

The Amino Acid Sequence of Rat Kidney 5-Oxo-L-Prolinase Determined by cDNA Cloning*

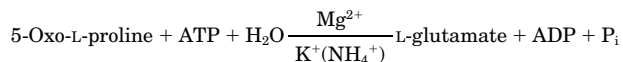
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5-Oxoprolinase (EC 3.5.2) catalyzes a reaction in which the endergonic cleavage of 5-oxo-L-proline to form L-glutamate is coupled to the exergonic hydrolysis of ATP to ADP and inorganic phosphate. Highly purified preparations of the enzyme have been obtained from rat kidney and *Pseudomonas putida*. The rat kidney enzyme is composed of two strongly interacting, apparently identical subunits ($M_r = 142,000$), whereas that from *P. putida* is composed of two functionally different protein components that can readily be dissociated. Here we report the cloning of rat kidney 5-oxoprolinase with preliminary expression studies. cDNA clones encoding the enzyme were isolated by screening a λ gt11 cDNA library beginning with a degenerate oligonucleotide probe based on peptide sequence data obtained from the purified enzyme. The whole cDNA clone was completed by amplifying its 5' end from a premade library of rat kidney Marathon-Ready™ cDNAs using polymerase chain reaction methodology. The composite cDNA (4,016 bases) revealed an uninterrupted open reading frame encoding 1,288 amino acid residues ($M_r = 137,759$). The deduced amino acid sequence contains all four of the peptide sequences that were independently found in peptide fragments derived from the enzyme. Expression of the full-length clone in *Escherichia coli* yielded a product of the same size as the rat kidney enzyme and which reacted with antibodies directed against the rat kidney enzyme. The predicted amino acid sequence is almost 50% identical throughout its entire length to that of a hypothetical yeast protein YKL215C. It is also 26% identical in half its length to the bacterial hydantoinase HyuA and 26% identical in the other half to the bacterial hydantoinase HyuB. The results suggest unexpected evolutionary relationships among the hydantoinases and rat kidney 5-oxoprolinase which share the common property of hydrolyzing the imide bond of 5-membered rings but which do not all require ATP.

5-Oxo-L-prolinase (5-OPase)¹ catalyzes the ATP-dependent cleavage of 5-oxoprolinase to L-glutamate:



This reaction is highly unusual in that hydrolysis of ATP is required for the cleavage of a specific peptide bond (1). 5-OPase has been found in mammalian tissues (2), plants (3), and microorganisms (4, 5). Apparently homogeneous preparations of 5-OPase from rat kidney (6) and *Pseudomonas putida* (7) had been obtained in this laboratory and were used for physical characterization and for studies of catalytic mechanism. 5-OPase from rat kidney is composed of two apparently identical subunits that exhibit a molecular mass of 142 kDa on SDS-polyacrylamide gel electrophoresis (6). The enzyme is evidently a "sulfhydryl enzyme" (8) and has a number of sulfhydryl groups/monomer (6). The relationship between the essential sulfhydryl groups of the enzyme and its various catalytic activities have been probed (6). Unlike rat kidney 5-OPase, *P. putida* 5-OPase is composed of two different, reversibly dissociable protein components, A and B (7). Component A catalyzes an initial step in the reaction that involves 5-oxoprolinase and ATP (9). Component B may function as a catalyst that converts a phosphorylated form of 5-oxoprolinase to glutamate, or it might alter the conformation of Component A so as to facilitate the reaction (10, 11). Data are lacking, however, on the amino acid sequence of the enzyme from any source. Knowledge of the amino acid sequence of the enzyme and the cloning of the encoding cDNA are essential for further studies on the structure, mechanism of action, and physiological function of the enzyme.

This study was undertaken to elucidate the primary structure of rat kidney 5-OPase as preparation for the analysis of relationships between its structure and function. Peptides obtained from the purified rat kidney enzyme by enzymatic cleavage with Lys-C were used to design oligonucleotide probes that permitted cloning and sequencing of the cDNA encoding the complete protein. Protein representing the composite whole cDNA and truncated cDNA clones of the enzyme was also expressed in *Escherichia coli*, as confirmed by immunodetection with the rabbit antiserum against isolated rat kidney 5-OPase.

EXPERIMENTAL PROCEDURES

Materials—Frozen rat kidneys were obtained from Pel-Freez. Ultrogel AcA34 was from LKB. Phenyl- and aminoethyl-Sepharose were obtained from Pharmacia Biotech Inc.. DEAE-cellulose (DE-52) was

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U70825 (BankIt69326).

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† Deceased April 6, 1995. His contributions illuminated the field of glutathione metabolism in which 5-oxoprolinase plays a central role and provided the basis of our understanding of the mechanisms by which key enzymes in this and in other areas of amino acid metabolism operate.

¹ The abbreviations used are: 5-OPase, 5-oxo-L-prolinase; 5' and 3' RACE, 5' and 3' rapid amplification of cDNA ends; PCR, polymerase chain reaction; LD-PCR, long distance polymerase chain reaction; IPTG, isopropyl- β -D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s); SSPE, a 1× solution represents 0.18 M NaCl, 10 mM sodium phosphate, pH 7.7, and 1 mM EDTA.

from Whatman. The rat kidney λ gt11 cDNA library, premade rat kidney Marathon-Ready™ cDNAs and *Taq*-start antibody were purchased from Clontech. DNA polymerase for PCR amplification (LD-PCR system) was from Boehringer Mannheim. Restriction endonucleases were from New England Biolabs Inc.. All radioisotopes were obtained from Amersham Corp. All chemicals and biologicals were purchased from Sigma unless noted otherwise.

General Methods—Standard molecular biology techniques were used (12). Recombinant λ DNA purification was performed as described (13). cDNA clones were subcloned into a Bluescript II KS vector (Stratagene) and sequenced by the dideoxy chain termination method (14) with Sequenase (United States Biochemical Corp.) using T3, T7 primers or specific internal primers. Automated DNA sequencing was carried out at the Cornell University DNA Synthesis and Sequencing Facility.

Determination of Enzyme Activity—Enzyme activity was determined (6) in reaction mixtures containing (final volume, 0.5 ml) 100 mM Na-Hepes buffer (pH 8.0), 2 mM 5-oxo-L-proline (containing 100 cpm/nmol 5-oxo-L-[U-¹⁴C]proline), 5 mM ATP, 8 mM MgCl₂, 2 mM P-enolpyruvate, 150 mM KCl, 2 mM dithiothreitol, pyruvate kinase (5 units), and 5-OPase. After incubation at 37 °C for 30 min, the reaction mixtures were treated with 0.1 volume of 1 M HCl and placed at 0 °C for 5 min; an equal volume of 1 M Tris was then added. Denatured proteins were removed by centrifugation. Portions of the neutralized reaction mixtures were then analyzed for [¹⁴C]glutamate by liquid scintillation counting after removal of unreacted 5-oxoprolinase by chromatography on Dowex 50 (H⁺) as described (6). One unit of activity is defined as the amount of enzyme needed for the production of 1.0 μ mol of glutamate/h under standard assay conditions.

Purification of 5-OPase—Rat kidney 5-OPase was isolated from rat kidney homogenates using a procedure developed by Williamson and Meister (6). The homogenate was first centrifuged (16,200 \times g for 90 min), and the proteins in the supernatant solution were fractionated by ammonium sulfate precipitation. The fraction containing the enzyme activity was then processed by a series of column chromatography steps, which sequentially involved DEAE-cellulose (DE-52), Ultrogel AcA34, phenyl-Sepharose, AH-Sepharose, and a second Ultrogel AcA34 step, as described (6). The preparation of the enzyme was further processed by a second DE-52 step and a third Ultrogel AcA34 chromatography step. An almost homogeneous (>90% pure) preparation of the enzyme with a specific activity of 72 units/mg was obtained in about 20% yield. Every step of the purification was followed by SDS-PAGE analysis. Quantitative assay for protein was done by the method of Bradford (15).

Preparation of Antibody against Rat Kidney 5-OPase—The 5-OPase (300 μ g) was dialyzed for 24 h against two changes of phosphate-buffered saline and was concentrated to a final volume of 600 μ l by vacuum dialysis against phosphate-buffered saline. An equal volume of complete Freund's adjuvant was added to the enzyme and mixed vigorously until the solution became very viscous. Two anesthetized New Zealand White rabbits (~1.8 kg) were injected intradermally at 20 sites (20–25 μ l of emulsion each). A test bleed was performed 2 weeks after the primary immunization. The titer and specificity of the antibodies were determined by enzyme-linked immunosorbent assay and Western blot analysis, respectively.

Amino Acid Sequences of Peptides from 5-OPase—Approximately 5 μ g (1.5 pmol) of the protein isolated from rat kidney was electrophoresed on a 7.5% SDS-polyacrylamide gel and electrotransferred to a polyvinylidene difluoride membrane (16). The membrane was stained with Amido Black, and the protein band corresponding to 5-OPase (~140 kDa) was cut out for solid phase enzymatic digestion (endopeptidase Lys-C, enzyme/substrate, 1:10) (17). The microbore high performance liquid chromatography purification and sequencing of the peptides were carried out at the Rockefeller University Protein & Peptide Sequencing Facility.

cDNA Cloning of Rat Kidney 5-OPase—A 29-mer degenerate oligonucleotide, oligonucleotide 1, (GTITTC(T)CAA(G)GAA(G)GAA(G)GCIGTACIGAA(G)GC) was synthesized based on the peptide sequence VFQEEAVTEA in peptide 1 (Table I, end-labeled with [³²P]ATP, and used to screen a λ gt11 rat kidney cDNA library. The probe is a mixture of 32 different 29-mer oligonucleotides corresponding to all codon combinations derived from peptide 1. Deoxyinosine (I) was substituted at the wobble positions in four of the codons. Duplicate filters were prehybridized at 42 °C for 3 h in prehybridization solution (6 \times SSC, 5 \times Denhardt's solution, 0.5% SDS, 0.01% sodium pyrophosphate, and 100 μ g/ml denatured fragmented salmon sperm DNA), followed by hybridization at 50 °C for 18 h in the same solution containing 1.6 \times 10⁵ cpm of ³²P-labeled probe/ml of hybridization buffer. The filters were washed with 2 \times SSC containing 0.5% SDS for 20 min at room temperature

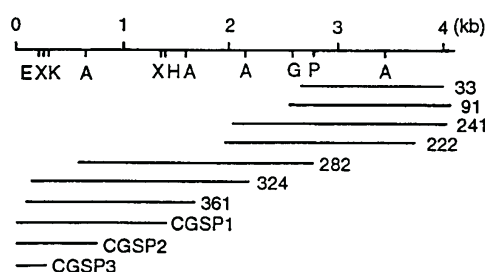


FIG. 1. Restriction map and overlapping rat kidney 5-oxoprolinase cDNA clones. Letters indicate restriction sites for *Eco*RI (E), *Xba*I (X), *Kpn*I (K), *Apa*I (A), *Hinc*II (H), *Age*I (G), and *Pst*I (P). Clones 33 and 91 were derived from the first screening of the rat kidney library; 241, 222, and 282 were derived from the second screening; 324 and 361 from the third screening. CGSP1, CGSP2, and CGSP3 were obtained from PCR amplification of rat kidney Marathon-Ready cDNAs.

with one change of the wash buffer, followed by washing with 2 \times SSC and 0.2% SDS at 50 °C for 40 min with one change of the buffer. Of the ~5 \times 10⁵ clones that were screened, three positives were identified, plaque-purified, and subcloned into Bluescript II KS (Stratagene). These three clones, designated as CL33, CL91, and CL99, contained inserts of 1.3, 1.5, and 0.8 kb, respectively; CL91 also contained a poly(A) tail. CL33 and CL91 were sequenced completely. The position of these clones in the ultimately derived sequence and that of the relevant clones described below are shown in Fig. 1.

A 0.39-kb *Pst*I/*Eco*RI fragment was prepared from the 5' end of CL33 and labeled with [³²P]dCTP by nick translation (1 \times 10⁸ cpm/ μ g) for use as a probe for the second screening of the same library. Duplicate filters were hybridized at 55 °C in 6 \times SSC hybridization solution (same as above) with 1.0 \times 10⁵ cpm of ³²P-labeled probe/ml of hybridization solution. After rinsing briefly in 2 \times SSC, 0.5% SDS at room temperature, the filters were washed in 0.4 \times SSC, 0.1% SDS for 40 min at 55 °C with one change of the wash buffer. Of the ~5 \times 10⁵ clones that were screened, 32 positives were identified, plaque-purified, and DNA prepared. Their insert sizes ranged from 1.1 to 3.2 kb. Southern blot analysis revealed that 14 of them hybridized with an oligonucleotide probe (17 mer) which is close to the 3' end of CL33 and CL91. This result indicated that these 14 clones overlapped completely with CL33 and CL91. One of these 14 clones, CL241, was chosen for subcloning and 5' and 3' end sequencing analysis because it represented a relatively large insert (~2.0 kb). Another four clones (all contain inserts >2.0 kb), CL222, CL252, CL273, and CL282, were chosen from the 18 clones that did not hybridize with the oligonucleotide probe. CL273 and CL282, the two apparently most 5'-extended clones, as well as CL241, were subjected to restriction mapping and complete sequencing. CL273 proved to be an artifact, as described further under "Results."

An 0.8-kb *Eco*RV/*Xba*I fragment was prepared from the 5' end of CL282 and used as a probe for further screening of the library. Fourteen positive clones were obtained. In order to quickly screen the most 5'-extended clones, a 24-mer primer (antisense primer) complementary to nucleotides 100–123 of CL282 (nucleotides 744–764 in Fig. 2), was synthesized and used to amplify the 5'-extended region of the 14 positive clones with PCR according to the manufacturer's instructions. The sense primer used here was one of the two primers of the λ gt11 cDNA insert Amplifier (Clontech). Three positive clones, CL341, CL342, and CL361, seemed to contain relatively longer sequences in the 5'-end region and were subjected to subcloning, restriction mapping, and sequencing, although CL341 proved to be another artifact (see under "Results").

In order to obtain a complete cDNA clone, three antisense gene-specific primers (GSP1, GSP2, and GSP3, which are complementary to nucleotides 254–277, 744–764, and 1414–1434, respectively; Fig. 2) were designed and used to amplify the 5'-cDNA end of 5-OPase from a premade library of rat kidney Marathon-Ready cDNAs (Clontech) with the provided adapter specific primer AP1 (18). PCR (30 cycles) was performed according to the manufacturer's instruction with the LD-PCR System (Boehringer Mannheim) in a Perkin-Elmer DNA Thermal Cycler 480 using the following program: 94 °C for 1.0 min, then 30 cycles of 94 °C for 30 s, 63 °C for 45 s, and 68 °C for 1.5 min. 5 μ l of the PCR products were subjected to electrophoretic examination on a 1.2% agarose-EtBr gel. A secondary PCR reaction was performed following the same procedure described above except that 5 μ l of the diluted (1:10) primary PCR product were used in place of the Marathon-Ready cDNAs as template, and 25 cycles were applied. After the secondary PCR

Construction of Expression Plasmids for 5-OPase (Full-length and Terminal Deleted Mutants)—An N-terminal truncated cDNA clone of 5-OPase was constructed by joining the overlapped clones of CL361,

CL282, and CL241 using the unique restriction sites of *HincII* and *AgeI* (Fig. 1). The assembled 3.8-kb cDNA clone, which contains a stop codon at its 3' end, was excised from the recombinant Bluescript II KS by *EcoRI* digestion and ligated to pT7-7 (19) which had been digested with *EcoRI*. The resulting clone, pFCL361-241, which is truncated at the N-terminal of 5-OPase by 54 residues, is in the proper reading frame in the *EcoRI* site of pT7-7 and would subsequently be translated as a fusion protein linked through its amino terminus to the first 4 amino acids of the gene 10 protein.

Another N-terminal deleted clone (truncated by 850 residues at the N terminus of 5-OPase), pFCL33, was constructed directly by joining the cDNA insert of CL33 into pT7-7 at the *EcoRI* site. The resulting clone, pFCL33, would also subsequently be translated as a fusion protein linked through its amino terminus to the first 4 amino acids of the gene 10 protein.

Creation of the *NdeI* site at the start codon of 5-OPase was achieved by PCR amplification from rat kidney Marathon-Ready cDNAs using 5'-CTCCAGCTTCAACCATATGGGCAGC-3' as the gene-specific primer. The LD-PCR amplification resulted in a full-length cDNA clone of 5-OPase (4.0 kb, as shown in Fig. 3B). This clone was digested with *NdeI/PstI*, yielding a 2.9-kb cDNA fragment that was truncated at the C-terminal region of 5-OPase by 302 amino acid residues and was ligated to pT7-7 which had previously been digested with *NdeI/PstI*. For subsequent expression as a fusion protein, the resulting clone (pFCPCR) was linked at its 3' end to the DNA sequence encoding the hexapeptide AQAYHR followed by a stop codon.

The full-length clone was constructed by ligation of the *NdeI/KpnI* fragment of pFCPCR to pFCL361-241 which had been digested with *NdeI/KpnI*. The constructed full-length clone, pPROPASE, is immediately downstream from the T7 promoter ($\phi 10$). Sequences representing the full-length and truncated recombinant 5-OPase were expressed in *E. coli* (BL21:DE3) as described (20).

RESULTS

Isolation of 5-OPase from Rat Kidney and Its Peptide Sequences—The enzyme preparation obtained from step 9 of the purification procedure yields, as monitored by SDS-polyacrylamide gel electrophoresis, a dominant band of 140 kDa that comprises >90% of the protein and two minor bands of lower molecular weight. Approximately 600 μ g of protein could be obtained from 500 g of frozen rat kidney with a specific activity of 72 units/mg. Table I shows the sequences of four Lys-C enzymatic peptides obtained from the purified enzyme. The amino acid sequences corresponding to these four peptides were subsequently found in the cDNA clone (Fig. 2).

Isolation of cDNA Clones Coding for 5-OPase—Fig. 1 outlines the position in the cDNA sequence of the relevant clones used for sequence determination. By screening a rat kidney cDNA library with the end-labeled degenerate oligonucleotide probe encoding the partial sequence of peptide 1, three positive clones were initially obtained. Two of them, CL33 and CL91, were found to contain the longest sequence of the 3'-untranslated region. CL91 also included a poly(A) tail. Screening of the same library using the 0.39-kb 32 P-labeled *EcoRI/PstI* fragment prepared from the 5' end of CL33 gave 32 positives. Of these, CL241, CL222, and CL282 were completely sequenced. Though CL273 was the largest (3.2 kb) and the most 5'-extended, this clone was found to be an artifact at its 5' region on the basis of restriction mapping and sequencing; *i.e.* about two-thirds of the 5' terminal region of CL273 was from an unknown cDNA clone, as judged in part by the presence of a poly(A) tail in this region. CL341, the most 5'-extended cDNA clone (2.4 kb) from the third screening (which used a probe from the 5' end of CL282), was also found to be incorrect; restriction mapping and sequencing of this clone indicated that a fragment of 0.6 kb at its 5' end was an artifact, as evidenced by the presence of a number of internal stop codons. CL324 and CL361 from the third sequence allowed determination of most of the remaining sequence.

The complete cDNA clone for the enzyme was obtained by LD-PCR amplification using the premade rat kidney Mara-

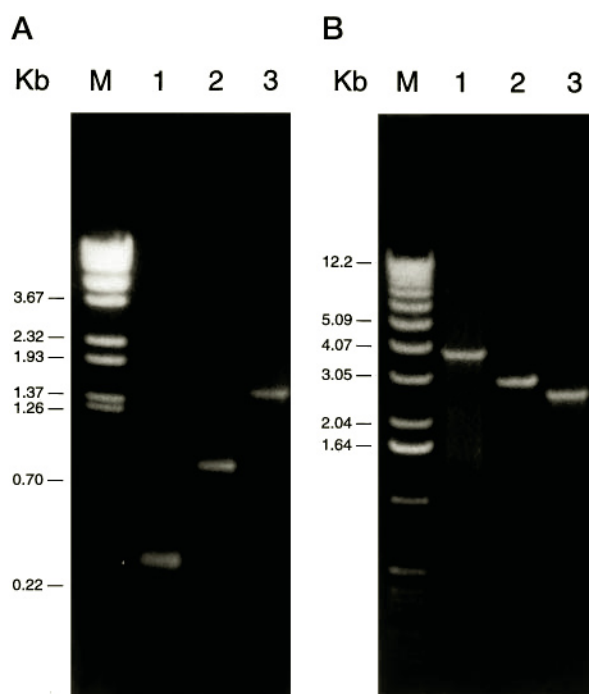


FIG. 3. 5' and 3' Marathon RACE of 5-oxoprolinase from rat kidney Marathon-Ready cDNAs. 5' and 3' Marathon RACE were performed using the provided adapter-specific primer (AP1) and the indicated gene-specific primer. A, 5' RACE products. Lanes 1, 2, and 3 are 5' RACE products using GSP1, GSP2, and GSP3 as the gene-specific primers, respectively (see under "Experimental Procedures"). B, 3' (lanes 1 and 2) and 5' (lane 3) Marathon RACE products. Cycling parameters: 1 min at 94 °C followed by 30 cycles (25 cycles for secondary PCR amplification) of 30 s at 94 °C, 45 s at 62 °C, and 5 min at 68 °C. The gene-specific primers used were: lane 1, nucleotides 92–114, containing an *NdeI* restriction site introduced at the start codon in this primer; lane 2, nucleotides 1067–1084, and lane 3, an antisense primer complementary to nucleotides 2581–2599. The expected size of the products can be deduced by the position of the gene-specific primer used in each case. For example, the expected size of the products in B are: lane 1, ~4.0 kb; lane 2, 2.95 kb (4016–1066); lane 3, 2.599 kb.

thon-Ready cDNAs as templates. After secondary PCR amplification as described under "Experimental Procedures," the resulting PCR product generated a band of the predicted size after electrophoresis on a 1.2% agarose-EtBr gel (Fig. 3A). Sequencing of the clones of CGSP2 and CGSP3 revealed two completely overlapping cDNA fragments of the 5' end of 5-oxoprolinase, in which CGSP3 was more 5'-extended than CGSP2 by 4 bases.

The sequence of the assembled 4.016-kb cDNA for rat kidney 5-OPase is shown in Fig. 2. It revealed an uninterrupted open reading frame encoding 1288 amino acid residues with a calculated molecular weight of 137,759, which agrees well with that previously estimated by SDS-PAGE ($M_r = 142,000$) (6). The 5'-untranslated region of 105 nucleotides contains an in-frame stop codon. The first ATG (position 106) is presumed to be the initiation codon and conforms to the consensus sequence for an initiation codon context (21). The coding sequence ends with a termination codon (TGA) at position 3969, followed very closely by the polyadenylation signal AATAAA beginning at position 3981 and a 14-nucleotide poly(A) tail (Fig. 2).

Analysis of the Predicted Amino Acid Sequence of Rat Kidney 5-OPase—All four independently determined peptide sequences are found in the deduced amino acid sequence (Fig. 2). The sequence also indicates five potential sites for N-glycosylation (N-X-S/T), although actual glycosylation has not been demonstrated. The calculated amino acid composition is in fair agreement with the amino acid analysis of the isolated protein (6), except for Asx, Glx, and Lys (data not shown). The pre-

TABLE I

Sequences of peptides derived from purified rat kidney 5-OPase and comparison with the sequence of the protein deduced from its cDNA

Sequences are identical except where noted. Underlined residues are those derived from the cDNA sequence that align with residues which were not identified with certainty by peptide sequencing.

Peptide	Amino acid sequence	Positions in cDNA sequence
1	IQTGPPHVEK	652–661
2	LVQGGVFQEEAVTEALRAP (G) K	886–906
3	??A(C) (S) G(T) N(N) (L) HDN(S) DLRAQVAANQK	
4	ISG C S T R N L S	907–932
	??RTVNLGGK	1223–1232
	DG	

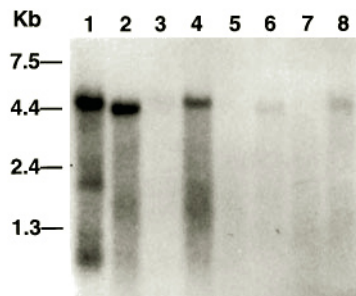


FIG. 4. Tissue distribution of mRNA for 5-oxoprolinase. Hybridization with the 32 P-labeled probe (CL33) was carried out as described under "Experimental Procedures." The filter was exposed to Kodak XAR-5 film with two intensifying screens at -79°C for 3 days. Lanes 1–8 were mRNAs from rat testis, kidney, skeletal muscle, liver, lung, spleen, brain, and heart, respectively.

dicted protein sequence was found to be unique when compared with the protein sequences given in the GenBank and SwissProt data bases, but strong sequence similarities to three other proteins were found (see under "Discussion").

Tissue mRNA Expression—Northern blot hybridization studies with nick-translated probe prepared from a fragment of 5-OPase (CL33) showed that the mRNA for the enzyme is well expressed in testis, kidney, and liver, whereas the mRNA levels in other tissues examined (brain, heart, lung, spleen, and muscle) were about 10% or less of that found in testis or kidney (Fig. 4). These findings are in general agreement with determinations of the activities of 5-OPase in these tissues (8). The size of the transcript in kidney (~ 4.4 kb) is a little smaller than that found in testis and liver (~ 5.0 kb). Minor bands of smaller size, particularly evident in the testis sample, probably arise from mRNA degradation or from nonspecific hybridization. It should be noted that the rat multiple tissue Northern blot (Clontech) used in this study had been probed several times before, so the strength of the hybridization signal does not represent the real level of the mRNA.

Expression of Recombinant 5-OPase—The expressed recombinant rat kidney 5-OPase and the expressed N-terminal and C-terminal truncated proteins gave bands of the predicted sizes when analyzed by SDS-gel electrophoresis and Western blot using the antibodies against the purified 5-OPase (Fig. 5, A and B). Also, recombinant 5-OPase and the 5-OPase isolated from rat kidney homogenate gave bands of apparently same size when detected by Western blot. These data provide further evidence that the cDNA clone obtained in this study corresponds to that of rat kidney 5-OPase. The C-terminal truncated recombinant 5-OPase (missing a C-terminal peptide of 243 residues) encoded by pFCPCR gave no detectable band on the Western blot (Fig. 5B), but its expression was readily seen when gels were stained with Coomassie Blue (Fig. 5A). This result suggests that the polyclonal sera principally contain

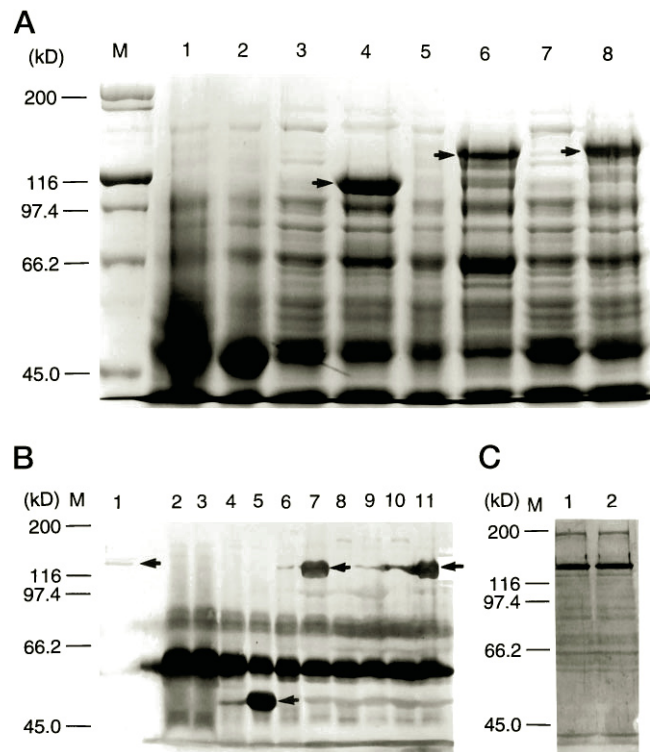


FIG. 5. SDS-PAGE analysis and immunodetection of expressed rat kidney whole and truncated 5-OPases. Samples were electrophoresed on a 7.5% SDS-PAGE gel. For immunodetection (B and C), the proteins were electroblotted onto a nitrocellulose filter. Antigen-specific signals were probed with a 1:250 dilution of rabbit anti-5-OPase antiserum. *E. coli* carrying the vector of pT7-7 was used as a control. A, gel stained with Coomassie Blue after electrophoresis. Lane M, marker; lanes 1, 2, 4, 6, and 8, lysates of *E. coli* (BL21:DE3) transformed with the plasmids of pT7-7, pFCL33, pFCPCR, pFCL361-241, and pROPASE, respectively, and induced with IPTG for 3 h; lanes 3, 5, and 7, lysates of *E. coli* (BL21:DE3) transformed with pFCPCR, pFCL361-241, and pROPASE, respectively (no IPTG induction). Arrows indicate the bands of proteins expressed. B, lane 1, rat kidney homogenate; lanes 2, 4, 6, 8, and 10, lysates of *E. coli* (BL21:DE3) transformed with pT7-7, pFCL33, pFCL361-241, pFCPCR, and pROPASE, respectively (no IPTG induction); lanes 3, 5, 7, 9, and 11, lysates of *E. coli* (BL21:DE3) with pT7-7, pFCL33, pFCL361-241, pFCPCR and pROPASE, respectively, and induced with IPTG for 3 h. Arrows indicate the detected bands of 5-OPase in rat kidney homogenate and expressed target proteins. C, specificity analysis of the rabbit antiserum against isolated 5-OPase by Western blot. Lanes 1 and 2, fresh rat kidney homogenate.

antibodies that recognize denaturation-resistant epitopes within the C-terminal region of 5-OPase. Enzymatic activity analysis of the expressed recombinant rat kidney 5-OPase in the lysate of *E. coli* using standard procedures (see under "Experimental Procedures") indicated no significant increase in activity above the control.

DISCUSSION

The present work is the first carried out on the cloning and expression of 5-oxoprolinase. The deduced amino acid sequence of the rat kidney enzyme consists of 1288 residues with a calculated molecular weight of 137,759, which is close to the weight of 142,000 previously estimated from SDS-PAGE analysis of the isolated enzyme (6). Previous work (4, 6) in this laboratory also suggested that the native enzyme ($M_r = 325,000$, estimated by gel filtration) is composed of two subunits of identical molecular weight that are not held together by disulfide bonds. Since all of the four independently determined peptide sequences were found within the predicted protein sequence and the calculated amino acid composition was in fair agreement with the amino acid analysis of the isolated rat kidney enzyme, the data support the suggestion that the two

YKL215C	1	..MQKGNIRI	AIDKGGTFTD	CVGNGTGGKQ	EHDTVIKLLS	VDPKNYPDAP	YKL215C	701	DNKWVDVAV	.FKIDDLPAQ	TIIEGPAILA	DGTQNTIILP	NSQATILN.S
5-OPase		MGSPEGRFHF	AIDRGGTFTD	VFAQCPCGG..	.HVRVLKLLS	EDPANYPDAP	5-OPase		EGGYQETPV	.YLLGELGYG	HQLQGCLII	DNNSTILVEP	GQAEVTDTC
HyuA	MKLF	GVDVGSTFTD	II.....FS	DTETRVTAIH	KVPTLLDDPS	HyuA		DGSLVKVEIN	FVQREKIPVG	AEFNGPCIVL	QKDTTIVIPP	NCTAYIEEYG
YKL215C	51	LEGIRRLLEV	LEHKTIPRGI	PLDISNVRSI	RMGTTLATNC	ALERNGERCA	YKL215C	751	HIFIKINQKA	AKTLSKSGYE	LDIDPILLSI	FSHRFMDIAL	QMGTOQLRKS
5-OPase		TEGIRRLIEQ	EEGVLLPRGR	PLDTSRIASI	RMGTTVATNA	LLERQGERVA	5-OPase		DIRISVGAEG	P.....SMAD	TRLDPICLSI	FSHRFMSIAE	QMGRIQLQRTA
HyuA		TGVVGQILEL	CDRQYIDRT.AIDHV	PHGTTIATNA	ILEYDGAKTG	HyuA, B		NMIKVGCV*	MSKIHTDL	KKIDPITVQV	VLGSLENVAV	EMGHKLARMS
YKL215C	101	FITTKGFKDS	LLIGDQTRPD	IFNLNIKKVV	PLYDTVVEID	ERVTFLEDFSE	YKL215C	801	VSTNVKERLD	FSCALFDSKG	NLVANAPH.V	PVHLGSMSTC	ISQAQKLREG
5-OPase		LLVTRGRFRL	LHIGTQARPD	LFDLALPMPE	VLVEEVLEVD	ERVVL..YRG	5-OPase		ISTNIKERLD	FSCALFGPDG	GLVSNAPH.I	PVHLGAMQET	VQFQIQHLGA
HyuA		MITTEGYRDI	IHIGRHQRQP	NYSI...MQE	IFWQDRPLVQ	RRHRL.....	HyuB		YSSIRESSED	FGCALVDVVRG	QQLCESSHST	PLQSGPIPGY	IKGIREIMED
YKL215C	151	DPYFTKSSPN	EQEGILEGNS	GEMVRVKKP	DESSVRSLK	VLYASGIKSI	YKL215C	851	K...LKPQDV	LITNHPDIGG	THLPDITVIT	PSFSST.GEL	IFYVASRAHH
5-OPase		EP.....	GAGSPVKGR	GDLEIQQPV	DLEALRGKLE	GLLSRGIHSL	5-OPase		D...LHPGDV	LLSNHPSAGG	SHLPDLTVIT	PVFVPGQTRP	VFYVASRGHH
HyuA	AIA	ERMGPVKG..QVITPV	QEDQVRGAVA	TLKERGVDSI	HyuB		RNDTFNQGDV	IMHNSPYHGA	SHGPDVGFCI	PVFY..KDEL	IGFSVTTAHH
YKL215C	201	AIAPFLHSYTF	PDHERIV.GN	IAREIGFSHV	SLSSEVSPMI	KFLPRAHSSV	YKL215C	901	ADTGGILPGS	VPPNSKELYE	EGTAIYSELV	VKEGIFQEEL	IYKLVFEDPG
5-OPase		AVVLMHSYTW	AQHEQGV.GT	LARELGFTHV	SLSSEVMPMV	RIVRGRHTAC	5-OPase		ADTGGITPGS	MPPHSTTLQQ	EGAVFLSPKL	VQGGVVFQEEA	VTEAL.RAPG
HyuA		IVNLFYSYTN	PEHEQRVKEI	IEEYPEAFV	TISSEVSPQF	REFERFTTAS	HyuB		LDIGSSTPGS	CGIVDAVDAY	AEGLQFKAIK	VYDQGVKNRY	VWDILKDN..
YKL215C	251	ADAYLTPVIK	KYLNISISAGL	.SHAEDTHIQ	FMQSDGGLVD	GGKFSG..LK	YKL215C	951	KYPGCSGSR	FSDNISDLKA	QVAANTKGIQ	LIGSLTKEYD	LATILKYMMA
5-OPase		ADAYLTPTIQ	RYVQGFRRGF	QGQLKNVQVL	FMRSDGSLAP	MDAFSG..SR	5-OPase		KISGCSGTRN	LHDNLSDLRA	QVAANQKGIQ	LVLGELIQVYG	LDVVQAYMGH
HyuA		INGFVGFKVK	NYIQNLQESL	KDSGISAEIH	IMCSNGGVAT	PKTVSEKPVN	HyuB	IRA	PKLVVGDMEA	QIAAARIGAQ	RYIEIIEKYG	LDTVQAASEE
YKL215C	301	SILSGPAGGV	IGYSSTCYDK	NNNIPLIGFD	MGSTSTDVSR	YGDGRLEH..	YKL215C	1001	IQTNASESIK	KMLAKMVEHF	GTTK.....	.FSGEDRLD	DGSLIKLQVI
5-OPase		AVLSGPAGGV	VGYSATTYHL	EGGQPVIGFD	MGSTSTDVSR	YA..GEFEH..	5-OPase		IQANAEALVR	DML...RAF	GTSRQARGLP	LEVSADHDMD	DGSPICLRVQ
HyuA		TLLSGPAAGI	LG..GAWAGEL	TNRQKLITFD	VGSTSDADIGI	ITDSGYGESS	HyuB		LMNYSEKMMR	DAIKKLDPGE	YTAEGFLDGY	LDSDPAKKDLRINVT
YKL215C	351	VFEVTVAGII	IQSPQLDIHT	VAAGGSILS	W.KNGLFRVG	PDSAADDPG	YKL215C	1051	IRPEKEEYIF	NFDGTSPQV.YG	NLNAPAEITN	SAILYCLRCL
5-OPase		VFEASTAGVT	LQAPQLDINT	VAAGGSRLF	F.RSGLVFVG	PESAGAHPGP	5-OPase		INLSQGSASF	DFTGSGSEV.FG	NLNAPRAITL	SALIYCLRCL
HyuA		ARDTWIAGYP	VMVPMIDIHT	IGAGGGSIAH	IDEGGAFKVG	PRSGSRPGP	HyuB		VKVDGSDLTV	DLTGTSPPQVT	DKPINMPLIG	TVDAIYLTIL	RSIL..LDST
YKL215C	401	AAYRKGG.PL	TITDANFLG	RLVPEFFPKI	FGPNEDESLD	LETTTLKFRE	YKL215C	1101	VGEDIPLNQ	CLKPLTIKIP	AGSLLSPRSG	AAVVGGNVLT	SQRVTDVILK
5-OPase		ACYRKGG.PV	TITDANFLVG	RLLPASFPCI	FGPGEDQPLS	PEASRKALEA	5-OPase		VGRDIPLNQ	CLAPVRVLIIP	KGSILDSPE	AAVVGGNVLT	SQRVVDVILG
HyuA		ACYGHGGLKP	TVDANVVLG	RIDEHNF...	..LGSEMKIY	TNAAYKVIDE	HyuB		VYGNFPQNSG	LIRPIKIVAP	KGTLCNPIFP	APTI..ARFNS	GNAVADTIMK
YKL215C	451	LTDVINKDL.NSNLT	MEEVAYGFIK	VANECMARPV	RAITEAKGHV	YKL215C	1151	TFNVMAVSQ	DCNNTFTGTG	GNSGNKTDKQ	IKGFGYYETI	CGSGAGADS
5-OPase		VAMEVNSFLT	NGPCPASQLS	LEEAMGFVR	VANECMARCP	RALTQARGHD	5-OPase		AFGACASQ	CMNNVTLGNARMGYETV	AGSAGAGP..
HyuA		LAGQL.....DLS	RERTABGVLO	IMNNMNAI	REKTIQKGBD	HyuB		ALAQVVPVHQ	SAGVGNLQVV	AFSGQSNEY	VWYMDIMEGS	YGR.....
YKL215C	501	VSQHRLVSFG	GAGGQHAIAV	ADSLGIDTVL	IHRYSILSA	YGIPLADVIE	YKL215C	1201	WRSGGWNQSD	AVHTNMTNTR	MTDTEVFERR	YPVLLKEFSI	RRSGGKGKY
5-OPase		PSAHVLACFG	GAGGQHACAI	ARALGMDTVH	IHRHSGLLSA	LGLALADVIE	5-OPase		...GWHGRS	GVHSHMTNTR	ITDPEILLES	YPVILRRFEL	RPGSGGRGRF
HyuA		PREFSLVAFG	GAGPLHAEV	AQILNIPEVI	IPLYPGINSA	TGLLTITDLKY	HyuB		...YKDGMD	AVDTLYANTR	NNPIEDIESH	YPLRVNRYEL	RDNSDAPGW
YKL215C	551	ENQEPSCFIL	GEPETILKVK	KRFLELSKNS	IKNLSQSFS	REDIVLERYL	YKL215C	1251	TGGNGVVRDV	QFRKAVTASI	LSERVVIGPH	GKGGQDGSR	GENLWVRHST
5-OPase		EAQEPCLSY	T.PETFAQLD	QRLSRLBQC	VDALQVQGF	RSQISTESFL	5-OPase		RGGQGVVREL	VFREALLSV	LTERRAFQPY	GLHGGEFGAR	GLNLLIR.KD
HyuA		DVIKT.EFMM	STNMDPSGLN	EDLAGLETQL	INQLKEDGVS	QKDIRILRSA	HyuB		RGIGSIREV	SFLADGSFSV	EADGHKYPW	GFDDQDQGVV	GSLSIRDNET
YKL215C	601	NLRYEGTETS	LMILQKYDDQ	WNFREW....	...FSEAHKK	EFGFSFDDRR	YKL215C	1301	GALINVGGK.	NTIYAQPGDR	FIKTPGGGG	FGQYKD....
5-OPase		HLRYQGTDC	LMVSAHQHPA	TACSPRAGDF	GAAVERYMR	EFGFIIPERP	5-OPase		GRTVNLGGK.	TSVTVPYGDV	FCLHTPGGGG	YGDPEDPAPP	PGSPPLFPAP
HyuA		DCRYAGQGYE	LRV..DLPDV	FLDEETIVDA	LNNFHESHKA	EYGHNTDPS	HyuB		NELVQLPSKL	PNRHAQSGST	IQLVGFPCGG	YGNPLEREPE	KVLSDYLDGF
YKL215C	651	IIIDDIRIRA	IG..KSGVRKE	KTVDEQLIEI	SHEFKADVSK	DASFTQKAYF	YKL215C	1351
5-OPase		VVDVVRVRG	TG..RSLQLLE	DTPKIQ.TGP	PHVEKVT...QCYF	5-OPase		PERGSVFYR	RAQEAV....
HyuA		IEFVNIRVTG	TGYMPKIEKQ	AIHHEYQLED	ALLKTGDATF	N.....I	HyuB		ITKEKALVEY	GVTTITDSEEI	DYEKTNELRK	V	

FIG. 6. Comparison of the amino acid sequence of rat kidney 5-oxoprolinase with that of the yeast sequence YKL215C and the bacterial hydantoinases HyuA and HyuB. Vertical double dotted lines indicate identities; vertical single dotted lines indicate replacements by closely related amino acids. Horizontal dotted lines indicate deletions. The sequence of each protein is residue 1. Comparisons were performed by using the Pileup program made available by the University of Chicago, Kovler Viral Oncology Laboratories.

subunits are identical.

Most of the cDNA sequence of 5-OPase presented here represents the overlapping cDNA clones that were obtained from successive screening of a λ gt11 rat kidney cDNA library. However, further screening of the same library with probes directed to the 5'-terminal region failed to produce positive results. Accordingly, to determine the 5'-terminal sequence of the enzyme, we turned to the rat kidney Marathon-Ready cDNAs. Marathon-Ready cDNAs are premade libraries of adapter-ligated double strand cDNA ready for use as templates in Marathon cDNA amplification, a unified method for performing

rapid amplification of both 5'- and 3'-cDNA ends (RACE) from the same template (18) with the LD-PCR method (22). In the present studies, we completed the cDNA of rat kidney 5-OPase by amplifying its 5' end from a library of rat kidney Marathon-Ready cDNAs. Three primers, which were complementary to the known sequences at various regions of the cDNA, were designed and used as gene-specific primers to perform the 5' RACE. They each gave the product of the predicted size (Fig. 3A). Sequencing of CGSP2 and CGSP3 revealed that they were completely overlapped except for a several base extension at the 5' end of CGSP3. Sequencing of the 5' end of another 5'

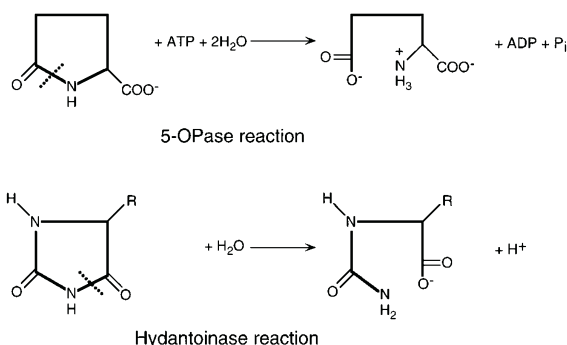


FIG. 7. Comparison of the reactions catalyzed by 5-oxoprolinase and the hydantoinases HyuA and HyuB.

RACE product (Fig. 3B, lane 3) gave the same results as CGSP3.

The full-length (beginning just before the start codon; Fig. 3B, lane 1) and the 3' end cDNA of 5-OPase (Fig. 3B, lane 2) have also been amplified from Marathon-Ready cDNAs and their sequences confirmed by restriction mapping and DNA sequencing, giving the same results as obtained from the conventional screening of the λ gt11 cDNA library. This indicates that the library of rat kidney Marathon-Ready cDNAs contains the full-length cDNA of 5-OPase and that the cDNA clones from conventional screening of the λ gt11 cDNA library are consistent with those obtained from Marathon-Ready cDNAs by LD-PCR amplification. Thus, the cDNA clone of rat kidney 5-OPase obtained in this study has been checked by two different methods.

Western blot and standard SDS-PAGE analysis of the proteins encoded by the recombinant 5-OPase clones demonstrated that the full-length recombinant 5-OPase expressed in *E. coli* migrates at a position identical to that of endogenous rat kidney 5-OPase (Fig. 5), further demonstrating that the isolated cDNA corresponds to that encoding the rat kidney enzyme. Similar analysis of proteins encoded by the truncated recombinant cDNA clones revealed that recombinant proteins with intact C-terminal regions are specifically detected by the antiserum against the isolated rat kidney 5-OPase, whereas the C-terminal truncated recombinant proteins were not detected by the antiserum. The results indicate that the C-terminal 243 residues of 5-OPase contain the reactive denaturation-resistant epitopes for the anti-5-OPase serum and that other regions of the enzyme either do not contain such epitopes or that their epitopes are unreactive in the absence of the C-terminal region.

It is perhaps not surprising that expression of the rat kidney 5-OPase cDNA in *E. coli* did not result in a detectable increase in 5-OPase activity. The rat kidney enzyme is a very large homodimer in which the two subunits interact tightly without any disulfide bonds and might well not fold to give an enzymatically active protein in *E. coli*, at least under the conditions used here. Obtaining functional recombinant 5-OPase is clearly a prerequisite for mechanistic and further structural studies of this enzyme.

The unexpected result from this study is the demonstration of strong sequence similarities among rat kidney 5-OPase, the two bacterial hydantoinases, and the yeast protein YKL215C (Fig. 6). YKL215C is a hypothetical protein of 1287 residues encoded by the URA1-RSD1 intergenic region (23). The similarity between this sequence and that of the rat kidney enzyme is sufficiently strong (48.4% identity) to suggest that the yeast protein is also a 5-oxoprolinase. To understand the similarity to the bacterial hydantoinases, a comparison of the 5-oxoprolinase reaction and that catalyzed by the hydantoinases indi-

cates the relationship between the chemical reactions involved (Fig. 7). Both reactions involve the hydrolysis of 5-membered rings via hydrolysis of their internal $-\text{CO}-\text{NH}-$ bonds. HyuA is specific for D-5-substituted hydantoin, while HyuB is specific for the corresponding L isomers (24, 25). The amino-terminal half of rat kidney 5-oxoprolinase shows 26.6% identity with the amino-terminal region of HyuA, while the carboxyl-terminal half of the rat kidney enzyme shows 25.9% identity with the amino-terminal region of HyuB. These somewhat surprising relationships partially reflect the fact that HyuA and HyuB show no significant sequence similarities to each other (24, 25). Thus it is possible that the rat kidney enzyme evolved from an evolutionary fusion of these functionally similar but structurally different enzymes, or that the hydantoinases arose from an enzyme related to rat kidney 5-oxoprolinase. However, it is relevant that neither HyuA nor HyuB require ATP for ring hydrolysis, in contrast not only to 5-OPase, but also to a different bacterial hydantoinase that cleaves 5-substituted hydantoin (26) as well as to one that cleaves *N*-methylhydantoin (27). The difference among the bacterial hydantoinases in their requirement for ATP is surprising, and it will clearly be of interest to learn the structural relationship of rat kidney 5-OPase to the ATP-requiring hydantoinases.

The above raises the question of bacterial 5-oxoprolinase. 5-OPase from bacteria (*P. putida*) (4, 7), unlike that from rat kidney, is composed of two functionally different protein components, Component A and Component B. Neither component alone catalyzes the 5-OPase reaction, but the reaction is effectively catalyzed when the components are mixed. Component A is composed of two subunits, $M_r = 65,500$ and $50,000$, and exhibits 5-oxo-L-proline-dependent ATPase activity, indicating this component can interact with both ATP and 5-oxo-L-proline. The role of Component B ($M_r = 80,000$) is uncertain, since it does not exhibit ATPase activity, and there is no evidence that it binds 5-oxo-L-proline. Of relevance here is the fact that the antiserum directed against the rat kidney enzyme strongly reacts with an *E. coli* component of ~ 60 kDa (Fig. 5B), suggesting that this component might be a subunit of *E. coli* 5-OPase Component A and raising the question of homology between the rat kidney and bacterial 5-oxoprolinases and the relationship of the bacterial 5-oxoprolinases to the bacterial hydantoinases. The sequence of bacterial 5-oxoprolinase will be an important clue in this evolutionary puzzle.

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