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Inhibition of 5-Oxoprolinase by 2-Imidazolidone-4-Carboxylic Acid

(pyroglutamate/pyrrolidone carboxylate/glutathione/γ-glutamyl cycle/kidney/aminoacid transport)

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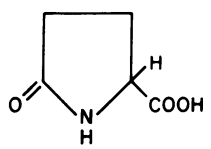
ABSTRACT **L-2-Imidazolidone-4-carboxylic acid is an effective competitive inhibitor of the reaction catalyzed by 5-oxoprolinase, in which 5-oxo-L-proline (L-pyroglutamic acid, L-2-pyrrolidone-5-carboxylic acid, L-5-oxopyrrolidine-2-carboxylic acid) is converted to L-glutamate, with concomitant cleavage of ATP to ADP and orthophosphate. L-2-Imidazolidone-4-carboxylate decreased the rate of metabolism of 5-oxo-L-[^{14}C]proline to $^{14}CO_2$ by rat-kidney slices but had no effect on the metabolism of [^{14}C]glutamate. Mice injected with L-2-imidazolidone-4-carboxylate exhibited greatly reduced ability to metabolize 5-oxo-L-proline, but metabolized glutamate at an essentially normal rate. The findings provide an approach to an animal model for the human condition 5-oxoprolinuria, in which there is apparently a deficiency of renal 5-oxoprolinase activity. The evidence indicates that 5-oxoproline is a normal metabolite.**

Previous studies in this laboratory have led to the finding of a new enzyme, 5-oxoprolinase, which catalyzes the conversion of 5-oxo-L-proline* to L-glutamate, with concomitant stoichiometric cleavage of ATP to ADP and orthophosphate (1, 2):

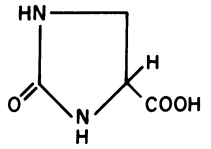
$$\text{5-Oxo-L-proline} + \text{ATP} + 2\ H_2O \xrightarrow[K^+(NH_4^+),Mg^{++}(Mn^{++})]{\text{5-oxoprolinase}} \text{L-glutamate} + \text{ADP} + P_i$$

The need for energy in this reaction is consistent with the 5-oxoproline–glutamate equilibrium, which markedly favors cyclization. This enzyme, which has been found in several animal tissues, has been purified from rat kidney (1, 2). While 5-oxoproline has previously been found at the N-terminus of several proteins, its metabolic role as a free compound has only recently become apparent. 5-Oxoproline is formed by the action of γ-glutamylcyclotransferase on γ-glutamylamino acids (3–5). The conversion of 5-oxoproline to glutamate catalyzed by 5-oxoprolinase thus links reactions involved in the utilization of glutathione (catalyzed by γ-glutamyltranspeptidase and γ-glutamylcyclotransferase) to those that are required for the synthesis of glutathione (catalyzed by γ-glutamylcysteine synthetase and glutathione synthetase). It has been suggested that these reactions, functioning in a cyclic manner, mediate the transport of amino acids across the renal tubule (5).

Earlier work in this laboratory (1, 2, 5) and independent studies by Ramakrishna *et al.* (6) have shown that 5-oxo-L-[^{14}C]proline is rapidly metabolized by intact animals; subsequently, similar observations have been reported in man (7). It has also been shown that slices of rat-kidney cortex can rapidly metabolize 5-oxo-L-[^{14}C]proline to $^{14}CO_2$ (1, 2, 6). In the present work we have prepared and studied L-2-imidazolidone-4-carboxylic acid; this compound is a competitive inhibitor of 5-oxo-L-prolinase. Mice treated with L-2-imidazolidone-4-carboxylate exhibit markedly reduced capacity to metabolize 5-oxo-L-proline. The findings are consistent with the view that 5-oxo-L-proline is a metabolite.



5-OXOPROLINE



2-IMIDAZOLIDONE-4-CARBOXYLIC ACID

EXPERIMENTAL

Materials. L-[*U*-^{14}C]-5-Oxoproline and L-[*U*-^{14}C]glutamic acid were obtained as described (2). BALB/c mice (18–22 g) were used. L- and D-2-Imidazolidone-4-carboxylic acids were prepared from the corresponding isomers of *N*-carbobenzoxyasparagine by the Hofmann reaction (8).

Methods. Enzyme activity was determined in reaction mixtures (final volume, 0.5 ml) containing purified rat-kidney 5-oxo-L-prolinase (2), 2 mM ATP, 1 mM $MgCl_2$, 0.15 M KCl, 0.05 M Tris·HCl (pH 7.8), 2 mM dithiothreitol, and 5-oxo-L-[^{14}C]proline (2 mM; 200,000 cpm/μmol). After incubation at 37° for 30–60 min, 0.2 ml of 0.1 N acetic acid was added and the precipitated protein was removed by centrifugation. An aliquot of the supernatant solution was applied at 5° to the top of a column (1 ml) of Dowex 50 (H^+); the unbound ^{14}C was washed through with about 4 ml of water. The absorbed [^{14}C]glutamate was eluted with 3 ml of 3 N NH_4OH, and an aliquot of the effluent was counted in a scintillation counter.

The mice were placed in a metabolic chamber, which permitted quantitative collection of respiratory carbon dioxide (9). The various compounds were administered by subcutaneous or intraperitoneal injection; the results were substantially the same by either route. The control animals (i.e., those that received only glutamate or 5-oxoproline) were given injections of 0.325 M NaCl equivalent in volume to that of the 0.25 M sodium 2-imidazolidone-4-carboxylate solution injected into the other mice.

* L-pyroglutamic acid, L-2-pyrrolidone-5-carboxylic acid, L-5-oxopyrrolidine-2-carboxylic acid.

TABLE 1. *Effect of L-2-imidazolidone-4-carboxylate on the oxidation of 5-oxo-L-[^{14}C]proline and of L-[^{14}C]glutamate to $^{14}CO_2$ by rat-kidney slices*

Exp. no.	^{14}C-labeled compound	Imidazolidone-4-carboxylate	$^{14}CO_2$ nmol/mg per hr	% Inhibition
1	5-Oxoproline	Absent	15.4	—
	5-Oxoproline	Present	7.3	47
2	Glutamate	Absent	12.7	—
	Glutamate	Present	14.2	0

Kidney slices (from Sprague–Dawley rats), prepared with a Stadie–Riggs slicer, were placed in the main compartment of a double-arm Warburg vessel in 2 ml of Krebs–Ringer phosphate buffer containing (as indicated) 2.5 mM imidazolidone-4-carboxylate (Exp. 1) or 10 mM imidazolidone-4-carboxylate (Exp. 2). [^{14}C]-5-Oxoproline (5.8 × 10^5 cpm; 0.2 ml of 0.1 M) or [^{14}C]-glutamate (5.8 × 10^5 cpm; 0.1 ml of 0.01 M) was placed in one side-arm, and 0.2 ml of 50% trichloroacetic acid was placed in the other. Phenethylamine (0.2 ml) was placed in the center well. After equilibration at 37° under oxygen, 5-oxoproline was tipped in. After the flask had been shaken for 2 hr, the reaction was terminated by tipping in the trichloroacetic acid. Aliquots of the phenethylamine solution were counted with a liquid scintillation counter.

RESULTS

Studies on 5-oxoprolinase

When the reaction catalyzed by 5-oxoprolinase was performed in the presence of L-2-imidazolidone-4-carboxylate, there was marked inhibition of the conversion of 5-oxoproline to glutamate. Similar studies with D-2-imidazolidone-4-carboxylate revealed no inhibition. As indicated by the data given in Fig. 1, inhibition by L-2-imidazolidone-4-carboxylate is competitive with respect to 5-oxo-L-proline. The data lead to an apparent K_m value of 0.05 mM for 5-oxo-L-proline and a K_i value of 0.11 mM for L-2-imdazolidone-4-carboxylate.

Metabolic studies

The data given in Table 1 (Exp. 1) indicate that the conversion of 5-oxo-L-[^{14}C]proline to $^{14}CO_2$ by rat-kidney slices is markedly inhibited by 2.5 mM L-2-imidazolidone-4-carboxylate. In similar studies in which the oxidation of [^{14}C]-glutamate was studied in the presence of 10 mM L-2-imidazolidone-4-carboxylate (Exp. 2), there was no inhibition.

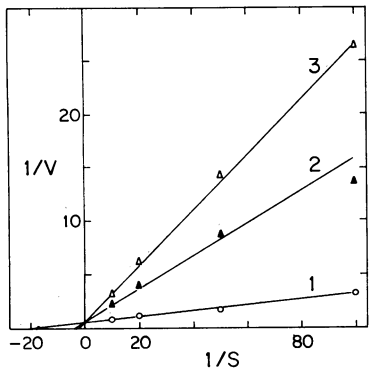


FIG. 1. Inhibition of 5-oxo-L-prolinase by L-2-imidazolidone-4-carboxylic acid. Enzyme activity was determined as described under *Methods. Curve 1,* no inhibitor; *Curves 2 and 3,* L-2-imidazolidone-4-carboxylate, 0.5 and 1.0 mM, respectively. *Abscissa:* 1/S, where S is the mM concentration of 5-oxo-L-proline. *Ordinate:* 1/V, Where V is nmol of glutamate formed per min.

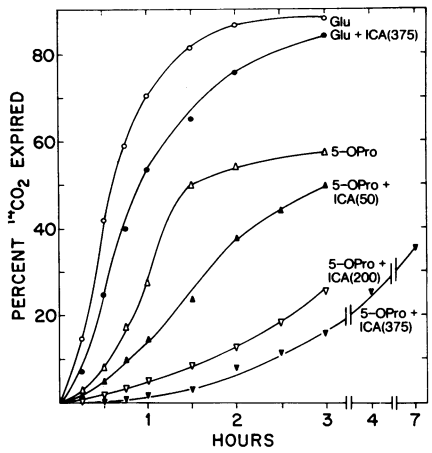


FIG. 2. Metabolism of 5-oxo-L-[^{14}C]proline by mice. The animals were injected with 5-oxo-L-[*U*-^{14}C]proline (20 μmol) (*5-OPro*), L-[*U*-^{14}C]glutamate (20 μmol)(*Glu*), or L-2-imidazolidone-4-carboxylate (50–375 μmol)(*ICA*) as described in the text. The respiratory $^{14}CO_2$ was collected and counted.

In the experiments described in Fig. 2, mice were injected with either 5-oxo-L-[^{14}C]proline or L-[^{14}C]glutamate, and in some cases also with L-2-imidazolidone-4-carboxylate. The two uppermost curves indicate that the administration of L-2-imidazolidone-4-carboxylate had little effect on the conversion of [^{14}C]glutamate to respiratory $^{14}CO_2$. (In other similar experiments, there was even less difference between the results obtained with treated and untreated mice.) In contrast, the conversion of 5-oxo-L-[^{14}C]proline to respiratory $^{14}CO_2$ was markedly affected by administration of L-2-imidazolidone-4-carboxylate. Fig. 2 gives results obtained with three different doses of L-2-imidazolidone-4-carboxylate. In the experiment with the largest dose (375 μmol), urinary excretion of [^{14}C]-5-oxoproline was observed. It has also been found that administration of L-2-imidazolidone-4-carboxylate leads to urinary excretion of 5-oxo-L-proline in amounts substantially greater than the trace amounts found in the urine of untreated mice.†

DISCUSSION

These studies indicate that L-2-imidazolidone-4-carboxylate, a structural analog of 5-oxo-L-proline and a competitive inhibitor of 5-oxoprolinase, inhibits the metabolism of 5-oxoproline *in vivo*. Such inhibition is evidently analogous to the metabolic status of patients with 5-oxoprolinuria; thus, Jellum *et al.* (10) described a mentally retarded and partially paralyzed patient whose average daily urinary excretion of 5-oxoproline is about 30 g.‡ We suggested (2) that this

† Very recent studies in this laboratory in which 5-oxo-L-prolinase was used for the determination of tissue 5-oxo-L-proline indicate that normal mouse tissues (liver, kidney, brain) contain measurable amounts of 5-oxo-L-proline; the values thus far obtained are in the range 0.01–0.1 mM; skin contains a much larger concentration of 5-oxo-L-proline. Mice treated with 2-imidazolidone-4 carboxylate exhibit tissue concentrations of 5-oxo-L-proline that are several times higher than the normal concentrations.

‡ Another patient with 5-oxoprolinuria was recently found by Dr. Agne Larsen (Dept. of Pediatrics, Karolinska Institute, Stockholm); we are indebted to Dr. Larsen for communicating these findings to us.

patient might have an error of metabolism in which the enzymatic conversion of 5-oxoproline to glutamate is blocked or markedly reduced. Subsequent study (7) has shown that administered [^{14}C]-5-oxoproline is metabolized very slowly by this patient as compared to the rate of metabolism of [^{14}C]-5-oxoproline by a healthy human control. After the patient was given [^{14}C]-5-oxoproline, only 1.7% of the injected radioactivity was expired as $^{14}CO_2$ in 3 hr, and about 65% was excreted, mainly as 5-oxoproline, in the urine in 24 hr. In an experiment on the control, respiratory $^{14}CO_2$ accounted for 17% of the administered dose in 2.5 hr, and 6% of the radioactivity, mostly in the form of glutamate, was excreted in the urine. Both the patient and the control metabolized [^{14}C]-glutamate efficiently and at about the same rate. The available information indicates that the patient probably has a deficiency of renal 5-oxoprolinase activity.

The present findings seem to offer an approach to an animal model for this disease. The evidence derived from enzymatic studies, metabolic experiments, and the clinical observations support the conclusion that 5-oxoproline is a normal metabolite. Indeed, the data on 5-oxoprolinuria would seem to indicate that the formation and utilization of 5-oxoproline is quantitatively quite large, perhaps of the order of 0.5 mol/day, in man.

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