Trapping of an Intermediate in the Reaction Catalyzed by 5-Oxoprolinase*

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Bacterial 5-oxoprolinase is composed of two protein components: Component A, which catalyzes 5-oxoproline-dependent ATP-hydrolysis and Component B, which couples the hydrolysis of ATP with the decyclization of 5-oxoproline to form glutamate (Seddon, A. P., Li, L., and Meister, A. (1984) J. Biol. Chem. 259, 8091–8094). Studies on this unusual enzyme system have led to evidence that an intermediate is formed by Component A. Application of the isotope-trapping method demonstrated an activated 5-oxoproline intermediate, whose formation requires ATP, Mg2+, and Component A. The amount of ATP-dependent trapping was close to the number of enzyme active sites. The intermediate formed by Component A was shown to be reducible by potassium borohydride to proline in low yield; when Component B was added, the formation of proline was abolished. Treatment of reaction mixtures containing Component A, 5-oxoproline, and [γ-32P]ATP with diazomethane led to appearance of a 32P-labeled compound (found on thin layer chromatography), whose formation was significantly reduced when Component B was present. The new compound, which is labile, breaks down to form dimethyl[32P]phosphate. The total amount of dimethyl[32P]phosphate formed after breakdown is close to the number of active sites of Component A. The data are consistent with the conclusion that a phosphorylated form of 5-oxoproline is formed by Component A and suggest that Component B is required for conversion of this intermediate to glutamate.

5-Oxoprolinase catalyzes the ATP-dependent decyclization of 5-oxo-L-proline (1–7):

5-Oxo-L-proline + ATP + 2H2O → L-glutamate + ADP + P1

(1)

The mechanism of this interesting reaction, in which the endergonic cleavage of the internal amide bond of 5-oxoproline is coupled to the exergonic cleavage of ATP, has remained enigmatic. Although plausible mechanisms have been considered (2, 4, 7), there has been no direct experimental evidence for formation of intermediates in this enzyme-catalyzed reaction.

The present work was stimulated by the finding that, unlike rat kidney 5-oxoprolinase, the 5-oxoprolinase of Pseudomonas putida can readily be dissociated into two proteins (Components A and B) (7). Component A was found to catalyze 5-oxoproline-dependent cleavage of ATP to ADP and P1; Component A does not catalyze cleavage of 5-oxoproline. Reaction (1) is catalyzed by a mixture of Components A and B; Component B alone catalyzes neither the cleavage of ATP nor that of 5-oxoproline. We previously suggested (7) that Component B might alter the conformation of Component A in such a manner as to facilitate the coupled reaction or, more likely, that Component B might act as a catalyst in converting a phosphorylated form of 5-oxoproline (formed by Component A) to glutamate.

In the present work we have obtained direct evidence for the formation of an intermediate by Component A. Thus, application of the isotope-trapping method (6–14) showed formation of a productive complex involving Component A, ATP, and 5-oxoproline. Evidence was obtained that this intermediate can be (a) reduced by borohydride to proline and (b) esterified by treatment with diazomethane.

EXPERIMENTAL PROCEDURES

Materials—5-Oxo-L-[U-14C]proline, L-[U-14C]proline, L-[U-14C]glutamate, sodium[1H]borohydride, [γ-32P]ATP (tetraethylammonium) salt and [2,8-3H]ATP were supplied by New England Nuclear. Tris, HEPES, 5-oxo-L-proline, L-glutamate, L-proline, β-hydroxyisocitrate hydrochloride (mixture of DL- and DL-allo forms), Sephadex G-25 (50–150 μm), and potassium borohydride were obtained from Sigma. Dimethyl and trimethyl phosphate were obtained from ICN Biomedicals. Glutamic acid α,γ-dimethyl ester was purchased from Chemical Dynamics Corporation. N-Methyl-N′-nitro-N-nitroso-pentanamine and the apparatus for preparing millimolar quantities of diazomethane were obtained from Aldrich. Dowex AG 50W-X8 (H+), AG 1-X8, and Coomassie Blue protein dye reagent were obtained from Bio-Rad. Centricon (molecular weight cut-off 10,000) microconcentrators were obtained from Amicon and collection bags (molecular weight cut-off 10,000) were obtained from Schleicher & Schuell. Analytical silica gel 60 precoated thin layer chromatography plates were obtained from Pierce Chemical Co. Polyethyleneimine (PEI) Cel 300 and Polygram Cel 400 precoated plastic sheets for thin layer chromatography were supplied by Brinkmann Instruments.

General Procedures—5-Oxo-L-proline-dependent ATPase and 5-oxo-L-proline activities were determined as previously described (7). Bacterial 5-oxo-L-proline was isolated from P. putida (strain ALA) as described (15). Component A (5-oxo-L-proline-dependent ATPase; specific activity 500 units/mg) and Component B were resolved and purified as described (7, 15).

Components A and B were freed of 5-oxoproline, KCl, and dithiothreitol by gel filtration on Sephadex G-25 or by exhaustive dialysis against Tris-HCl (50 mM, pH 7.5); HEPES (50 mM, pH 7.5), or potassium phosphate buffers (50 mM, pH 7.5) at 4°C. Enzyme solutions were concentrated either by vacuum dialysis or by centrifugation with Centricon 10 microconcentrators. Protein concentrations were determined as described (18) using bovine serum albumin as the standard. To check the validity of the standard curve in estimating the concentration of Component A, a sample of this protein was exhaustively dialyzed against high purity water followed by lyophilization to constant weight. The results obtained by the two methods agreed within ±10%. Protein estimations by the Lowry method (17)

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using the same standard were in close agreement with assays by the Coomassie dye-binding procedure and by the gravimetric method. A minimum hemodimeric molecular weight value of 115,000 (51,000 plus 64,000) was used to calculate the concentration of Component A. All experiments (unless otherwise indicated) were conducted at a concentration of Component B that saturated Component A, and to an activity of 5-oxoproline (46 min), 6-hydroxynorvaline (54 min) and proline (66 min) were eluted. Under these conditions, glutamate eluted at 135-155 ml. Fractions containing radioactive material (1-2 x 10^6 cpm) were co-chromatographed with unlabeled proline on microcrystalline cellulose thin layer plates (20 x 20 cm). The plates were developed by ascending chromatography in a solvent consisting of tert-butanol, methylthyl ketone, water and ammonium hydroxide (40:30:20:10, v/v) (22). Proline was located (blue spot), after spraying with a solution of isatin (0.2%) in n-butyl alcohol/acetic acid (25:1, v/v) (23). The plates were divided into 1-cm sections, which were analyzed for radioactivity. The formation of labeled proline was also confirmed by chromatography on a Durrum model 500 amino acid analyzer using a sodium citrate buffer system (pH 3.24, 0.172 M) and authentic [14C]proline as a standard; retention time for proline was 28 min. Attempts to Detect Phosphorylated or Adenylylated Enzymes—Experiments were conducted in the presence and absence of a saturating amount of Component B and in the presence and absence of 5-oxoproline. Reaction mixtures contained (final volume, 0.05 ml): Component A (1.6 nmol, 180 M), Component B, MgCl2 (0.2 mM), KCl (2 mM), 5-oxoproline (0.1 mM), [guanidino-14C]ATP (0.1 mM, G x 10^6 cpm/ml), or [gamma-32P]ATP (0.1 mM, 7 x 10^6 cpm/ml) in HEPES buffer (4.2 mM, pH 8.0). Mixtures were incubated for 5 min at 37 °C then immediately placed on ice. The solutions were applied to G-25 columns (1 x 15 cm) that had been previously equilibrated with HEPES buffer (100 mM, pH 8.0) at 4 °C. Columns were eluted with the buffer and fractions (0.27 ml) were collected. Portions (0.1 ml) of each fraction were taken for scintillation counting. Eluted enzyme(s) were detected by assayng for 5-oxoproline dependent ATPase, analyzing for inorganic phosphate formation or 5-oxoproline residues by paper chromatography. Under these conditions, authentic samples of dimethyl and trimethyl phosphate were separated (fractions 6-7 and 14-26, respectively). Similar experiments were conducted with Component A alone and [gamma-32P]ATP in potassium phosphate buffer (100 mM, pH 7.0) in the presence and absence of 5-oxoproline. Attempts were made to detect a phosphorylase enzyme under the conditions described above except that separation of Component A from [gamma-32P]ATP was affected by the rapid centrifugation chromatography method of Penefsky (20). Mechanism of 5-Oxoproline 11539

**Mechanism of 5-Oxoproline**

**Evidence for an Enzyme-bound Intermediate**

The experiments described below, in which the isotope-trapping or pulse-chase method was used, provide evidence for the formation of a phospho- or adenylylated enzyme. These experiments were carried out with the enzyme (Component A or Components A + B) rapidly mixed (5-10 s) at 37 °C with reaction mixtures as described (Table II). After addition of diazomethane in n-butyl alcohol/acetone (2:1, v/v), the clear supernatant solution (0.1 ml) were applied to silica gel plates (20 x 20 cm) containing Dowex AG 1-X8 ion exchange resin. Portions (1 ml) of the eluates were removed for scintillation counting, 95-100% of the radioactivity applied to the columns was recovered in the eluents. The total amount of radioactivity was normalized to 100% to correct for small differences in recoveries.

**Stabilization of a Borohydride Reducible Product by Dimethyl Sulfoxide—**The method was essentially that of Todhunter and Purich (21), except that aqueous (20%, v/v) dimethyl sulfoxide was used; this solution was cooled to -20 °C in a solid carbon dioxide/ethanol bath. The enzyme solution (0.3 ml) was then added by forced delivery from an automatic pipette to the cold aqueous dimethyl sulfoxide (1.2 ml) with rapid mixing. (Use of this technique prevents heat denaturation of the enzyme and thermal destruction of the labile intermediates, which occurs to an appreciable extent when enzyme solutions are diluted with dimethyl sulfoxide.) This solution (1.5 ml) was then treated with 5 mg of sodium borohydride. The solutions were left overnight at room temperature and then diluted with water (8.5 ml) and 10% acetic acid to remove any unreacted dimethyl sulfoxide. The residue was dissolved in water (0.5 ml) and the remaining borohydride was destroyed by adding formic acid (0.05 ml). The pH was then adjusted to 8.5. Borate was removed by complex formation with Sephadex G-25 at pH 8.5 as follows. Samples were loaded on Sepha- dex G-25 columns (0.4 x 5 cm) with HEPES buffer (40 mM, pH 8.5) until no further radioactivity eluted. Radioactive fractions were pooled and lyophilized and the residue was taken up in the minimal volume of water and applied to Dowex 50 (H+) columns to remove residual 5-oxoproline as described above. The fraction eluted from the column with 3 M NH4OH was lyophilized and the residue dissolved. Solid sulfosalicylic acid was then added to give a 10% (w/v) solution and centrifuged to remove residual proteins. Samples were analyzed on a Durrum model 500 amino acid analyzer using a lithium citrate buffer (0.238 M, pH 2.7). Under these conditions, glutamic acid, trimethyl phosphate, and glucuronate (5-methyl, 5-acetyl) were well resolved. Standards were also run in the presence of sulfosalicylic acid (10%, w/v). Borohydride Reduction of an Enzyme-formed Product—Reaction mixtures contained either sodium [14C]borohydride and unlabeled 5-oxoproline or potassium borohydride and [14C]-labeled 5-oxoproline (see Figure 2 for experimental conditions). After incubation for 3 h at 37 °C, the samples were acidified by adding formic acid to destroy residual borohydride and then adjusted to pH 8.5 by adding KOH. Prior to chromatography, borate was removed by complex formation with Sephasex G-25 at pH 8.5 (as described above). Separation of radiolabeled components was accomplished by anion exchange chromatography on columns (0.9 x 27 cm) containing Dowex AG 1-X8 (HCO). The columns were developed with a linear gradient established between 250 ml each of water and 0.4 M NH4HCO3. The columns were calibrated using 14C-labeled samples of proline, 5-oxoproline, and glutamate, which eluted at 45-65, 60-95, 325-350 ml, respectively. Inorganic phosphate eluted at 135-155 ml. Fractions containing radioactivity were pooled and lyophilized. Because the elution of proline and 5-oxoproline overlap to some extent, the [gamma-32P]ATP were first passed through columns (1 x 6 cm) of Dowex 50 (H+) to remove contaminating 5-oxoproline. Proline was then eluted with 3 M NH4OH. The ammonia effluent was lyophilized, and the residue was dissolved in the minimal volume of water. Aliquots of solutions containing radioactive material (1-2 x 10^6 cpm) were co-chromatographed with unlabeled proline on microcrystalline cellulose thin layer plates (20 x 20 cm). The plates were developed by ascending chromatography in a solvent consisting of tert-butanol, methylthyl ketone, water and ammonium hydroxide (40:30:20:10, v/v) (22). Proline was located (blue spot), after spraying with a solution of isatin (0.2%) in n-butyl alcohol/acetic acid (25:1, v/v) (23). The plates were divided into 1-cm sections, which were analyzed for radioactivity. The position of authentic samples of dimethyl and trimethyl phosphate were located by hydrolyzing scraped-off segments of silica gel plates in HCl (5 M, 100 °C) 1 h. The HCl solutions were neutralized by addition of sodium hydroxide and the inorganic phosphate determined as described (26). In this system dimethyl phosphate remained at the origin and trimethyl phosphate moved close to the solvent front.

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**RESULTS**

Evidence for an Enzyme-bound Intermediate

The experiments described below, in which the isotope-trapping or pulse-chase method was used, provide evidence for the formation of a phospho- or adenylylated enzyme.
for the formation of an enzyme-bound intermediate containing 5-oxoproline; formation of this complex requires ATP and Mg\textsuperscript{2+}. Component A was incubated for 5 s in a solution containing \textsuperscript{14}C-labeled 5-oxoproline, ATP, and Mg\textsuperscript{2+}, and this pulse mixture was then treated with a chase solution containing a large excess (about 300-fold) of unlabeled 5-oxoproline and Component B. After 5-15 s (as indicated), the reaction was stopped and the radioactivity of the glutamate formed was determined. As indicated (Table I, Experiment A.1), much more radioactivity was found in the glutamate than would be expected if the labeled and unlabeled 5-oxoproline had equilibrated.

In Experiment A.2, the amount of radioactivity found in the glutamate fraction at the end of the pulse period was, as expected, very low. In Experiment A.3, the unlabeled 5-oxoproline was added to the pulse solution and the labeled 5-oxoproline was added to the chase solution; here again relatively little label was found in glutamate. In Experiment A.4, ATP and Mg\textsuperscript{2+} were present only in the chase solution. In Experiment A.5, Mg\textsuperscript{2+} was omitted from the pulse solution and added to the radioactive glutamate in Experiments A.4 and A.5 was relatively low. The result obtained in Experiment A.1 is consistent with formation of an activated form of 5-oxoproline; the results obtained in Experiments A.4 and A.5 indicate that the formation of such a 5-oxoproline-containing enzyme-bound intermediate requires both ATP and Mg\textsuperscript{2+}. Experiments A.1, 3, 4, and 5 were carried out with saturating amounts of Component B. When nonsaturating levels of Component B were used, the labeling of glutamate was substantially lower. For example, in an experiment comparable to A.1 in which the concentration of Component B was 10% of that required for saturation, only 5,490 cpm were found in glutamate. Experiment A.3 provides a measure of the amount of labeled glutamate formed after addition of the chase solution. Subtraction of the value obtained in Experiment A.3 from that observed in Experiment A.1 gives a value (41,000 cpm), which corresponds to the binding of about 10 nmol of 5-oxoproline. This value is about twice that of the amount of Component A suggesting that Component A (which is a dimer?) can bind 2 mol of 5-oxoproline per mol. When similar experiments were carried out at 4 °C, the results were about the same. Thus, in Experiment B.1, excess binding of 5-oxoproline of 6,070,000 cpm was found; this value is equivalent to the binding of 2 mol of 5-oxoproline per mol of Component A. In Experiment C, carried out under slightly different conditions at 37 °C, the excess binding of label found (1,102,000 cpm) corresponds to 1.7 mol/mol of Component A. Studies in which Mg\textsuperscript{2+} was replaced by Mn\textsuperscript{2+} (Experiment D.2) gave results comparable to those obtained with Mg\textsuperscript{2+}.

The results described above support the view that an activated form of 5-oxoproline is formed on Component A and that this intermediate is converted to glutamate. We have considered the possible involvement of a phosphorylated protein. We therefore made a number of attempts to detect phosphorylated or adenylated enzymes in experiments with \textsuperscript{32}P-labeled ATP and [2,8-H]Mg\textsuperscript{2+} under a variety of experimental conditions (see "Experimental Procedures"). No phosphorylation or adenylilation of Component A was observed in these studies which were performed in the presence and absence of Component B and in the presence and absence of 5-oxoproline.

**Conversion of an Enzyme-bound Intermediate to Proline**

**Attempts to Stabilize a Borohydride-reducible Product by Dimethyl Sulfoxide**—One would think that an enzyme-bound phosphorylated intermediate such as \textgamma-glutamyl phosphate might be reducible by borohydride. The following experiment was carried out in the presence of dimethyl sulfoxide, which

### Table I

**Pulse-chase studies**

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Pulse solution</th>
<th>Chase solution</th>
<th>\textsuperscript{14}C Glutamate</th>
<th>&quot;Excess&quot; \textsuperscript{14}C label</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP, Mg\textsuperscript{2+}, 5-Oxoproline</td>
<td>B, 5-Oxoproline</td>
<td>45,200</td>
<td>41,000 (10.4)</td>
</tr>
<tr>
<td>A. 1</td>
<td>ATP, Mg\textsuperscript{2+}, 5-Oxoproline</td>
<td>None</td>
<td>234</td>
<td></td>
</tr>
<tr>
<td>B. 1</td>
<td>ATP, Mg\textsuperscript{2+}, 5-Oxoproline</td>
<td>B, 5-Oxoproline</td>
<td>4,200</td>
<td></td>
</tr>
<tr>
<td>C. 1</td>
<td>ATP, Mg\textsuperscript{2+}, 5-Oxoproline</td>
<td>B, 5-Oxoproline, ATP, Mg\textsuperscript{2+}</td>
<td>6,000</td>
<td></td>
</tr>
<tr>
<td>D. 1</td>
<td>ATP, Mg\textsuperscript{2+}, 5-Oxoproline</td>
<td>B, 5-Oxoproline, Mg\textsuperscript{2+}</td>
<td>5,270</td>
<td></td>
</tr>
</tbody>
</table>

In Experiment A, the pulse solutions (0.06 ml) contained ATP (250 nmol), Mg\textsubscript{Cl} \textsubscript{2} (500 nmol), 5-oxoproline (27 nmol, 3.96 \times 10^4 cpm/nmol), KCl (5 \mu mol), Tris-HCl buffer (6 \mu mol, pH 8.2), and Component A (5.2 nmol, 800 \mu g, added last); 37 °C. The chase solutions (0.1 ml) contained Component B at a saturating concentration (680 \mu g), unlabeled 5-oxoproline (8 \mu mol) in Tris-HCl buffer (pH 8.2). The reactions were terminated after 5 s by adding ice-cold HClO\textsubscript{4} (see "Experimental Procedures"). In Experiment B, the temperature was 4 °C. The chase solution contained 5-oxoproline (10.7 nmol, 1.51 \times 10^5 cpm/nmol and the amount of Component A was 2 nmol. The chase solution contained 2.6 \mu mol of unlabeled 5-oxoproline. The incubation time after adding the chase solution was 15 s. In Experiment C, the temperature was 37 °C. The pulse solution contained 5-oxoproline (25 nmol, 1.091 \times 10^5 cpm/nmol) and 2.4 nmol of Component A. Chase solution contained unlabeled 5-oxoproline (6.25 \mu mol) and the incubation time after adding the chase solution was 15 s. In Experiment D, the temperature was 37 °C. The pulse solution contained 5-oxoproline (25 nmol, 1.091 \times 10^5 cpm/nmol) and 0.9 nmol of Component A. The chase solution contained unlabeled 5-oxoproline (1.54 \mu mol) and the incubation time after adding the chase solution was 15 s. The incubation time after adding the chase solution was 5 s.
was added in the hope of stabilizing such an intermediate. Component A (5 nmol) was incubated for 2 min at 37 °C in a reaction mixture (final volume, 0.3 ml) containing 10 mM ATP, 20 mM MgCl₂, 100 mM KCl, 2 mM 5-oxo-[¹⁴C]proline (2 × 10⁵ cpm/nmol), and 100 mM HEPES buffer (pH 8.2), and then treated with 1.2 ml of an 80% (v/v) aqueous solution of dimethyl sulfoxide. Sodium borohydride (5 mg) was then added, and the mixture was processed as described under "Experimental Procedures." Analysis on the amino acid analyzer revealed no evidence for the formation of 5-hydroxyornovaline. However, a labeled product was found that chromatographed with proline. Similar results were obtained in studies in which the reaction mixtures contained Component A plus a saturating level of Component B. No product was formed when Component A was omitted. The amount of product formed in these experiments with Component A alone or with Components A plus B were 0.5–1 and 1.5–2%, respectively, of the number of available enzyme active sites.

Catalytic Formation of a Product Reducible by Borohydride—In the course of further studies in which attempts were made to trap the enzyme-bound intermediate, we discovered that the rates of the reactions catalyzed by Component A (5-oxoproline-dependent ATPase) and by Components A plus B (Reaction (1)) were not greatly affected by the presence of relatively high concentrations of borohydride. Potassium borohydride (200 mM) did not affect the rates for the first 2 min; the rates were about 30% inhibited (after correction for pH changes) after 20 min. We therefore examined the formation of proline in a reaction mixture containing 0.54 nmol of Component A as a function of borohydride concentration (Fig. 1). At the highest concentration of borohydride used (500 mM), about 40 nmol of proline was formed, and the curve plateaus, probably reflecting protein denaturation. During the course of the reaction, the pH increased from 8.2 to 9.0 (at 25 mM) and to 9.8 (at 500 mM). Attempts to buffer the solutions more effectively by increasing the concentration of HEPES or by use of a Tris/glycine buffer led to lower yields of proline. Although some inhibition and denaturation of enzyme undoubtedly occurred in the studies described in Fig. 1, the data indicate that product formation is catalytic. We calculate, based on the amount of enzyme and ATP present, that proline formation is about 2–4% of substrate turnover.

In the studies described above (and also those below), proline was identified on the amino acid analyzer and also by thin layer chromatography. (In the latter analyses, radioactivity was detected only in the area stained by isatin.) Studies were also carried out with unlabeled 5-oxoproline and sodium borotritide as follows. Component A was incubated with ATP, Mg²⁺, 5-oxoproline, and sodium borotritide (Fig. 2). After destruction of residual borotritide and removal of borate, the reaction mixtures were applied to a column of AG 1-X8 (HCO₃⁻), and the labeled products were separated (see "Experimental Procedures"). Chromatography revealed four labeled components (Fig. 2); peak II was identified as proline. Analogous reactions were carried out with Component A plus Component B and appropriate controls as indicated in Fig. 2. Proline was formed only in Experiment 1. The label present in peak IV was volatilized during lyophilization. Peaks I and III, which were not identified, appeared in all of the experiments and may be ascribed to impurities.

Experiments 1, 2, and 4 were repeated under the same conditions except that the reaction mixtures contained unlabeled potassium borohydride and ¹⁴C-labeled 5-oxoproline (5 mM, 2.3 × 10⁶ cpm/nmol). Since chromatography on AG 1-X8 did not resolve proline and 5-oxoproline, the labeled 5-oxoproline present was removed by chromatography on Dowex 50 (H⁺) as described under "Experimental Procedures," and the proline formed (about 10 nmol) was quantitated. Again, proline was found only in the reaction mixture that contained Component A alone (Experiment 1). When Components A and B were present, no proline was formed and glutamate was formed as expected (Experiment 2). However, in Experiment 1, an additional ¹⁴C-labeled product was found on the amino acid analyzer; this product contained 25% of the total ¹⁴C present (the remainder being [¹⁴C]proline) and eluted at 24 min (proline eluted at 28 min). (In the comparable study with sodium borotritide, all of the ³H was present in proline.) The additional ¹⁴C-labeled product eluted in the position of Δ¹-pyrroline-5-carboxylate, but is probably not identical to this compound because we found that authentic Δ¹-pyrroline-5-carboxylate disappears completely under these conditions. Δ¹-Pyrroline-5-carboxylate is reported to be unstable when chromatographed in a citrate buffer system (19). Strecker (28) and also Gallop et al. (29) previously observed

![Fig. 1. Formation of proline as a function of borohydride concentration.](image1)

![Fig. 2. Formation of proline.](image2)
that borohydride reduction of Δ1-pyrroline-5-carboxylate gave an 80% yield of proline, and it has been suggested that the diminished yield is due to polymer formation.

We reinvestigated the borohydride reduction of Δ1-pyrroline-5-carboxylate examining the products formed by high pressure liquid chromatography analysis of the phenyl thio-carbamyl derivatives (30, 31). Authentic Δ1-pyrroline-5-carboxylate gave a single peak (retention time, 7.67 min). Reduction with borohydride and removal of borate as described under "Experimental Procedures" gave proline (retention time, 6.06 min) together with a small peak at 7.24 min with an area of about 24% that of proline. This product was not formed in a comparable study in which the borohydride was destroyed prior to addition. These results suggest that Δ1-pyrroline-5-carboxylate may be formed in the borohydride reduction of the enzyme-bound intermediate and that the additional 14C-labeled product may thus is a polymeric side product. That a labeled product is not detected when enzyme mixtures are treated with NaB₃H₄ suggests that the tritium introduced upon reduction is exchangeable with water.

**Trapping of a Phosphate-containing Intermediate by Esterification with Diazomethane**—When reaction mixtures containing Component A, 5-oxoproline, and [γ-32P]ATP/Mg²⁺ were immediately methylated with diazomethane, a 32P-containing product was formed (Table II). The Rₚ value of the enzyme-formed compound on thin layer chromatography was 0.5 and different from those of P, (Rₚ 0), dimethyl phosphate (Rₚ 0), trimethyl phosphate (Rₚ 0.95) and ATP (Rₚ 0). The amount of labeled product (Rₚ 0.5) formed was about 0.4 nmol/nmol of active site. This product was not formed in controls in which enzyme or 5-oxoproline were separately omitted (Table II, Experiments 3 and 4). When Component B was included in the reaction mixture, the amount of the 32P-containing product decreased substantially (Table II, Experiment 2).

Chromatographic analysis of methylated mixtures that had been kept at 0°C for 1 h after methylation showed about a 10% decrease in the amount of the new 32P-labeled product. Aliquots of the mixtures (Table II, Experiments 1–4) were evaporated at room temperature, redissolved in 1,2-dimethoxyethane, and analyzed again by thin layer chromatography. In the controls (Experiments 3 and 4), little or no radioactivity was found at the origin indicating the absence of inorganic phosphate, ATP, and dimethyl phosphate in these studies. In the re-chromatography of Experiments 1 and 2, substantial radioactivity was found at the origin consistent with formation of dimethyl phosphate in amounts close to those of the number of enzyme active sites present. The expected breakdown products of the methylated phosphate derivative of 5-oxoproline are dimethyl[32P]phosphate and the methyl ester of 5-oxoproline. The amount of radioactivity found in the dimethyl phosphate area was consistently equivalent to the number of active sites. The finding of only 0.4 nmol of intermediate (Rₚ 0.5) per nmol of active site is most likely due to nonenzymatic breakdown of the methylated intermediate, or possibly to some degree of its coprecipitation with the enzyme.

**DISCUSSION**

The isotope-trapping method used here permits recognition of and provides information about tightly bound intermediates that are formed on and transformed by an enzyme (8–14). The procedure was first successfully applied to glutamine synthetase (9) and later to other enzymes including argininosuccinase synthetase (10), glutathione synthetase (11), carbamyl phosphate synthetase (12, 13), and hexokinase (14). The method is applicable to the study of intermediates formed in reactions involving two or more substrates. The present application differs from those used previously in that the reaction is brought to completion by addition of a second protein (Component B). The data show that an enzyme-bound activated form of 5-oxoproline is formed on Component A; formation of this intermediate requires ATP and Mg²⁺, and its efficient utilization for glutamate formation requires Component B. The amount of ATP-dependent trapping of 5-oxoproline on Component A is close to that of the available active sites.

The chemical-trapping techniques (reduction by borohydride and methylation by diazomethane) used here were successfully applied to identify carbonic phosphoric anhydride (carboxy phosphate) in the reaction catalyzed by glutamine-dependent carbamyl phosphate synthetase (24, 25). In the present work the observed formation of proline after treatment of reaction mixtures with borohydride is in accord with the view that an enzyme-bound phosphorylated form of 5-oxoproline is formed (Fig. 3, II). The efficiency of the reduction reaction is relatively low; thus, the formation of proline

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**Table II**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>32P-Labeled intermediate</th>
<th>Dimethyl[32P] phosphate</th>
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</thead>
<tbody>
<tr>
<td>1. A + [γ-32P]ATP + 5-oxoproline</td>
<td>0.40</td>
<td>0.80</td>
</tr>
<tr>
<td>2. A + [γ-32P]ATP + 5-oxoproline + B⁺</td>
<td>0.10</td>
<td>0.93</td>
</tr>
<tr>
<td>3. [γ-32P]ATP + 5-oxoproline</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>4. A + [γ-32P]ATP</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* Component B was present at a level that gave 35% saturation of Component A.

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**Fig. 3.** Proposed scheme for enzymatic conversion of 5-oxoproline to glutamate (I→II→III→IV), for reduction of the intermediate (II) to proline (VIII), and for formation and breakdown of the esterified intermediate (V). See the text.
is equivalent to only 2-4% of substrate turnover. Although the enzyme can turn over substrate in the presence of borohydride, it undergoes denaturation. In an apparently analogous chemical reaction, the reduction by borohydride of the ethyl ester imidate corresponding to 5-oxoproline \( [-N=C(O-ethyl)] \) has been reported to go smoothly (32). The relatively low efficiency of the borohydride reaction in the enzyme system may reflect disruption of the enzyme by borohydride with consequent hydrolysis of the intermediate and possibly also hindrance by the negatively changed phosphate moiety of the intermediate. The phosphorylation of the carbonyl group of 5-oxoproline postulated here is in accord with model studies of Satterthwait and Westheimer (33, 34) on the phosphorylation of acetophe none by monomeric metaphosphate anion. A notable point in these studies is that monomeric metaphosphate anion reacts preferentially with the keto form rather than the enol form of acetophenone (34).

The finding of a labile phosphate-containing product (presumably V, Fig. 3) after treatment of enzyme reaction mixtures with diazomethane is also in accord with the proposed intermediate (II, Fig. 3). Although the efficiency of this trapping procedure is substantially greater than that of the borohydride method, the findings indicate that the ester (V) is not very stable and that it breaks down to yield dimethyl phosphate. Notably the amount of dimethyl phosphate found is 80-90% of the number of active sites present (Table II). Dimethyl phosphate might also be formed in the breakdown of other intermediates (e.g. \( \gamma \)-glutamyl phosphate) that may be formed in the reaction.

The tentative scheme for the mechanism of action of 5-oxoprolinase (Fig. 3) involves conversion of phosphorylated 5-oxoproline (II) to glutamate (IV) by reactions mediated by Component B. The finding in model studies that a neighboring phosphoryl moiety accelerates amide hydrolysis (35, 37) seems relevant to the mechanism of action of the enzyme. Although the data presented here are consistent with the formation of the proposed intermediate (II), one cannot conclude this unequivocally, because information concerning its kinetic competence has not yet been obtained. It may be noted that chemical studies of the type reported here for 5-oxoprolinase previously showed formation of \( \gamma \)-glutamyl phosphate in the glutamine synthetase reaction (21) and carboxy phosphate in the reaction catalyzed by carbamyl phosphate synthetase (24, 25). The kinetic competence of these intermediates, initially proposed on the basis of chemical-trapping studies, was later shown by positional isotope exchange studies (38, 39). Direct chemical evidence for the existence of phosphorylated 5-oxoproline (II) must be supplemented by studies which elucidate its kinetic role in the overall reaction, the function of Component B, and the details of the steps involved in conversion of II to IV.

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REFERENCES