DEPENDENCE OF ENERGY COUPLING ON NUCLEOTIDE BASE STRUCTURE IN THE REACTION CATALYZED BY 5-OXO-L-PROLINASE

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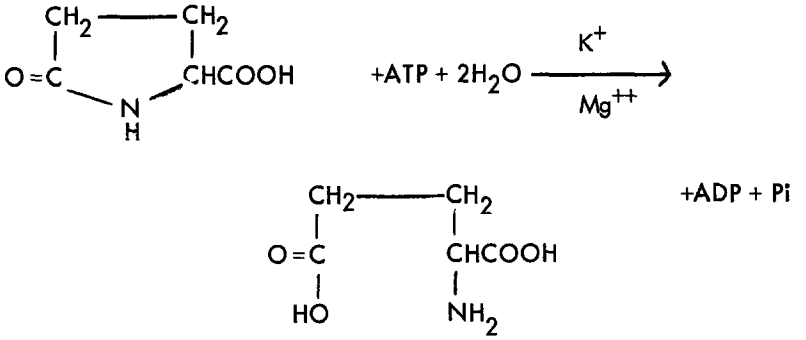
SUMMARY

5-Oxo-L-prolinase catalyzes the virtually complete hydrolysis of 5-oxo-L-proline (L-pyroglutamate) to L-glutamate. The thermodynamic driving force for this endergonic amide hydrolysis is supplied by the coupled stoichiometric hydrolysis of ATP to ADP and Pi. We report here that the efficiency of the coupling between nucleotide and amide hydrolysis is dependent on the nucleotide base. Thus, with both ATP and dATP there is one to one stoichiometry between nucleotide cleavage and 5-oxoproline hydrolysis. With ITP, GTP, or UTP, however, the hydrolysis of NTP exceeds amide hydrolysis by 6 to 50-fold. In the absence of 5- oxoproline, the enzyme catalyzes a slow ATPase reaction, but it catalyzes very rapid ITPase, GTPase and UTPase reactions. These NTPase reactions, which under some conditions are faster than the ATP-mediated overall coupled reaction, are inhibited by 5-oxoproline and by analogs of 5-oxoproline that bind to the enzyme.

5-Oxo-L-prolinase (L-pyroglutamate hydrolyase) catalyzes the coupled double hydrolysis shown in reaction (1). This reaction accounts for the utilization of 5-oxo-



(1)

L-proline formed by γ-glutamyl cyclotransferase in the γ-glutamyl cycle (1). The requirement for energy in the enzymatic process is mandated by the unusual stability of the 5-oxoproline amide bond. Thus, at neutral pH, an equilibrium mixture of 5-oxoproline and glutamate contains only a few percent of glutamate (2-4); the complete

enzymatic hydrolysis of 5-oxoproline is therefore thermodynamically dependent on the concomitant exergonic hydrolysis of ATP. In this respect the 5-oxoprolinase reaction is unique; it is the only known ATP-dependent hydrolase in which the requirement for ATP is based on energetic grounds as well as on enzymatic specificity. The nature of the coupling between ATP cleavage and 5-oxoproline hydrolysis has been the focus of our interest in 5-oxoprolinase. Earlier studies in this laboratory showed that reaction (1) proceeds with close to exact stoichiometry between 5-oxoproline and ATP hydrolysis over the entire pH range of enzymatic activity (pH 5.4-11.3) (5). However, when 5-oxoproline is replaced by analogs, the hydrolysis of ATP substantially exceeds that of the amide (5).

We have now found that stoichiometry is also lost when any of several non-adenosine nucleoside triphosphates is used in place of ATP. Thus, when ITP, GTP, or UTP is substituted for ATP in reaction (1), hydrolysis of 5-oxoproline is minimal whereas hydrolysis of the nucleotide proceeds rapidly. In the absence of 5-oxoproline, 5-oxoprolinase catalyzes even more rapid hydrolysis of ITP, GTP, and UTP. Data are presented here which indicate that these nucleotidase activities co-purify with 5-oxoprolinase, that they are inhibited not only by 5-oxoproline but also by analogs of 5-oxoproline, and that they share, in common with the overall coupled reaction, a dependence on K^+ and Mg^{++}.

MATERIALS AND METHODS

5-Oxo-L-[^{14}C] proline was prepared by cyclization of generally labeled L-[^{14}C] glutamate [New England Nuclear Corp](5). L-2-Imidazolidone-4-carboxylic acid (6), 4-oxy-5-oxoproline, 3-oxy-5-oxoproline and 2-piperidone-6-carboxylate were prepared as described (5). Nucleotides, Na phosphoenolpyruvate and pyruvate kinase were obtained from Sigma. Dithiothreitol was obtained from RSA Corp., Ardsley, New York.

Hydrolysis of 5-oxoproline was determined by measuring the formation of L-[^{14}C] glutamate from 5-oxo-L-[^{14}C] proline; separation of the radioactive compounds was achieved on small Dowex 50 (H^+) columns (5). The standard assay mixture (assay 1) contained (final vol., 0.5 ml) Tris HCl (70 mM; pH 7.8), KCl (150 mM), ATP (5 mM), $MgCl_2$ (10 mM), dithiothreitol (4 mM), 5-oxo-L-[^{14}C] proline (4 mM), phosphoenolpyruvate (5 mM), pyruvate kinase (5 units) and 5-oxoprolinase. In assay 2, ITP was used in place of ATP, and IDP and glutamate were determined. Inorganic phosphate was determined by the method of Fiske and Subbarow (7). Nucleoside diphosphates were determined in a coupled assay using pyruvate kinase and lactate dehydrogenase. When pyruvate kinase and phosphoenol-

Table I: Summary of Nucleotide Specificity*

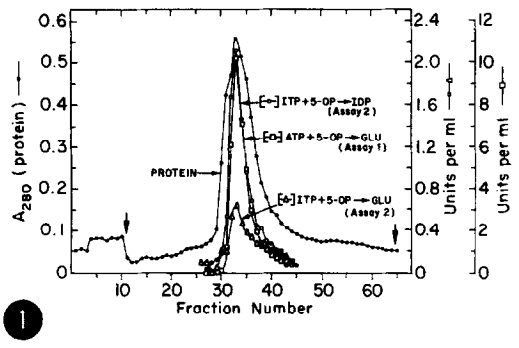
Exp.	Nucleotide	L-Glutamate formed	NDP formed	NDP / L-Glu Ratio
		nmoles	nmoles	
1	ATP	707	771	1.1
2	ITP	32	241	7.5
3	GTP	40	243	6.1
4	XTP	2	77	39.
5	CTP	13	118	9.1
6	UTP	9	455	51.
7	dATP	204	206	1.0

*The reaction mixtures (final vol., 0.5 ml) contained nucleotide (2 mM), $MgCl_2$ (4 mM), 5-oxo-[^{14}C] proline (5.1 mM), KCl (150 mM), Na phosphoenolpyruvate (2 mM), Tris·HCl (125 mM; pH 7.8), dithiothreitol (5 mM), pyruvate kinase (10 units) and 5-oxoprolinase (1.4 unit); incubated for 30 minutes at 37°.

pyruvate were present, nucleoside diphosphate formation was equivalent to pyruvate formation. Pyruvate was assayed with lactate dehydrogenase and DPNH. 5-Oxo-L-prolinase was purified as described (5) or by a modified method in which the heat step was replaced by chromatography on DEAE-cellulose (8). Similar results were obtained with enzyme purified by either procedure. As noted previously (5) successful storage of the enzyme requires the presence of 5-oxoproline; the enzyme was freed of 5-oxoproline (in the studies given in Table II) by gel filtration just prior to use.

RESULTS

It was previously shown that the enzymatic hydrolysis of 5-oxo-L-proline to L-glutamate takes place at a significant rate only in the presence of ATP or dATP. Table I shows an extension of this study in which the hydrolysis of 5-oxoproline and that of NTP were simultaneously determined. As shown previously, significant hydrolysis of 5-oxoproline occurred only with adenine nucleotides, but measurements of NDP formation indicated that ITP, GTP, and UTP were hydrolyzed significantly in the presence of 5-oxoproline. As shown in Table I, the ratio of NDP to L-glutamate formation was close to unity in the presence of ATP or dATP, but varied from about 6 to 50 in the



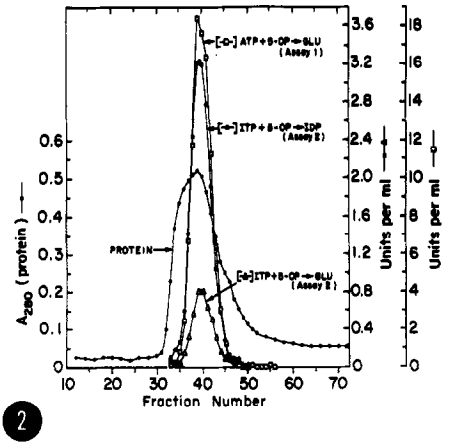


Figure 1. Chromatography of 5-oxoprolinase on a column of DEAE-cellulose (2.0 x 15 cm); eluted with a linear gradient (0-300 mM NaCl) in Tris HCl (50 mM; pH 7.2) with 5 mM 5-oxo-L-proline (arrows indicate start and end of gradient). Fraction vol. 14.4 ml. Assay 1 and assay 2 are described in methods.

Figure 2. Chromatography of 5-oxoprolinase on a column of Sephadex G-200 (2.5 x 90 cm) using reversed flow elution. The buffer was Tris HCl (50 mM; pH 7.2) containing 5 mM 5-oxo-L-proline. Fraction vol., 4.8 ml.

presence of other nucleoside triphosphates. Similar data were obtained when Pi formation was determined.

The 5-oxoprolinase used in these experiments was purified about 1600-fold from rat kidney and exhibited a specific activity of 50 μmoles hr^{-1} mg^{-1} (5). Experiments were carried out to determine whether the NTPase activities co-purify precisely with 5-oxoprolinase on chromatography on columns of DEAE-cellulose (Fig. 1) and Sephadex G-200 (Fig. 2); these procedures are steps 5 and 6 of the purification method for 5-oxoprolinase (5). As shown in Figs. 1 and 2, the activity that catalyzes ATP-dependent conversion of 5-oxoproline to glutamate (assay 1) coincides with the activity observed with ITP (assay 2; exp. 2, Table I). Furthermore, the ratios of the three activities shown in Figs. 1 and 2 remain essentially constant during the separations shown and also during the final Sephadex G-200 procedure (5).

The results given in Table II provide additional evidence that the hydrolysis of

Table II: Properties of the ITPase Reaction

Exp.	Reaction Conditions ±/	IDP		Glutamate
		nmoles	%	nmoles
1	ITP, Mg^{++}, K^+	2420	(100)	-
2	ITP, Mg^{++}, K^+, 5-OP	230	8.3	30.
3	ITP, Mg^{++}, K^+, ICA	330	13.7	-
4	ITP, Mg^{++}, K^+, Pip-Ca	540	22.2	-
5	ITP, Mg^{++}, K^+, 3-OH-5-OP	790	32.7	-
6	ITP, Mg^{++}, K^+, 4-OH-5-OP	1490	61.5	-
7	ITP, Mg^{++}, K^+, Glu	1970	81.5	-
8	ITP, Mg^{++}, Na^+	150	6.3	-
9	Mg^{++}, K^+	<20	0	-
10	ATP, Mg^{++}, K^+	340*	14.0*	-
11	ATP, Mg^{++}, K^+, 5-OP	970*	40.2*	800.

* ADP formation

±/ The reaction mixtures (final vol., > 0.5 ml) contained Tris-HCl buffer (120 mM; pH 7.8), KCl or NaCl (150 mM), as indicated, $MgCl_2$ (10 mM), ITP or ATP (5 mM), phosphoenolpyruvate (4 mM), dithiothreitol (4 mM), pyruvate kinase (10 units), and 5-oxoprolinase (1.94 units); incubated at 37° for 30 min. Where indicated the mixtures also contained 10 mM 5-oxo-L-[^{14}C] proline (5-OP), L-2-imidazolidone-4-carboxylate (ICA), 2-piperidone-5-carboxylate (Pip-Ca), 3-oxy-5-oxoproline (3-OH-5-OP), or 4-oxy-5-oxoproline (4-OH-5-OP). There was no IDP or 5-oxoproline formation in the absence of 5-oxoprolinase; omission of phosphoenolpyruvate decreased IDP formation only minimally. In experiments 1-7, 9-11, NDP formation was assayed with lactate dehydrogenase and DPNH, i.e., the NDP formed was equal to the pyruvate present at the end of the incubation. In experiment 8, IDP was assayed by adding (after incubation) pyruvate kinase, phosphoenolpyruvate, K^+ and lactate dehydrogenase (this approach was used because pyruvate kinase is also K^+-dependent).

ITP is an intrinsic property of 5-oxoprolinase. In addition, the data show that the ITP hydrolysis demonstrated in Table I and Figs. 1 and 2 is only a small fraction of the potential ITPase activity of 5-oxoprolinase. Thus, comparison of experiments 1 and 2 (Table II) shows that ITPase activity is inhibited about 90 % by 10 mM 5-oxoproline. (ITPase in Table 1 and Figs. 1 and 2 was measured under conditions similar to that of

exp. 2, Table II). As shown in experiments 3-6, analogs of 5-oxoproline also inhibit the ITPase activity; the degree of inhibition corresponds in general with the ability of the analogs to inhibit 5-oxoproline hydrolysis in the presence of ATP (5). L-Glutamate (exp. 7) inhibits only slightly; this is in accord with its minimal effect on the overall reaction (8). Experiment 8 shows the K^+-dependence of the ITPase reaction. Experiments 10 and 11 are controls showing the ATPase activity and the ATP-dependent overall reaction. Comparison of experiments 1, 10, and 11 shows that ITPase activity in the absence of 5-oxoproline is 2.5 fold faster than the overall reaction, and that ATPase in the absence of 5-oxoproline is slower than both the ITPase and the overall reaction. The ADP:glutamate ratio of 1.2 found in exp. 11 reflects some damage to coupling, which occurs irreversibly when the enzyme is freed of 5-oxoproline.

DISCUSSION

The present and previous (5) findings show that coupling of amide hydrolysis and nucleotide cleavage is reduced or abolished by replacement of the adenine moiety of ATP or by modification of the structure of the amide substrate. The results presented in Table II show that ITP hydrolysis in the presence of 5-oxoproline is only a small fraction of the maximal ITPase activity, and that full ITPase activity is expressed only in the absence of 5-oxoproline. The inhibition of ITP hydrolysis by 5-oxoproline suggests that 5-oxoproline may exclude water from its site of attack on ITP. This phenomenon may be associated with a conformational change in the enzyme due to the binding of 5-oxoproline; thus, addition of 5-oxoproline to the enzyme leads to a time-dependent decrease in Vmax value and an increase in apparent Km value for ITP (8). Similar data have been obtained with GTP and UTP.

The previous observation that 5-oxoprolinase loses activity rapidly on storage in the absence of 5-oxoproline seems to be explained, at least in part, by the extremely fragile nature of the coupling between the hydrolyses of ATP and 5-oxoproline. Thus,

storage in the absence of 5-oxoproline leads to loss of the overall coupled reaction but only to minimal loss of ATPase and ITPase. Therefore, the precise ratio of ITPase to overall activity depends on the conditions under which the enzyme is stored.

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