated release of the bound ^{14}C-labeled *threo*-β-hydroxyglutamate moiety (17).



Acivicin



threo-β-Hydroxy-L-glutamic acid

Although acivicin was found to bind to Thr-523 of the rat enzyme, studies on the pig enzyme (which is also inactivated by acivicin) showed that this enzyme does not have a threonine at this position, but has an alanine residue instead (10). In the present work, we have obtained evidence that the binding site of acivicin on the pig enzyme is Ser-405. The human enzyme, which has a Thr-524 (corresponding to rat enzyme Thr-523), binds acivicin at Ser-406 (corresponding to rat and pig Ser-405). In this work, we have also prepared and studied several human enzyme mutants in which the Ser-406 and Thr-524 residues are replaced by alanine residues. The results obtained further elucidate the nature of the inhibition by acivicin of γ-glutamyl transpeptidase.‡

EXPERIMENTAL PROCEDURES

Materials

γ-Glutamyl transpeptidase was isolated from frozen rat kidneys (Pel-Freez Biologicals) (3); the purified preparation had a specific activity of 1100 units/mg (a unit is defined below). The transpeptidase was also isolated from fresh pig kidneys

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‡A preliminary account of some of these findings has appeared (18).

(obtained from a local slaughterhouse) and from human kidneys (obtained from New York–New Jersey Regional Transplantation Program, New York Blood Center, New York; the kidneys were perfused continuously until used). These isolated enzyme preparations had specific activities of 560 and 850 units/mg, respectively. The enzyme preparations were solubilized by papain digestion of brush border membranes, which removes the N-terminal membrane-spanning domain (3). [^{14}C]Acivicin {L-($\alpha S,5S$)-α-amino-3-chloro-4,5-dihydro-5-[3-^{14}C]isoxazoleacetic acid} having specific radioactivity of 31 Ci/mol (1 Ci = 37 GBq) and unlabeled acivicin were kindly supplied by R. S. Hsi of Upjohn (acivicin was previously known as AT-125).

Endoproteinase Glu-C (EC 3.4.21.19) and endoproteinase Lys-C (EC 3.4.99.30) were purchased from Boehringer Mannheim. Other compounds were obtained from Sigma unless noted. Low molecular weight standards for gel electrophoresis and protein assay dye were purchased from Bio-Rad. Boric acid was obtained from Fisher Scientific and Monofluor scintillation liquid, from National Diagnostics. HPLC solvents were obtained from VWR Scientific. Guanidinium hydrochloride and methylamine were obtained from Schwarz/Mann and Eastman Kodak, respectively. Pierce supplied the constant-boiling 6 M HCl, triethylamine, amino acid standards and phenylisothiocyanate that were used for Pico-Tag amino acid analysis. Hydroxylamine was purchased from Mallinckrodt and compounds used in peptide sequencing were obtained from Applied Biosystems.

Methods

Enzyme Activity Assay. Assay for activity was carried out in a total volume of 1 ml at 37°C with L-γ-glutamyl-*p*-nitroanilide (1 mM) and glycylglycine (20 mM) in Tris·HCl buffer (0.1 M, pH 8) (3, 19). The release of *p*-nitroaniline was monitored at 410 nm (ε = 8800 $M^{-1}\cdot cm^{-1}$) by using a Varian Cary 219 spectrometer (1 unit is equivalent to the release of 1 μmol of *p*-nitroaniline per min). Protein was determined by the Bio-Rad protein assay dye, using bovine serum albumin as standard (20).

Deglycosylation of γ-Glutamyl Transpeptidase. Deglycosylation of the enzymes was conducted as described (21), using a mixture of glycosidases; 80–90% of the initially bound carbohydrate was removed after 10 days at 37°C. The treated enzymes were isolated from the reaction mixtures by passing them through a Sephadex G-75 column (3.5 × 10 cm) equilibrated with Tris·HCl (50 mM, pH 8). The fractions were pooled and concentrated; enzyme activity and protein concentration were determined before use for acivicin inhibition studies or proteolytic digestions.

Inactivation Studies. The enzyme (≈250 units) was incubated at 37°C in a solution (100 μl) of Tris·HCl (0.1 M, pH 8) containing various concentrations of acivicin (50 μM–5 mM). At various intervals, portions (5 μl) were assayed for residual activity.

Inactivation with [^{14}C]acivicin was carried out in sodium phosphate (50 mM, pH 7.5) at 37°C, with 10-fold molar excess of [^{14}C]acivicin to enzyme protein. Portions were assayed for activity at various intervals. After complete inactivation, the excess [^{14}C]acivicin was removed on a Sephadex G-25 (fine) column (1 × 5 cm) equilibrated with sodium phosphate (50 mM, pH 8.5) as described by Penefsky (22). The recovered radiolabeled inactivated enzyme was then used as stated below.

The various mutant enzymes and the wild-type enzyme (2 μg) were incubated at 25°C without and with acivicin (0.25–4 mM) in 100 μl of Tris·HCl (0.1 M, pH 8.0). The residual activities were measured in the standard assay at various intervals.

Gel Electrophoresis. SDS/10% polyacrylamide gels were prepared (23) and run at 100 V for 1–1.5 h on a Mighty Small II (SE 250) gel electrophoresis apparatus (Hoefer). The molecular weights of the subunits were estimated by using low molecular weight standards; Coomassie blue (R-250) was used to visualize the proteins. The lanes of other gels were cut out and each was cut into five equal parts, which were shaken with Tris·HCl (0.5 ml, 0.1 M, pH 8) containing SDS (0.1%) for 24 h. Liquid scintillation fluid (4 ml) was added and the radioactivity was determined.

^{14}C-Labeled Enzyme Linkage Stability Studies. The stability of the linkage between labeled compounds and the enzymes was studied under various conditions. These solutions were incubated at 37°C in a total volume of 100 μl for 24 h; portions (5 μl) were removed and assayed for enzyme activity. The remaining protein was precipitated, after addition of bovine serum albumin (1 mg), by adding trichloroacetic acid to a final concentration of 20%. After 30 min at 0°C, the samples were centrifuged in a Beckman B microcentrifuge for 5 min. The protein precipitate was washed twice with trichloroacetic acid (5%), and the combined supernatant solution and washings were analyzed for radioactivity.

Proteolytic Digestions. The acivicin-inhibited enzyme preparations were subjected to proteolytic digestion at 37°C for 24 h with endoproteinase Lys-C (10% by weight) in Tris·HCl (100 mM, pH 8.2) containing urea (2 M) and methylamine (20 mM) or with endoproteinase Glu-C (10% by weight) in sodium phosphate (50 mM, pH 7.8). Digestions with α-chymotrypsin (EC 3.4.21.1) (5% by weight) in Tris·HCl (50 mM, pH 7.8) containing $CaCl_2$ (5 mM) were carried out for 6 h. The studies involving protease digestion were carried out at least in duplicate.

Separation of Peptides. Peptides generated by proteolytic digestion were separated on a Waters μBondapak C_{18} column (3.9 mm × 30 cm) with a Waters system, using 0.1% trifluoroacetic acid as solvent A and 95% (vol/vol) acetonitrile (0.1% trifluoroacetic acid) as solvent B. A flow rate of 0.7 ml/min was used with a linear gradient established between 100% A and 60% B after 60 min; thereafter 100% B was used for 10 min. Elution of the peptides was monitored at 214 nm. Fractions (1 min) of the eluate were collected and assayed for radioactivity on an LKB-1218 Rackbeta scintillation counter.

Amino Acid Analysis. The analyses were performed by using the Pico-Tag system (24). Peptides were hydrolyzed with 6 M HCl for 24 h under nitrogen; after neutralization, the resulting amino acids were derivatized to form phenylthiocarbamyl-conjugated amino acids. Amino acid sequencing was performed by the Rockefeller University Protein Sequencing Facility, using automated Edman degradations on an Applied Biosystems gas-phase sequencer (25). Portions of the amino acid phenylthiohydantoin fractions were assayed for radioactivity.

DNA Sequence Analysis. A subclone containing nucleotides 1037 to 1799 (Ser-346 to the 3′ noncoding sequence) within γ-glutamyl transpeptidase cDNA isolated from porcine brain capillaries designated psGT 18/12 was a kind gift from H. Gassen and G. Weber (10). Sequencing of a portion of this 762-bp subfragment was conducted by annealing the primer, 5′-GAACCCCGGTTGCACAAC-3′ (Operon Technologies, Alameda, CA), and sequencing double-stranded DNA by the dideoxynucleotide chain-termination method (26), using Sequenase (United States Biochemical) according to the supplier's instructions.

Construction of Transfer Plasmid. An *Nco* I–*Eco*RI 1.8-kb fragment containing the whole coding region of the human enzyme was prepared from the expression plasmid previously described (14). The fragment was then ligated to pBluescript SK (+) (Stratagene) in which an *Nco* I site had been created at a *Sma* I site. The insert was excised from the resulting plasmid by using *Not* I and *Eco*RI and ligated to a transfer

vector, pVL1392. The transfer plasmid carrying the cDNA for the human enzyme was purified by CsCl density gradient centrifugation and was used to express the wild-type enzyme.

Site-Directed Mutagenesis. A 3′ *Bam*HI–*Eco*RI 0.6-kb fragment from the wild-type sequence was subcloned in pBluescript KS (+). The uracil-substituted single-stranded DNA was prepared from *Escherichia coli* CJ236 transformed with the plasmid. The uracil-substituted template was used with the oligonucleotide primers 5′-TACTTTGGCGCCAAGGTCC-3′ and 5′-AGGCAGTGGCTGCAGCC-3′ to generate the mutant sequences in which Ser-406 and Thr-524, respectively, in the human enzyme were replaced by Ala, according to Kunkel (27). The mutations were confirmed by dideoxynucleotide sequencing (26). The double mutant in which both Ser-406 and Thr-524 were replaced by Ala was prepared by combination of those single mutants by using a *Bsp*EI site located between the two mutated regions. The resulting mutant sequences were inserted into the corresponding regions of the wild-type sequence by replacement. The transfer plasmid carrying the mutants, designated as T524A and S406A, and the double mutant (S406A + T524A) were constructed and purified as done for the wild type.

Preparation of the Recombinant Viruses. *Spodoptera frugiperda* Sf21 cells were used as host cells for the baculovirus expression system (28). The cells were maintained at 28°C in Grace's insect medium (BRL) supplemented with 10% fetal bovine serum, "yeastolate" (BRL) at 3.3 g/liter, lactalbumin hydrolysate at 3.3 g/liter, and gentamicin at 50 μg/ml. The purified transfer plasmids were cotransfected with BaculoGold DNA (PharMingen), which was employed as an *Autographa californica* nuclear polyhdedrosis virus genome, using Lipofectin (BRL). The generated recombinant viruses were recovered 5 days after transfection and amplified to 5×10^7 plaque-forming units/ml after a single round of plaque assay.

Expression and Purification of the Recombinant Enzymes. Sf21 cells (2×10^8) were infected by the recombinant viruses at a multiplicity of infection of 4. Infected cells were harvested 80 h after infection. The enzymes were solubilized from microsome fractions of the infected cells by treatment with 1% Triton X-100 and papain. The extracted enzymes were purified by a series of chromatographic steps, including hydroxylapatite (Bio-Rad), chromatofocusing (PBE94 and polybuffer74; Pharmacia), and Sephacryl S-200 HR (Pharmacia) gel filtration. SDS/gel electrophoresis analyses (as described earlier) of the purified wild-type and mutant enzymes showed only two bands, corresponding to the heavy (44-kDa) and light (24-kDa) subunits.

RESULTS

Inhibition Studies. The enzymes isolated from kidneys were rapidly inactivated at pH 8 and 37°C when incubated with 1 mM acivicin. Inactivation of the rat enzyme was complete within 30 min; the enzymes from human kidney and pig kidney were, respectively, 95% and 85% inactivated after 1 hr. The second-order kinetic constants for inactivation of the rat, pig, and human enzymes were, respectively, 8.0, 1.0, and 1.6 $M^{-1} \cdot s^{-1}$. Inactivation was completely prevented by adding 25 mM each of L-serine and sodium borate (but not by adding either of these alone) (16, 29), and the inactivation rate was increased on addition of 50 mM sodium maleate, by about 4.5-fold (human, pig) and by about 9-fold (rat). Maleate is known to enhance hydrolysis and to decrease transpeptidation, presumably by binding to the acceptor site of the enzyme (30). Complete inactivation of the enzymes by [^{14}C]acivicin (see *Methods*) was associated with essentially stoichiometric binding of acivicin (Table 1). About 87–88% of the ^{14}C was associated with the light subunit after separation of the subunits by SDS/gel electrophoresis. The relative stabilities of the ^{14}C-labeled enzyme linkage for the pig and human enzymes to hydrochloric acid, sodium hydroxide, and hydroxylamine were very similar to those found for the rat enzyme (17); treatment with sodium hydroxide led to release of *threo*-β-hydroxyglutamate in all cases.

Table 1. Binding of [^{14}C]acivicin to the enzymes

Enzyme source	[^{14}C]Acivicin equivalents/mol
Rat	1.03 ± 0.14
Human	0.98 ± 0.11
Pig	0.96 ± 0.11

Results are mean ± SD (n = 4).

Binding Site of Acivicin. The reactive hydroxyl group was confirmed to be that of Thr-523 in the rat enzyme by use of a procedure alternative to that used previously (15). The deglycosylated labeled enzyme (21) was treated with endoproteinase Glu-C (see *Methods*), and a peptide corresponding in composition to residues 520–534 (Table 2, rat) was obtained. N-terminus sequencing showed that about 90% of the peptide was blocked by cyclization of Gln-520; the unblocked portion was sequenced through five cycles, giving the sequence Gln-Val-Val-Thr-Ala. The radioactivity (≈80% recovery) eluted in cycle four (Thr-523), confirming previous results (15).

The sequence of the pig enzyme reported by Papandrikopoulou *et al.* (10) has no Thr-523, but has instead Ala-523. We confirmed this by sequencing a portion of a subclone (kindly supplied by H. Gassen) corresponding to a segment from Ser-346 (nt 1037) to part of the 3′-noncoding sequence (nt 1799). The relevant nucleotides were identified as GCT, thus confirming alanine at position 523 (10).

To identify the ^{14}C-binding site in the pig enzyme, the [^{14}C]acivicin-inhibited deglycosylated pig enzyme was prepared and subjected to proteolytic digestion by Endoproteinase Glu-C. A radiolabeled peptide was separated and repurified on a C_{18} column; amino acid analysis indicated that the peptide corresponded to residues 390–418 (Table 2, pig). Automated Edman degradation of this peptide confirmed the sequence as Gly-Ser-Ala-Val-Ser-Ala-Thr-Ser-Thr-Ile for the first 10 cycles, after which the yield significantly decreased; no radioactivity was detected. The isolated radiolabeled peptide was then subjected to digestion with α-chymotrypsin, and the resulting mixture of oligopeptides was fractionated on a C_{18} column. Two peptides containing all of the radioactivity were isolated; the larger corresponded to the enzyme sequence 390–418 (40% of the radioactivity). The smaller peptide (60% of radioactivity) gave the initial sequence Gly-Ser-Lys-Val-Arg; the radioactivity (731 cpm, 76% recovery) was eluted, on automated Edman sequencing, with cycle two, corresponding to Ser-405.

[^{14}C]Acivicin binding in the human kidney enzyme was also studied; the human enzyme sequence (11) contains both Thr-524 and Ser-406. Endoproteinase Glu-C digestion of the [^{14}C]acivicin-inhibited deglycosylated human enzyme revealed a single radiolabeled peptide whose composition agreed with that of peptide 391–420 of the human sequence (Table 2). After treatment with α-chymotrypsin, a smaller peptide containing about 74% of the total radioactivity was separated by chromatography on a C_{18} column. The amino acid composition of this peptide was similar to that obtained with the pig enzyme under these conditions, indicating linkage of ^{14}C to Ser-406. No radioactivity was associated with the peptide obtained from the Glu-C digestion of the [^{14}C]acivicin-inhibited deglycosylated human enzyme that corresponded to residues 521–528. The findings indicate that acivicin inhibits the human enzyme by binding to Ser-406 and not to Thr-524.

Human Enzyme Mutants. The findings given above were further pursued by construction of several mutants of the human enzyme, which were expressed in *Spodoptera frugiperda* (Sf21) cells as described in *Experimental Procedures.* A mutant

Table 2. Amino acid compositions (mol/mol) of radiolabeled peptides obtained from endoproteinase Glu-C digestions of [^{14}C]acivicin-inhibited γ-glutamyl transpeptidases

Amino acid residue	Rat		Pig		Human	
	Found	Seq. A	Found	Seq. B	Found	Seq. C
Asx	0.1 ± 0.1	0	2.7 ± 0.3	3	2.8 ± 0.3	3
Glx*	2.8 ± 0.2	2	1.1 ± 0.2	0	1.9 ± 0.2	1
Ser	0.2 ± 0.1	0	4.9 ± 0.5	6	5.5 ± 0.5	6
Gly	1.1 ± 0.1	1	3.1 ± 0.2	3	2.8 ± 0.3	3
His	2.7 ± 0.2	3	0.1 ± 0.0	0	0.1 ± 0.0	0
Arg	0.9 ± 0.1	1	1.9 ± 0.1	2	0.8 ± 0.3	1
Thr	3.1 ± 0.2	3	1.8 ± 0.1	2	1.7 ± 0.2	2
Ala	0.8 ± 0.1	1	1.4 ± 0.1	2	1.8 ± 0.0	2
Pro	0.3 ± 0.1	0	0.4 ± 0.1	0	0.9 ± 0.1	1
Tyr	0.0 ± 0.1	0	0.8 ± 0.1	1	1.0 ± 0.0	1
Val	2.0 ± 0.2	2	2.2 ± 0.2	2	2.7 ± 0.2	3
Met	0.1 ± 0.0	0	0.2 ± 0.0	0	0.2 ± 0.1	0
Ile	0.1 ± 0.1	0	3.2 ± 0.1	3	1.9 ± 0.1	2
Leu	0.8 ± 0.1	1	1.8 ± 0.2	2	2.2 ± 0.2	2
Phe	0.0 ± 0.0	0	2.5 ± 0.1	2	1.8 ± 0.1	2
Lys	0.9 ± 0.1	1	1.1 ± 0.0	1	0.9 ± 0.0	1

Sequence A = rat, residues 520–534: QVVTAGLKTRHHHTE. Sequence B = pig, residues 390–418: GSAVSATSTINLYFGSKVRSRISGILFND. Sequence C = human, residues 391–420: GSAVSATSTINLYFGSKVRSPVSGILFNNE.

*Glx includes glutamate, hydrolyzed glutamine, and *threo*-β-hydroxyl-L-glutamate (from acivicin).

in which Thr-524 was replaced by alanine (T524A) was obtained and purified (Table 3). Mutant T524A was active and was effectively inhibited by acivicin; inactivation followed pseudo-first-order kinetics at several concentrations of acivicin. Second-order kinetic constants for the wild-type and the T524A mutant are given in Table 3. A mutant in which Ser-406 was replaced by alanine (S406A) was also expressed and purified. This mutant was active and was inhibited by acivicin (Table 3). The double mutant (S406A plus T524A) was also expressed and purified. This mutant enzyme was also active and was inhibited by acivicin (Table 3).

It was previously found that the isolated rat kidney enzyme, after inhibition by acivicin, can be at least 85% reactivated by incubation with hydroxylamine (17). This supports the idea that the inhibitory species is bound to the enzyme by ester linkage. In the present work, the recombinant wild-type human enzyme and the double mutant enzyme were incubated with 1 M hydroxylamine at pH 8.0 at 37°C (Fig. 1); the reactivation of both acivicin-inhibited forms was about the same and was about 70% complete after 72 hr. Only 0.5% of the activity of the wild-type and 2% of the double mutant returned in the absence of hydroxylamine. Treatment of the purified enzymes with hydroxylamine under these conditions led to loss of 45% and 51%, respectively, of the initial activities of the wild type and mutant.

Table 3. Specific activities and inhibition rate constants of several γ-glutamyl transpeptidases

Enzyme	Activity, units/mg protein	Rate constant for inhibition by acivicin,* $M^{-1}\cdot s^{-1}$
Rat kidney (isolated)	1100	8.0
Pig kidney (isolated)	560	1.0
Human kidney (isolated)	850	1.6
Human (recombinant; wild type)	530	1.5
Human mutant S406A	490	1.4
Human mutant T524A	340	1.0
Human mutant T524A/S406A	380	1.2

*Second-order rate constants. Inhibition by 6-diazo-5-oxo-L-norleucine (DON) and L-azaserine followed a similar pattern. Thus, for DON the rate constants were 2.7, 0.41, and 0.24 $M^{-1}\cdot s^{-1}$ for rat, human, and pig, respectively; the corresponding values for azaserine were 0.75, 0.11, and 0.05 $M^{-1}\cdot s^{-1}$.

DISCUSSION

The findings indicate that acivicin inhibits the rat enzyme by esterification of Thr-523 and that it inhibits the pig and human enzymes by esterifying Ser-405 and Ser-406, respectively. De-esterification by treatment with hydroxylamine restores most of the enzyme activity. It appears that the acivicin-inactivation and reactivation phenomena are facilitated by similar mechanisms in the several enzymes. However, although there is strong analogy between the reaction of the enzyme with acivicin and its reaction with γ-glutamyl substrates, the present findings support the conclusion that acivicin binds to a site close to, but different from, that at which γ-glutamylation occurs. It is quite reasonable to conclude that serine plus borate (or γ-glutamyl donors) can simultaneously block both sites.

Since site-specific replacement of Thr-524 and Ser-406 by Ala in the human enzyme does not lead to loss of enzymatic activity, it may be concluded that these enzyme residues are not required for catalysis. Nevertheless, their esterification appears to block the active site. That the double mutant

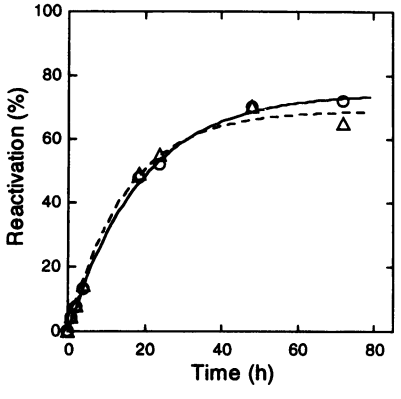

FIG. 1. Reactivation of wild-type and mutant S406A + T524A human enzymes by hydroxylamine. The enzymes (20 μg) were inactivated with 2.5 mM acivicin at 37°C until >99.9% of the activities disappeared. Excess acivicin was removed by gel filtration, and the inactivated enzymes were incubated at 37°C in 1 M hydroxylamine, 20 mM Tris·HCl (pH 8.0). The activities of the wild type (○) and the double mutant (△) were then determined by the standard assay.

(T524A + S406A) is enzymatically active confirms that these amino acid residues are not needed for catalysis. The findings that the double mutant is inhibited by acivicin and that inhibition is reversed by treatment with hydroxylamine indicate that still another hydroxyl group becomes esterified in the inhibition of this form of acivicin; such esterification can evidently block the active site or lead to a conformational change that hinders the function of the active site. This site may or may not be the postulated hydroxyl group that normally functions in the formation of the γ-glutamyl-enzyme. The quantities of double-mutant enzyme available do not permit identification of this residue at this time. The light subunit of the enzyme (human) has 30 hydroxyl groups, which potentially might interact with acivicin. One or more of these (or conceivably a hydroxyl group of the heavy subunit) might serve as the site that normally interacts with the γ-glutamyl substrate. This site, often postulated to be a hydroxyl group (3), must be reasonably close in the enzyme structure to the sites that have been found to bind acivicin. Although the site in the human enzyme that binds acivicin was found to be Ser-406, the human mutant lacking a hydroxyl group at this position was inactivated by acivicin at almost the same rate as the wild type. That the rates of inactivation by acivicin of the various human enzyme mutants are similar is consistent with the view that this rate is determined by the initial rate of binding of acivicin to an enzyme hydroxyl group. Possibly acivicin binds initially to a γ-glutamyl binding hydroxyl group and is then transesterified to another hydroxyl group present in the immediate environment, such as Thr-523(4) and Ser-405(6).

The remarkable reactivity of acivicin with the enzyme seems to depend only partially on its ability to interact with the enzyme sites that normally bind the α-carboxyl and α-amino groups of the γ-glutamyl substrates. Further reaction of the initially bound acivicin, which involves steps that are different from those that participate in normal catalysis, leads to loss of the chlorine atom of acivicin, opening of the isoxazole ring, and formation of *threo*-β-hydroxyglutamate (17).

Differences among the various γ-glutamyl transpeptidases have been noted previously, such as those involving substrate specificity and specific activity (2–5). Similarly, differences in specific activity and rates of inactivation were found in the present work as well as in the acivicin binding sites. Thus, the rat enzyme is more active than the others (Table 3), and the rate of inactivation by acivicin is greater for the rat enzyme than for the others. One might think that the γ-glutamyl binding site would be conserved among various γ-glutamyl transpeptidases, but this remains to be demonstrated. The data reported here indicate that the three-dimensional structures in the regions of the active centers of these enzymes are different; thus, for example, although the rat and human enzymes both have Thr-523(4) and Ser-405(6), acivicin is bound at different sites in the two enzymes.

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1. Meister, A. (1989) in *Glutathione: Chemical, Biochemical and Medical Aspects*, eds. Dolphin, D., Poulson, R. & Avramovic, O. (Wiley, New York), pp. 367–474.
2. Tate, S. S. (1980) in *Enzymatic Basis of Detoxification*, ed. Jakoby, W. B. (Academic, New York), Vol. 2, pp. 95–120.
3. Tate, S. S. & Meister, A. (1985) *Methods Enzymol.* **113,** 400–419.
4. Meister, A. & Anderson, M. E. (1983) *Annu. Rev. Biochem.* **52,** 711–760.
5. Meister, A., Tate, S. S. & Ross, L. L. (1976) in *The Enzymes of Biological Membranes*, ed. Martinosi, A. (Plenum, New York), Vol. 3, pp. 315–347.
6. Capraro, M. A. & Hughey, R. P. (1983) *FEBS Lett.* **157,** 139–143.
7. Nash, B. & Tate, S. S. (1982) *J. Biol. Chem.* **257,** 585–588.
8. Kuno, T., Matsuda, Y. & Katunuma, N. (1983) *Biochem. Biophys. Res. Commun.* **114,** 889–895.
9. Laperche, Y., Bulle, F., Aissani, T., Chobert, M.-N., Aggerbeck, M., Hanoue, J. & Guelaën, G. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 3159.
10. Papandrikopoulou, A., Frey, A. & Gassen, H. (1989) *Eur. J. Biochem.* **183,** 693–698.
11. Sakamuro, D., Yamazoe, M., Matsuda, Y., Kangawa, K., Taniguchi, N., Matsuo, H., Yoshikawa, H. & Ogasawara, N. (1988) *Gene* **73,** 1–9.
12. Suzuki, H., Kumagai, H., Echigo, T. & Tochikura, T. (1989) *J. Bacteriol.* **171,** 5169–5172.
13. Stole, E. & Meister, A. (1991) *J. Biol. Chem.* **266,** 17850–17857.
14. Ikeda, Y., Fujii, J. & Taniguchi, N. (1993) *J. Biol. Chem.* **268,** 3980–3985.
15. Stole, E., Seddon, A. P., Wellner, D. & Meister, A. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1706–1709.
16. Tate, S. S. & Meister, A. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 4806–4809.
17. Stole, E., Smith, T. K., Manning, J. M. & Meister, A. (1994) *J. Biol. Chem.* **269,** 21435–21439.
18. Ikeda, Y., Smith, T. K., Meister, A. & Taniguchi, N. (1994) *FASEB J.* **8,** A1357 (abstr.).
19. Orlowski, M. & Meister, A. (1963) *Biochim. Biophys. Acta* **73,** 679–681.
20. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
21. Smith, T. K. & Meister, A. (1994) *FASEB J.* **8,** 661–664.
22. Penefsky, H. S. (1977) *J. Biol. Chem.* **252,** 2891–2899.
23. Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244,** 4406–4412.
24. Bidlingmeyer, B. A., Cohen, S. A. & Tarvin, T. L. (1984) *J. Chromatogr.* **336,** 93–104.
25. Hewick, R. M., Hunkapiller, M. W., Hood, L. E. & Dreyer, W. J. (1981) *J. Biol. Chem.* **256,** 7990–7997.
26. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
27. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 488–492.
28. Piwnica-Worms, H. (1987) in *Current Protocols in Molecular Biology*, eds. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (Greene and Wiley, New York), pp. 16.8.1–16.11.7.
29. Revel, J. & Ball, E. G. (1959) *J. Biol. Chem.* **234,** 577–582.
30. Thompson, G. A. & Meister, A. (1980) *J. Biol. Chem.* **255,** 2109–2113.