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Stimulation of hepatic glutathione formation by administration of L-2-oxothiazolidine-4-carboxylate, a 5-oxo-L-prolinase substrate

(cysteine delivery system/phosgene/pyroglutamate)

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ABSTRACT 5-Oxo-L-prolinase, the enzyme that catalyzes the conversion of 5-oxo-L-proline to L-glutamate coupled to the cleavage of ATP to ADP and P_i, also acts on L-2-oxothiazolidine-4-carboxylate (an analog of 5-oxoproline in which the 4-methylene moiety is replaced by sulfur) and ATP to yield cysteine and ADP. The enzyme, which exhibits an affinity for the analog similar to that for the natural substrate, is inhibited by the analog *in vitro* and *in vivo*. L-2-Oxothiazolidine-4-carboxylate thus serves as a potent inhibitor of the γ-glutamyl cycle at the step of 5-oxoprolinase. Administration of L-2-oxothiazolidine-4-carboxylate to mice that had been depleted of hepatic glutathione led to restoration of normal hepatic glutathione levels. Since L-2-oxothiazolidine-4-carboxylate is an excellent substrate of the enzyme, it may serve as an intracellular delivery system for cysteine and thus has potential as a therapeutic agent for conditions in which there is depletion of hepatic glutathione.

5-Oxo-L-prolinase catalyzes the ATP-dependent hydrolysis of 5-oxo-L-proline according to the reaction given in Fig. 1. The requirement for energy in this reaction is consistent with the position of the equilibrium between 5-oxoproline and glutamate, which markedly favors the cyclic product (1–3). 5-Oxoprolinase activity has been found in a number of animal tissues and has been purified from kidney, a rich source of the enzyme. Earlier work in this laboratory established that 5-oxo-L-proline is a quantitatively significant metabolite of glutathione which is formed in the γ-glutamyl cycle by the action of γ-glutamyl cyclotransferase on γ-glutamyl amino acids (4). Thus, the enzyme-catalyzed hydrolysis of 5-oxo-L-proline links the reactions involved in the utilization of glutathione (catalyzed by γ-glutamyl transpeptidase, γ-glutamyl cyclotransferase, and cysteinylglycinase) with those involved in its synthesis (catalyzed by γ-glutamylcysteine synthetase and glutathione synthetase). In previous work (5) it was shown that L-2-imidazolidone-4-carboxylate, a competitive inhibitor of 5-oxoprolinase, markedly decreases the metabolism of 5-oxoproline *in vivo*.

Here we describe a new heterocyclic substrate of 5-oxoprolinase, L-2-oxothiazolidine-4-carboxylate, which is cleaved by the enzyme according to the scheme given in Fig. 1. In this pathway it is assumed that S-carboxy-cysteine, the initial product of hydrolysis, decarboxylates nonenzymatically. We have found that administration of L-2-oxothiazolidine-4-carboxylate to mice produces marked inhibition of the metabolism of 5-oxoproline but not that of glutamate. Administration of this compound also stimulates formation of glutathione in the liver.

EXPERIMENTAL PROCEDURES

Materials. The L and D isomers of 2-oxothiazolidine-4-carboxylate were synthesized by the method of Kaneko *et al.* (6) as modified (7). We are indebted to Sidney Weinhouse for a sample of the L isomer which was used in our initial studies. 5-Oxo-L-[U-^{14}C]proline and L-[U-^{14}C]glutamate were obtained from New England Nuclear. Male mice (NCS strain; 15–25 g) were obtained from The Rockefeller University. Pyruvate kinase, lactate dehydrogenase, glutathione reductase, phosphoenolpyruvate, NADPH, NADH, glutathione, dithiothreitol, ethanolamine, ATP, 5,5′-dithiobis(2-nitrobenzoate) (DTNB), and Hepes were obtained from Sigma. 2-Vinylpyridine was obtained from Aldrich.

5-Oxo-L-prolinase has been purified from rat kidney (3); the enzyme used in the present studies was isolated from rat kidney by a new procedure which led to a final specific activity of 84 units per mg. Immediately prior to use, the enzyme was freed of the 5-oxoproline present in the storage buffer by the gel filtration procedure described by Penefsky (8).

After incubation, the reaction mixtures (see Fig. 2 and Table 1) were treated with 0.1 vol of 1 M HCl and placed at 0°C for 5 min; an equivalent volume of 1 M Tris was added. Portions were analyzed for amino acids (glutamate, cystine, S-acetamidocysteine, and 2,3-diaminopropionic acid) by use of a Durrum model 500 amino acid analyzer. In some experiments, cysteine was determined by reaction with DTNB as described (9). The kinetic values reported in Table 1 were determined by following ADP formation continuously with the pyruvate kinase/lactate dehydrogenase coupled assay. In these studies, initial velocities were determined in reaction mixtures (final volume, 1.0 ml) containing 100 mM Na Hepes buffer (pH 8.0), 150 mM KCl, 8 mM $MgCl_2$, 2 mM phospho*enol*pyruvate, 20 units of pyruvate kinase, 20 units of lactate dehydrogenase, 5 mM ATP, 0.3 mM NADH, 2–9 μg of 5-oxoprolinase, and 5–200 μM substrate or substrate analog. Activity was followed at 37°C by measuring the rate of change of absorbance at 340 nm.

RESULTS

When the enzyme was incubated with L-2-oxothiazolidine-4-carboxylate in the presence of ATP there was rapid formation of ADP and cysteine (Fig. 2, curves 1 and 2). No reaction was observed when ATP was omitted (curve 4) or when the substrate was replaced by the corresponding D isomer (curve 3). That the ratio of ADP to cysteine produced increased with time may readily be explained by loss of cysteine due to oxidation. A more quantitative characterization of the reaction was obtained in studies in which the formation of cysteine was estimated by determining derivatives of this amino acid product. The formation of ADP and cysteine (determined as cystine after oxidation, or as the corresponding S-acetamido compound after reduction and derivatization) was stoichiometric (Table 1). It is notable that the apparent K_m value for the new substrate is somewhat lower than that found for the natural substrate, 5-oxo-

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Abbreviation: DTNB, 5,5′-dithiobis(2-nitrobenzoate).

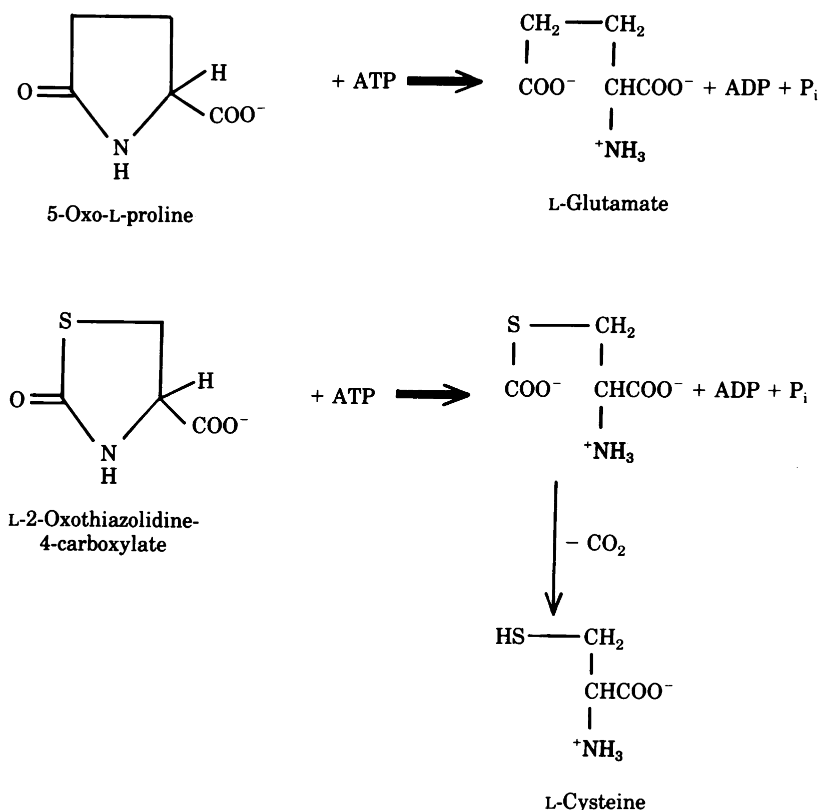

FIG. 1. Reactions catalyzed by 5-oxo-L-prolinase.

L-proline; however, the calculated V_{max} value for the natural substrate is higher than that found for the analog. As expected, L-2-oxothiazolidine-4-carboxylate is an excellent inhibitor of the utilization of 5-oxo-L-proline by the purified enzyme; in reaction mixtures containing 1 mM 5-oxo-L-proline, addition of L-2-oxothiazolidine-4-carboxylate at concentration of 1, 5, and 10 mM produced 73, 92, and 97% inhibition of glutamate formation, respectively. Under these conditions, D-2-oxothiazolidine-4-carboxylate did not inhibit.

The data in Fig. 3 show that L-2-oxothiazolidine-4-carboxy-

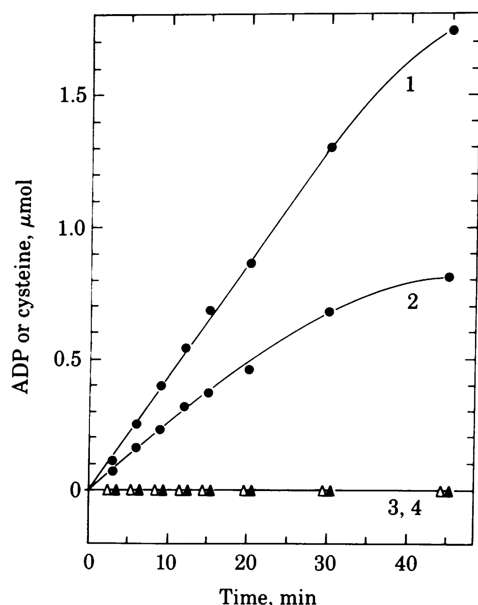

FIG. 2. Cleavage of L-2-oxothiazolidine-4-carboxylate by 5-oxo-L-prolinase. The reaction mixtures (final volume, 1.5 ml) contained 100 mM Na Hepes buffer (pH 8.0), 150 mM KCl, 8 mM $MgCl_2$, 2 mM phosphoenolpyruvate, 10 units of pyruvate kinase, 100 μg of 5-oxoprolinase, and: in curves 1 and 2, 5 mM L-2-oxothiazolidine-4-carboxylate and 5 mM ATP; in curve 3, 5 mM D-2-oxothiazolidine-4-carboxylate and 5 mM ATP; in curve 4, 5 mM L-2-oxothiazolidine-4-carboxylate but ATP was omitted. At the indicated intervals, portions were analyzed for ADP and for cysteine by reaction with DTNB (9).

Table 1. Action of 5-oxo-L-prolinase on L-2-oxothiazolidine-4-carboxylate

Substrate		Products formed, nmol		App. K_m, μM	V_{max}, μmol/min/mg
Compound	mM	ADP*	Amino acid		
5-Oxo-L-proline	2	924	916†	5	1.34
L-2-Oxothiazolidine-4-carboxylate	2	478	466‡	2	0.73
		499	463§		
D-2-Oxothiazolidine-4-carboxylate	5	96	0‡,¶	—	—
L-2-Imidazolidone-4-carboxylate	2	930	0‖	14	1.45

The reaction mixtures contained (final volume, 0.5 ml) 100 mM Na Hepes buffer (pH 8.0), 150 mM KCl, 8 mM $MgCl_2$, 2 mM phosphoenolpyruvate, 5 mM ATP, 10 units of pyruvate kinase, 20 μg of 5-oxoprolinase, and substrate as indicated; incubation was for 30 min at 37°C.

* Determined by use of pyruvate kinase/lactate dehydrogenase coupled assay; all values were corrected by subtracting a blank value of 54 nmol.

† Determined as glutamate.

‡ Determined after reduction with KBH_4 and reaction with iodoacetamide (10).

§ Determined after conversion to cystine by oxidation.

¶ <20 nmol.

‖ <5 nmol.

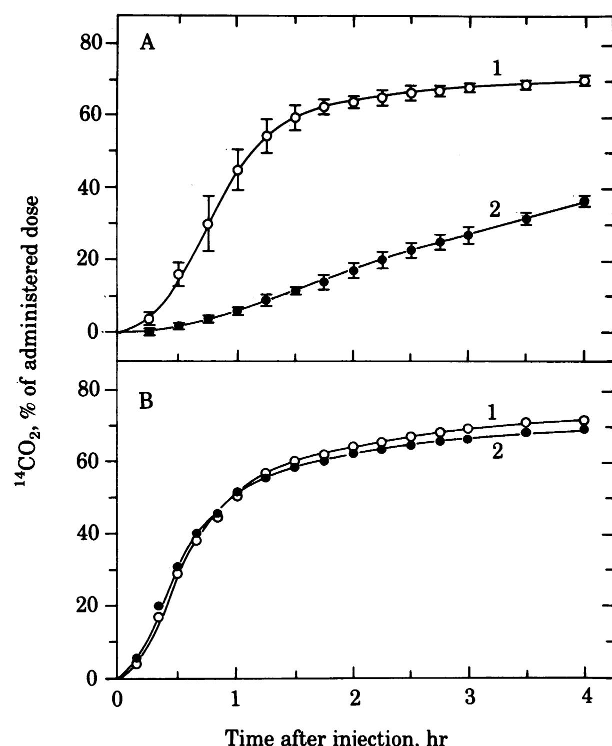

FIG. 3. Inhibition of 5-oxoproline metabolism by L-2-oxothiazolidine-4-carboxylate. Mice fasted overnight were injected intraperitoneally with NaCl (curve 1; controls) or with Na L-2-oxothiazolidine-4-carboxylate at 2.5 mmol/kg (curve 2). After 10 min, the mice were injected subcutaneously with 5-oxo-L-[^{14}C]proline (*A*) or with L-[^{14}C]glutamate (*B*) (0.02 mmol; 1.1×10^6 cpm). The mice were then placed in metabolic chambers and the respiratory CO_2 was collected in ethanolamine/methanol, 1:4 (vol/vol). In *A*, each curve represents the results on three animals; the data are shown as mean ± SD. In *B*, each curve represents the mean of closely agreeing results obtained from two animals.

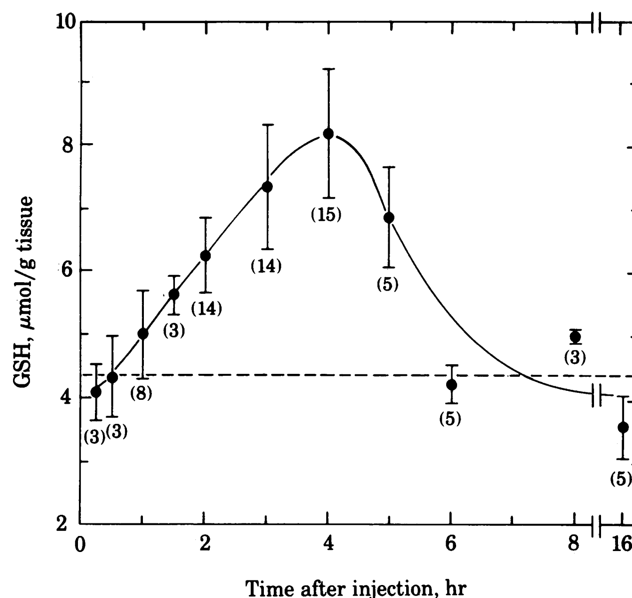

FIG. 4. Increase in hepatic levels of glutathione (GSH) after injection of L-2-oxothiazolidine-4-carboxylate. Mice fasted overnight were injected intraperitoneally with NaCl (controls; dashed line) or with Na L-2-oxothiazolidine-4-carboxylate (6.5 mmol/kg). At intervals, the mice were killed by decapitation and exsanguinated, and the excised liver was homogenized in 5 vol of 1% picric acid. After centrifugation, the protein-free supernatant solutions were analyzed in duplicate for glutathione by the method of Tietze (12). In a number of instances, these values were checked by the 2-vinylpyridine procedure (13); good agreement between the two methods was observed. The results are shown as mean ± average deviation from the mean. The numbers in parentheses give the number of animals used. The value for the controls was 4.40 ± 0.9 μmol/g.

late also inhibits *in vivo* utilization of 5-oxoproline. In the experiments described in Fig. 3*A*, animals were injected with 5-oxo-L-[^{14}C]proline and the respiratory $^{14}CO_2$ produced was collected for several hours and measured. There was a marked decrease in the formation of $^{14}CO_2$ in the animals given an injection of L-2-oxothiazolidine-4-carboxylate; these animals excreted about 16% of the injected oxoproline in their urine. No radioactivity was found in the urine of the controls. Injection of L-2-oxothiazolidine-4-carboxylate did not have a significant effect on the metabolism of glutamate (Fig. 3*B*). Previous studies in this laboratory showed that injection of L-2-imidazolidone-4-carboxylate also markedly inhibits the *in vivo* metabolism of 5-oxoproline but not that of glutamate. However, L-2-oxothiazolidine-4-carboxylate is a much more efficient inhibitor; the thiazolidine compound is at least 4 times more active on a molar basis (11).

Fig. 4 gives data on the effect of injection of L-2-oxothiazolidine-4-carboxylate on the level of hepatic glutathione. Administration of the thiazolidine stimulated the formation of glutathione to levels about twice those of the controls. The maximal effect was observed about 4 hr after injection. No such stimulation was observed when the animals were injected with equivalent doses of D-2-oxothiazolidine-4-carboxylate. These findings are indicative of *in vivo* formation of cysteine after administration of L-2-oxothiazolidine-4-carboxylate. We did not detect free cysteine in acid extracts of the livers of control or experimental animals by amino acid analyses carried out after reduction by dithiothreitol and derivatization with 2-vinylpyridine. This finding, not surprising in view of the low tissue levels of cysteine, indicates that cysteine formed by cleavage of the thiazolidine is rapidly utilized for glutathione synthesis.

DISCUSSION

These studies show that L-2-oxothiazolidine-4-carboxylate is an efficient substrate of 5-oxoprolinase and that its affinity for the enzyme is somewhat higher than that of the natural substrate. Because the thiazolidine inhibits 5-oxoproline metabolism in mice, this compound may prove useful as a tool for studies on the inhibition of 5-oxoprolinase *in vivo*. The thiazolidine is substantially more active as an inhibitor than the most active previously known inhibitor, L-2-imidazolidone-4-carboxylate. The latter compound serves as a partial substrate for the enzyme; thus, it stimulates rapid hydrolysis of ATP but is not converted to an amino acid product (3, 5, 11).

The findings indicate that L-2-oxothiazolidine-4-carboxylate is a substrate *in vivo* and thus leads to formation of cysteine which is used for glutathione synthesis. This suggests that the thiazolidine may be useful as a therapeutic agent by providing an intracellular delivery system for cysteine. Cysteine itself is known to be toxic when administered to animals. This observation and the fact that cysteine is rapidly degraded appear to limit the usefulness of this amino acid in therapy. We suggest that the thiazolidine might be considered in therapy designed to increase intracellular glutathione levels—for example, for hepatic toxicity produced by such drugs as acetaminophen. Preliminary studies in this laboratory have shown that the marked decrease in hepatic glutathione that occurs after administration of large doses of acetaminophen can be largely reversed by

administration of L-2-oxothiazolidine-4-carboxylate even several hours after acetaminophen is given.

The present findings also have implications in relation to the metabolism of carbon tetrachloride, chloroform, and phosgene. Previous studies on the metabolism of phosgene (which may be formed in the metabolism of chloroform and carbon tetrachloride) indicate that phosgene interacts with cysteine to form 2-oxothiazolidine-4-carboxylate (7, 14–17). There is also excellent evidence that phosgene is a major intermediate in the formation of carbon dioxide from carbon tetrachloride (7). The data reported here suggest that at least one pathway in which carbon dioxide is formed involves the action of 5-oxoprolinase on 2-oxothiazolidine-4-carboxylate. The overall process would then constitute an intracellular detoxification cycle for phosgene (and its precursors) in which cysteine functions catalytically and which requires energy supplied by cleavage of ATP.

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