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Enzymatic Conversion of 5-Oxo-L-Proline* (L-Pyrrolidone Carboxylate) to L-Glutamate Coupled with Cleavage of Adenosine Triphosphate to Adenosine Diphosphate, a Reaction in the γ-Glutamyl Cycle

(kidney/amino-acid transport/pyroglutamic acid/5-oxo-pyrrolidine-2-carboxylic acid)

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ABSTRACT **A new enzyme, 5-oxoprolinase, was found in rat kidney and in several other tissues; it catalyzes the conversion of 5-oxo-L-proline (L-5-oxo-pyrrolidine-2-carboxylic acid, L-2-pyrrolidone-5-carboxylic acid, L-pyroglutamic acid) to L-glutamic acid, with concomitant stoichiometric cleavage of ATP to ADP and orthophosphate. The reaction catalyzed by 5-oxoprolinase, in which 5-oxoproline formed from γ-glutamyl amino acids by the action of γ-glutamylcyclotransferase is converted to glutamate, appears to function in the γ-glutamyl cycle. 5-Oxoprolinase requires Mg^{++} (or Mn^{++}) and K^+ (or NH_4^+) for activity. The equilibrium is markedly in favor of glutamate formation at pH 7.8.**

A recent communication from this laboratory described a series of enzyme-catalyzed reactions in kidney that was termed the γ-glutamyl cycle; it was suggested that this cycle is involved in the transport of amino acids (1). The reactions of the cycle include: (*a*) the amino acid-dependent breakdown of glutathione to yield γ-glutamyl amino acid and cysteinylglycine, (*b*) the conversion of γ-glutamyl amino acid to free amino acid and pyrrolidone carboxylate (5-oxoproline)*, and (*c*) the resynthesis of glutathione from glutamate and the products of enzymatic hydrolysis of cysteinylglycine.

There is no evidence that 5-oxoproline (*a*) accumulates in the kidney or (*b*) is excreted to an appreciable extent in the urine. Therefore, the functioning of the γ-glutamyl cycle must involve a reaction in which 5-oxoproline is metabolized; indeed, these considerations led to the prediction that an enzyme must exist that rapidly utilizes 5-oxoproline. We suggested that the 5-oxoproline formed in the γ-glutamyl cycle is converted to glutamate (1); however, the details of such an enzyme-catalyzed reaction remain to be described.† The formation of 5-oxoproline in the γ-glutamyl cycle and the absence of evidence indicating its accumulation and excretion suggests that 5-oxoproline is a metabolite that turns over rapidly.

Experiments performed in our laboratory [cited in (1)], and independently by Ramakrishna *et al.* (3), showed that rat tissue-slices rapidly oxidize 5-oxoproline. Additional study of this phenomenon in our laboratory has now led to the recognition of a new enzyme that catalyzes the ATP-dependent conversion of 5-oxoproline to glutamate. The trivial name "5-oxoprolinase" is proposed for this enzyme.

EXPERIMENTAL

Materials. L-[U-^{14}C]- and DL-[5-^{14}C]-5-oxoprolines were prepared by cyclization of the corresponding [^{14}C]glutamic acids (New England Nuclear Corp.) at 140°, followed by purification on Dowex 50 (H^+). Phosphoenolpyruvate, pyruvate kinase, glutamate dehydrogenase, adenylate kinase, and lactate dehydrogenase were obtained from the Sigma Chemical Co. L-Methionine-SR-sulfoximine was obtained as described (4).

Methods. The tissue slices were prepared with a Stadie-Riggs slicer. The slices were placed in the main compartment of a double-arm Warburg vessel in 2 ml of Krebs–Ringer phosphate buffer. [^{14}C]-5-oxoproline (5.8×10^5 cpm; 0.1 ml of 0.1 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 of the flask at 37°C under oxygen, 5-oxoproline was tipped in. After the flask was shaken for 1–2 hr, the reaction was terminated by tipping in the trichloroacetic acid. Aliquots of the phenethylamine were counted with a liquid scintillation counter (5).

Enzyme activity was determined in reaction mixtures (0.5-ml final volume) containing 5 mM ATP, 5 mM phosphoenolpyruvate, 1 mM $MgCl_2$, 4 mM L-methionine-SR-sulfoximine, 0.15 M KCl, 0.05 M Tris·HCl (pH 7.8), pyruvate kinase (3.5 units), 2 mM dithiothreitol, 2 mM L-[^{14}C]-5-oxoproline (200,000 cpm/μmol), and enzyme. The reaction was initiated by the addition of enzyme; after incubation at 37°C for 30–60 min, the reaction mixture was placed at 100°C for 2 min. After removal of the precipitated protein by centrifugation, an aliquot of the supernatant solution was added to the top of a column (1 ml) of Dowex 50 (H^+); unabsorbed ^{14}C was washed through with about 4 ml of water. The absorbed [^{14}C]-labeled products were eluted with 3 ml of 3 N NH_4OH, and an aliquot of the eluate was counted. After the fourth step of the purification procedure, identical results were obtained when methionine sulfoximine, phosphoenolpyruvate, and pyruvate kinase were omitted. Inorganic phos-

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

† A review of the enzymology of 5-oxoproline (2), and an abstract reporting 5-oxoprolinase (62nd meeting of the American Society of Biological Chemists, San Francisco, June, 1971, Abstract No. 933) have appeared.

phate was determined by the method of Fiske and Subbarow (6). Protein was determined by the method of Lowry *et al.* (7) with bovine serum albumin as standard. ADP and AMP were determined by the method of Adam (8). A unit of enzyme activity is defined as the amount required to catalyze the formation of 1 μmol of L-glutamate from L-5-oxoproline per hr under the conditions given above.

The analysis of [^{14}C]aminoacids was performed on a Beckman aminoacid analyzer equipped with a column (0.9 × 59 cm) containing Durrum DC 1A resin. The column eluate was passed through a flow-cell adapter (Packard 3042) containing anthracene crystals before it was allowed to enter the ninhydrin reaction bath. The ^{14}C was monitored in a scintillation counter (Packard Tricarb 2002) equipped with a recorder, which was synchronized with the movement of the amino-acid analyzer chart.

Purification of the Enzyme. Step 1: Male Sprague-Dawley rats (250–300 g) were decapitated and exanguinated. Unless otherwise stated, all procedures were at 4°C. The freshly excised kidneys were homogenized with a Potter–Elvehjem apparatus equipped with a Teflon pestle in an equal volume of buffer A [0.05 M Tris·HCl (pH 7.8) containing 5 mM L-5-oxoproline], and the homogenate was diluted with an equal volume of buffer A, then centrifuged for 1 hr at 145,000 × *g*.

Step 2: Solid ammonium sulfate (20.9 g/100 ml) was added at 0°C. The precipitate that formed was collected by centrifugation and dissolved in buffer A.

Step 3: The solution was added to the top of a column (4.5 × 45 cm) of Sephadex G-200, which was eluted at a rate of 1 ml/min with buffer A containing 1 mM dithiothreitol. The fractions containing the activity were pooled.

Step 4: The pooled fractions were added to the top of a column (1.2 × 17 cm) of DE-52 DEAE-cellulose at a rate of 40 ml/hr with a Varioperpex peristaltic pump. The column was washed with 30 ml of the buffer used in *Step 3*, and the enzyme was then eluted by a linear gradient established between 200 ml of this buffer and 200 ml of the same buffer containing 0.3 M NaCl; flow rate, 40 ml/hr; 5-ml fractions were collected. The fractions containing the activity were pooled and the protein was precipitated by the addition of solid ammonium sulfate (56 g/100 ml). The precipitate thae formed was dissolved in buffer A. The specific activities of tht initial homogenate, and at the end of each of the above steps were, respectively, 0.033, 0.083, 0.373, 1.29, and 3.8 units per mg of protein. The overall yield of activity was 63%. Immediately before use, the enzyme was subjected to gel filtration on Sephadex G-25 in 0.05 M Tris·HCl (pH 7.8) buffer to remove 5-oxoproline.

RESULTS

Oxidation of L-5-oxoproline by tissue slices

The data given in Table 1 indicate that slices of various rat tissues convert [^{14}C]-5-oxoproline to $^{14}CO_2$; kidney, spleen, and liver were most active. The rates of formation of $^{14}CO_2$ from [^{14}C]-5-oxoproline and [^{14}C]glutamate by kidney slices were compared; the rate was about six times faster with L-glutamate. When conversion of [^{14}C]-5-oxoproline to $^{14}CO_2$ by kidney slices was determined in the presence of L-glutamate (7.5 mM), 2-oxoglutarate (7.5 mM), or malonate (40 mM), the rate of $^{14}CO_2$ formation was decreased 35, 62, and 97%, respectively. These observations are consistent with the conversion of 5-oxoproline to glutamate and with the participation of the citric-acid cycle in the utilization of 5-oxoproline; 2-oxoglutarate and glutamate would be expected to reduce the rate of $^{14}CO_2$ production from [^{14}C]-5-oxoproline by dilution of label, while malonate would be expected to inhibit competitively.

After the kidney slices were incubated with [^{14}C]-5-oxoproline, they were homogenized in 3% perchloric acid and the protein was removed by centrifugation; the perchloric acid was removed from the supernatant solution by treatment with 5 N KOH at 0°C, followed by centrifugation of the $KClO_4$. The solution was then placed on a small column of Dowex 50 (H^+) and washed with water; the amino acids that remained on the column were eluted with 3 N NH_4OH and, after lyophilization, were analyzed with an amino-acid analyzer equipped to determine radioactivity (see *Methods*). The major [^{14}C]-product was glutamate, which contained 49.2% of the total radioactivity recovered; ^{14}C was also found in glutamine (18.2%), alanine (15.6%), aspartate (3.7%), and glycine (5.8%).

Conversion of 5-oxoproline to glutamate by rat kidney preparations

Rat kidney homogenates were incubated with [^{14}C]-L-5-oxoproline in the presence of Mg^{++}, ATP, and an ATP-regenerating system; the deproteinized reaction mixtures were then passed through a column of Dowex 50 (H^+). The [^{14}C]-labeled material that remained bound to the column was eluted with 3 N NH_4OH and analyzed on an automated amino acid analyzer. Only [^{14}C]glutamine and [^{14}C]glutamate were found (Table 2, Expt. 1). No products were found when ATP and the ATP-generating system were omitted. When the experiment was performed in the presence of methionine sulfoximine [an irreversible inhibitor of glutamine synthetase (4)], only glutamate was found. When experiments of this type were done with purified preparations of the enzyme, obtained as described under *Methods*, the data given in experiments 2 and 3 (Table 2) were obtained. After *step 4* of the purification procedure, only glutamate was formed and addition of methionine sulfoximine had no effect on the formation of glutamate.

These findings indicate that kidney contains an enzyme (5-oxoprolinase) that catalyzes the conversion of 5-oxoproline to glutamate in the presence of ATP. The enzyme, which has been purified about 100-fold, does not contain glutamine synthetase. 5-Oxoprolinase activity was also found in rat liver, spleen, lung, and brain, as well as in pig kidney, sheep kidney, and human kidney.

TABLE 1. *Rate of oxidation of* L-[^{14}C]*-5-oxoproline to* $^{14}CO_2$ *by rat tissue slices**

Tissue	Relative rate	nmol CO_2 per mg dry wt per hr
Kidney	100	9.7
Spleen	29	2.7
Liver	16	1.6
Intestine	2.7	0.26
Heart muscle	2.7	0.25
Brain	1.4	0.14

* The tissue slices (150–200 mg) were suspended in 2.1 ml of Krebs-Ringer phosphate buffer containing L-[U-^{14}C]-oxoproline.

TABLE 2. *Conversion of 5-oxoproline to glutamate*

Expt. no.	Reaction mixtures* Enzyme preparation	Other components	Products Glutamine (μmol)	Glutamate (μmol)
1 *a*	Kidney extract	5-OP,ATP	0.28	0.05
b	Kidney extract	5-OP,ATP,MSO	0	0.51
c	Kidney extract	5-OP,MSO	0	0
2 *a*	Enzyme (*Step 2*)	5-OP,ATP	0.38	0.06
b	Enzyme (*Step 2*)	5-OP,ATP,MSO	0	0.50
c	Enzyme (*Step 2*)	5-OP,MSO	0	0
3 *a*	Enzyme (*Step 4*)	5-OP,ATP	0	0.63
b	Enzyme (*Step 4*)	5-OP,ATP,MSO	0	0.63
c	Enzyme (*Step 4*)	5-OP,MSO	0	0

* The complete reaction mixture contained enzyme preparation, [^{14}C]-5-oxoproline (5-OP), ATP, Mg^{++}, K^+, methionine sulfoximine (MSO), and an ATP-generating system (see *Methods*). In Expts. *1a*, *2a*, and *3a*, methionine sulfoximine was omitted. In Expts. *1c*, *2c*, and *3c*, ATP and the ATP-generating system were omitted. In Expt. *1*, 0.4 ml of centrifuged kidney homogenate was used. In experiments *2* and *3*, 0.50 and 0.63 unit, respectively, of enzyme were used. The reaction mixtures were incubated at 37°C for 1 hr.

In the presence of 2 mM Mg^{++}, purified kidney 5-oxoprolinase required either NH_4^+ or K^+ at optimal concentrations of 20 and 150 mM, respectively; these cations could not be replaced by Na^+. In the presence of 150 mM K^+ and 2 mM ATP, either Mg^{++} or Mn^{++} was required at optimal concentrations of 1–2 mM. The K_m value for ATP (with 2 mM Mg^{++}–150 mM K^+) was about 0.1 mM. The K_m value for 5-oxoproline was less than 0.1 mM. The optimal pH in Tris-maleate buffer was close to 7.8. When stored at 4°C, the enzyme preparation lost activity progressively; thus, 60% of the initial activity was lost in 6 days. The activity was completely restored upon the addition of 5 mM dithiothreitol.

Stoichiometry and time course of the reaction

The reaction was followed by simultaneous determinations of glutamate, ADP, AMP, and orthophosphate (Table 3). ADP, inorganic phosphate, and glutamate were formed in equimolar amounts; no AMP was formed.

The reaction proceeded to more than 90% of complete conversion of L-5-oxoproline to glutamate, as determined

TABLE 3. *Stoichiometry of the reaction**

Expt. no.	Inorganic phosphate (μmol)	Glutamate (μmol)	ADP (μmol)	AMP (μmol)
1	0.81	0.79	0.75	0
2	1.05	0.96	1.00	0

* The reaction mixtures contained 5 mM ATP, 1 mM $MgCl_2$, 0.15 M KCl, 0.05 M Tris·HCl buffer (pH 7.8), 2 mM dithiothreitol, 2 mM L-[U-^{14}C]-5-oxoproline (210,000 cpm/μmol) and enzyme [0.8 unit (Expt. 1) and 1.0 unit (Expt. 2)] in a final volume of 1 ml; incubated at 37°C for 1 hr.

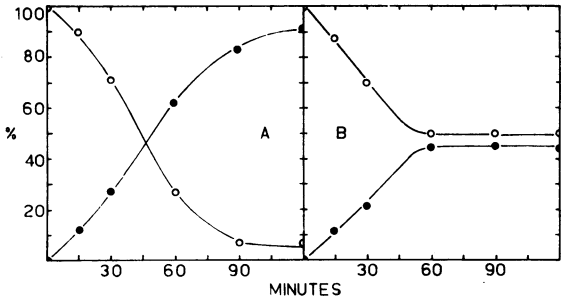
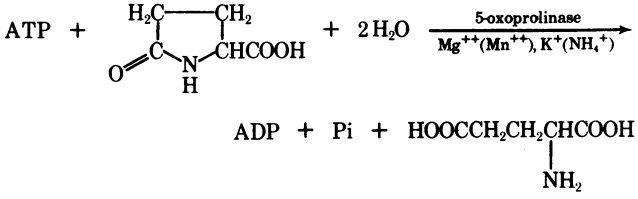


FIG. 1. Conversion of 5-oxoproline to glatamate. (*A*) The reaction mixtures were identical to those given in Table 3, except that the concentration of L-[U-^{14}C]-5-oxoproline (325,000 cpm/μmol) was 1 mM, 1.15 unit of enzyme was used. (*B*) Same as *A*, except that 1 mM DL-[5-^{14}C]-5-oxoproline (313,000 cpm/μmol) was used. The values for disappearance of 5-oxoproline (*open circles*) and formation of glutamate (*closed circles*) are expressed as percent of the initial amount of 5-oxoproline.

both by disappearance of substrate and formation of product (Fig. 1 *A*). When DL-[^{14}C]-5-oxoproline was used, only 50% of the [^{14}C]-labeled substrate was converted to [^{14}C]glutamate (Fig. 1 *B*). That the product is L-glutamate was established by the observation that it was completely oxidized by L-glutamate dehydrogenase.‡

DISCUSSION

The present studies demonstrate the presence of an enzyme in kidney and other mammalian tissues that catalyzes the conversion of 5-oxoproline to glutamate in accordance with the following reaction:



The reaction is unusual in that it involves hydrolysis of both ATP and an amide bond. The requirement of energy for the hydrolysis of this amide bond is, however, entirely consistent with the fact that the equilibrium of the glutamate-5-oxoproline reaction markedly favors cyclization (9). Studies on the mechanism of this interesting reaction are in progress. The occurrence of this reaction is in accord with, and gives support to, the concept of the γ-glutamyl cycle.

There is evidence that 5-oxoproline is not incorporated directly into proteins; it appears that the N-terminal 5-oxoproline residues of certain peptides and proteins are formed by cyclization of glutamate or a glutamate derivative during or after protein synthesis (2, 10, 11). Although [^{14}C]-5-oxoproline can be incorporated into proteins in certain experimental systems, the available data indicate that such incorporation involves prior conversion of 5-oxoproline to glutamate, probably catalyzed by the enzyme described here.

It seems relevant to cite a recent case report describing a mentally retarded patient whose daily urinary excretion of 5-oxoproline (normally nil) averaged about 30 g (12). It is

‡ We thank Mr. Ronald Sekura for performing this experiment.

tempting to consider the possibility that this individual is suffering from an inborn error of metabolism in which the enzymatic conversion of 5-oxoproline to glutamate is blocked or markedly reduced.

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