ISOLATION OF 5-OXOPROLINASE FROM A PROKARYOTE*

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SUMMARY

5-Oxoprolinase, one of the enzymes of the γ-glutamyl cycle, catalyzes the ATP-dependent decyclization of 5-oxo-L-proline (L-pyroglutamate, L-2-pyrrolidone-5-carboxylate) to glutamate. The enzyme, which has been found previously in mammalian tissues, was purified about 26-fold in high yield from a pseudomonad isolated from soil by enrichment culture. The purified bacterial enzyme exhibits properties that are similar though not identical to those of rat kidney 5-oxoprolinase. In contrast to the kidney enzyme, 5-oxoprolinase from pseudomonas is only slightly inhibited by L-2-imidazolidone-4-carboxylate.

Previous reports from this laboratory have described rat kidney 5-oxoprolinase, an enzyme that catalyzes the ATP-dependent hydrolysis of 5-oxo-L-proline (L-2-pyrrolidone-5-carboxylate, L-pyroglutamate) according to the following reaction (1-3):

$$\text{5-oxo-L-proline} + \text{ATP} + 2H_2O \xrightarrow{Mg^{++}(Mn^{++}),\ K^{+}(NH_4^{+})} \text{L-glutamate} + \text{ADP} + \text{Pi}$$

5-Oxoprolinase is one of several enzymes required in a series of reactions which accounts for the synthesis and degradation of glutathione, and which are collectively referred to as the γ-glutamyl cycle; it has been proposed that this cycle is involved in the transport of amino acids in the kidney and in other tissues (1, 4-7).

The role of free 5-oxo-L-proline as a metabolite in mammalian tissues has only recently become apparent. 5-Oxoproline is formed by the action of γ-glutamyl-cyclotransferase on γ-glutamylamino acids, which in turn are formed from glutathione and amino acids by the action of γ-glutamyltranspeptidase. The enzymatic hydrolysis of 5-oxo-L-proline to L-glutamate thus links the degradation of glutathione to the

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2-step synthesis of glutathione catalyzed by γ-glutamylcysteine synthetase and glutathione synthetase.

5-Oxoprolinase is competitively inhibited by L-2-imidazolidone-4-carboxylate, a structural analog of 5-oxo-L-proline. When mice are given this inhibitor together with 5-oxo-L-[U-^{14}C] proline there is a marked decrease in the rate of respiratory $^{14}CO_2$ formation (2). A similar effect was observed in comparable experiments with rat kidney slices. Administration of L-2-imidazolidone-4-carboxylate to mice leads to increased levels of 5-oxo-L-proline in various tissues (2,8). When any one of several L-amino acids is administered to mice together with the inhibitor, there is a greater accumulation of 5-oxoproline than is observed when the inhibitor is given alone (8). These observations are consistent with the function of the γ-glutamyl cycle in vivo and strongly indicate that 5-oxoproline is a metabolite in mammals. It is not yet known whether the γ-glutamyl cycle functions in prokaryotes.

In the present work, in which 5-oxoprolinase was found in a bacterial organism, the enzyme was purified from a pseudomonad isolated by enrichment culture. While the bacterial enzyme is similar to that previously obtained from rat kidney there are some significant differences between the two enzymes.

MATERIALS AND METHODS

Agar and casein hydrolysate were obtained from Difco Laboratories. L-2-Imidazolidone-4-carboxylic acid was synthesized by Dr. Ralph A. Stephani of this laboratory as described (2). Other materials were obtained as previously described (1, 2). Pseudomonas sp. was obtained from soil by enrichment culture (9) at 26°. The growth medium (5.5 mM KH_2PO_4, 3.5 mM Na_2HPO_4, 0.81 mM $MgSO_4$, 0.22 mM $CaCl_2$, 15 μM $FeCl_3$, 15 μM $MnCl_2$, 4.1 μM $NaMoO_4$ and 0.04 M 5-oxo-L-proline) was adjusted to pH 7 and sterilized. This medium was used to prepare 2% agar plates on which pure colonies were isolated. Culture stabs were maintained at 5° on solid

medium (0.9 g casein hydrolysate, 0.5 g NaCl and 0.75 g agar per 100 ml). Cells were grown in 12 liter quantities at 30° with aeration and stirring to the end of log phase, and harvested in a Sharples centrifuge. The cells were lyophilized and stored in a desiccator at 26°.

Enzyme activity was determined in reaction mixtures (final volume, 0.5 ml) containing 0.05 M Tris·HCl (pH 8.0), 2 mM ATP, 4 mM $MgCl_2$, 4 mM dithiothreitol, 80 mM KCl, 10 mM phosphoenolpyruvate, pyruvate kinase (2 units), 2 mM 5-oxo-L-[U-^{14}C] proline (200,000 cpm/μmole) and enzyme. The reaction was initiated by addition of enzyme; after incubation at 26° for 30-60 minutes, the reaction was stopped by placing the solution at 100° for 2 minutes. Quantitation of L-[U-^{14}C] glutamate was carried out as described (1). One unit of enzyme is defined as the amount that catalyzes the formation of 1 μmole of L-glutamate from 5-oxo-L-proline per hour under the conditions given above. Inorganic phosphate, ADP, AMP and protein were determined as previously described (1).

PURIFICATION OF THE ENZYME. All steps were performed at 0-5° excepted as noted. Step 1. Lyophilized cells (5 g) were suspended in 60 ml of a solution containing 0.05 M Tris-HCl (pH 8.0), 5 mM 5-oxo-L-proline and 5 mM dithiothreitol, and disrupted by sonication at 100 watts for 7 minutes using a Heat Systems-Ultrasonics W185D Sonifier Cell Disrupter; a temperature of 5-15° was maintained by use of a NaCl-ice bath. Solid material was removed by centrifugation at 35,000 x g for 15 minutes; the supernatant solution (which contained all of the activity), exhibited a specific activity of 0.13 unit/mg. Step 2. The supernatant solution was treated with 5% protamine sulfate to obtain a final concentration of 0.125%, and the precipitate that formed was removed by centrifugation; the specific activity of the supernatant solution was 0.16 unit/mg. Step 3. Ammonium sulfate (0.194 g/ml) was added to the supernatant solution from Step 2, and the precipitate which formed was removed by centrifugation. Ammonium

sulfate (0.087 g/ml) was added to the supernatant solution and the precipitate, which contained the activity, was collected by centrifugation and dissolved in the minimum amount of the same solution used for the initial extraction. The specific activity was 2.0 units/mg. Step 4. The enzyme solution obtained in Step 3 was heated to 45° in a water bath at 50° with rapid swirling and maintained at 45° for 5 minutes in a 45° bath. The precipitate which formed was removed by centrifugation. The supernatant solution, which was used in the studies described below, exhibited a specific activity of 3.4 units/mg. The overall yield was 95% and the overall purification was about 26-fold. This preparation was stable for up to 2 months when stored at 0°.

RESULTS

The reaction was followed by simultaneous determinations of glutamate, ADP, AMP and orthophosphate in mixtures (final volume, 1 ml) containing 50 mM Tris-HCl (pH 8.0), 80 mM KCl, 8 mM ATP, 8 mM MgCl, 5 mM dithiothreitol, 2 mM 5-oxo-L-[U-^{14}C]-proline (15,400 cpm/μmole) and 0.93 unit of purified enzyme. After incubation for 20 minutes at 37° there were formed (average of duplicates) 0.31 μmole of glutamate, 0.37 μmole of ADP, 0.36 μmole of Pi and 0.01 μmole of AMP. Glutamate was identified and quantitated by use of a Beckman amino acid analyzer. No glutamate was formed when ATP was omitted. The reaction proceeded to more than 90% of complete conversion of 5-oxo-L-proline to glutamate as determined by disappearance of substrate and formation of product (Fig. 1B). When 5-oxo-DL-proline was used only 50% of the added 5-oxoproline was converted to glutamate (Fig. 1A).

In the presence of 1 mM $MgCl_2$, the purified 5-oxoprolinase required either K^+ or NH_4^+ at optimal concentration of 80 mM. In the presence of 80 mM K^+ or NH_4^+ and 2 mM ATP, Mg^{++} was required at an optimal concentration of 4 mM. The apparent Km value for ATP with 4 mM Mg^{++} and 80 mM K^+ was about 1 mM. The apparent Km value for 5-oxo-L-proline in the presence of 80 mM K^+, 4 mM Mg^{++} and 2 mM ATP

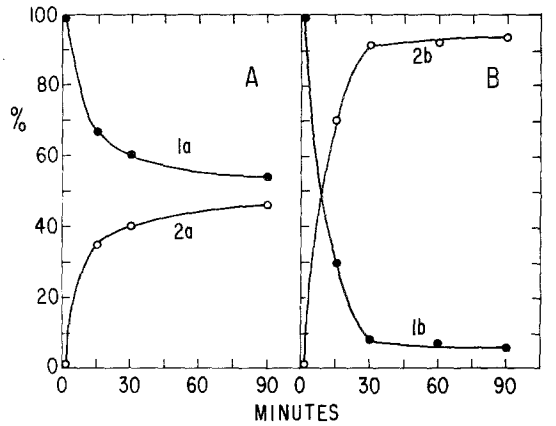

Figure 1. Conversion of 5-oxoproline to glutamate. (A) The reaction mixture contained 50 mM Tris·HCl (pH 8.0), 25 mM ATP, 5 mM MgCl, 80 mM KCl, 5 mM dithiothreitol, 12.5 mM phosphoenolpyruvate, 2 units of pyruvate kinase, 1 mM 5-oxo-DL-[5-^{14}C]-proline (468,000 cpm/μmole) and 2.8 units of 5-oxoprolinase in a final volume of 1.0 ml. At various intervals, 0.1 ml aliquots were placed on Dowex 50 (H^+) columns and analyzed as described (1). (B) The reaction mixture was the same as in A except that 5-oxo-L-[U-^{14}C] proline (1.86 x 10^6 cpm/μmole) was substituted for 5-oxo-DL-proline. The values for disappearance of 5-oxoproline (Curves 1a and 1b; ●) and formation of glutamate (curves 2a and 2b; 0) are expressed as percent of the initial amount of 5-oxoproline.

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was 0.14 mM. When 10 mM L-2-imidazolidone-4-carboxylate was added to the assay mixture the apparent Ki value was 30 mM. Optimal activity in Tris-HCl buffers was observed at pH 7.8-8.0; the enzyme was about 60% as active (at pH 7.8-8.0) when studied in 0.1 M imidazole-HCl and 17 mM Veronal-HCl buffers and about 30% as active in 0.1 M potassium phosphate buffer.

DISCUSSION

Table I summarizes several of the properties of the rat kidney and pseudomonas 5-oxoprolinases. Both enzymes require either K^+ or NH_4^+ for optimal activity, but the optimal concentrations of these cations are different, as is also the optimal Mg:ATP ratio. It is of interest that the specific activity of the pseudomonas enzyme (26-fold purified) is about the same as that obtained after 100-fold purification from rat kidney. In addition, the yield of the bacterial enzyme was far better than that obtained (1) in

TABLE I

Comparison of the Properties of Pseudomonas and Rat Kidney 5-Oxoprolinases

Property	Rat Kidney	Pseudomonas
Optimal $[K^+]$	150 mM	80 mM
Optimal $[NH_4^+]$	20 mM	80 mM
Optimal Mg^{++}:ATP ratio	1	2
pH optimum (Tris)	7.8-8.0	7.8-8.0
Apparent Km (ATP)	0.4 mM	1 mM
Apparent Km (5-oxo-L-Proline)	0.05 mM	0.14 mM
Apparent Ki (L-2-imidazolidone-4-carboxylate)	0.12 mM	30 mM

the purification of the kidney enzyme; this indicates that this microorganism may serve usefully as a good source of the enzyme.

It is notable that the pseudomonas enzyme exhibits somewhat higher apparent Km values for ATP and 5-oxoproline. However, the apparent Ki value for L-2-imidazolidone-4-carboxylate for the pseudomonas enzyme is about 300 times greater than that for the rat kidney enzyme. This property of the pseudomonas enzyme makes this enzyme very useful for the quantitative determination of 5-oxoproline in samples that contain L-2-imidazolidone-4-carboxylate (8).

The presence of 5-oxoprolinase in a bacterial organism is of interest in relation to the possibility that the γ-glutamyl cycle may function in prokaryotic organisms. The presence of glutathione and of γ-glutamyl transpeptidase in bacteria (10,11) is also notable in this respect.

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