Degradation of purines: only ureidoglycollate lyase out of four allantoin-degrading enzymes is present in mammals

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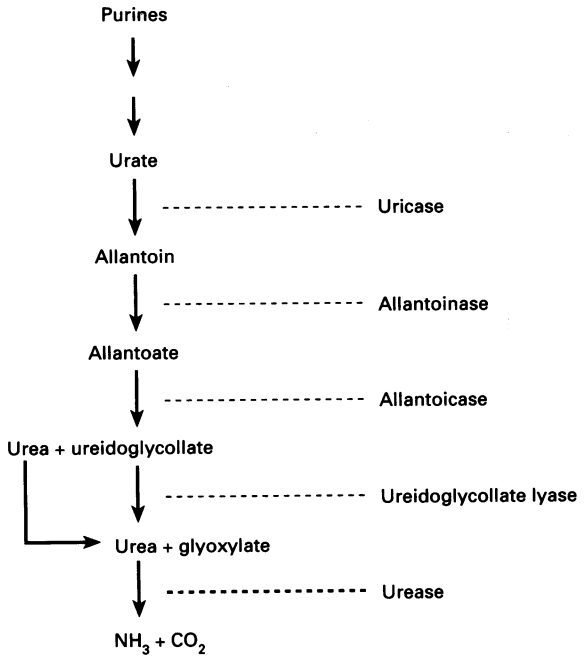
It is generally accepted that all of the allantoin-degrading enzymes (allantoinase, allantoicase, ureidoglycollate lyase and urease), used in purine degradation, were lost during mammalian evolution. However, surprisingly, ureidoglycollate lyase has been found in a mammalian tissue. Ureidoglycollate lyase was purified to homogeneity and characterized from rat-liver mitochondria. The apparent K_m (17 mM) of the rat enzyme for ureidoglycollate was much higher than that (0.33 mM) of fish-liver ureidoglycollate lyase. The rat-liver enzyme differed from the fish-liver enzyme in enzymic, physical and immunological properties.

INTRODUCTION

In lower animals such as marine invertebrates and crustaceans, urate is degraded to NH_3 and CO_2 as follows [1–5] (Scheme 1). Urate is degraded to allantoin by uricase (EC 1.7.3.3); allantoin is then degraded by allantoinase (EC 3.5.2.5) to allantoate, which is degraded to ureidoglycollate and urea by allantoicase (EC 3.5.3.4). Ureidoglycollate is further degraded to glyoxylate and another molecule of urea by ureidoglycollate lyase (EC 4.3.2.3). Urea formed is degraded to NH_3 and CO_2 by urease (EC 3.5.1.5). However, the degradation of urate is much less complete in higher animals [1–3,5]. It is generally accepted that during animal evolution, all of the urate-degrading enzymes (uricase, allantoinase, allantoicase, ureidoglycollate lyase and urease) were lost in humans, anthropoid apes and New World monkeys, and all of the allantoin-degrading enzymes (allantoinase, allantoicase, ureidoglycollate lyase and urease) were lost in other mammals [1,4–8].

However, in the present study, surprisingly, one of the four allantoin-degrading enzymes, ureidoglycollate lyase, has been found in a mammalian tissue. Ureidoglycollate lyase was purified to homogeneity and characterized from rat-liver mitochondria.



Scheme 1 Route of purine degradation in animals

EXPERIMENTAL

Animals

Male Sprague–Dawley rats (120–130 g) kept on a normal laboratory diet were used.

Enzyme assays

The assay of ureidoglycollate lyase was modified from that previously described [9]. The reaction mixture contained 50 mM sodium ureidoglycollate, 100 mM triethanolamine/HCl, pH 7.8, and the enzyme solution in a total volume of 50 μl. The reaction was initiated by the addition of the enzyme. After incubation at 37 °C for 10 min, the reaction mixture was buffered by the addition of 0.9 ml of 100 mM triethanolamine/HCl, pH 7.8, and the glyoxylate formed was determined by using lactate dehydrogenase (EC 1.1.1.27) and NADH. Catalase (EC 1.11.1.6) [10], allantoinase [6], allantoicase [6], urease [11], glutamate dehydrogenase (EC 1.4.1.3) [12] and acid phosphatase (EC 3.1.3.2) [13] were assayed as described in the cited references. A unit of enzyme activity is defined as the amount of enzyme that catalyses a formation of product or a decrease in substrate of 1 μmol/min at 37 °C.

Sucrose-density-gradient centrifugation of liver homogenates with a vertical rotor

All procedures were performed at 0–4 °C. Livers were cut into small pieces with scissors and homogenized by one treatment in 9 vol. of 0.25 M sucrose in 20 mM glycylglycine, pH 7.4, in a Potter–Elvehjem homogenizer with a Teflon pestle at 600 rev./min. The homogenate was filtered through two layers of cheesecloth and centrifuged at 300 *g* for 10 min to sediment nuclei and any whole cells. A portion (3 ml) of the resulting post-

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nuclear fraction was layered on a 32 ml sucrose gradient (24–54%, w/v) with 2.0 ml of 57% (w/v) sucrose solution as a cushion in the bottom, then centrifuged at 132000 *g* for 55 min in a Hitachi (Tokyo, Japan) 55P-72 centrifuge with a vertical rotor (RPV 50T-160). Fractions (2.2 ml) were collected from the bottom of the gradient.

Preparation of the antibody against rat-liver mitochondrial ureidoglycollate lyase

The mitochondrial ureidoglycollate lyase was purified to homogeneity from rat-liver mitochondria as described later. The antibody against this enzyme was prepared by subcutaneous injection of 0.15 mg of the purified enzyme in complete Freund's adjuvant into a rabbit (Nippon white rabbit strain, 1.5 kg). Two weeks later, the rabbit was again immunized with 0.1 mg of the enzyme as above. The serum was collected 1 month later and stored at −80 °C.

Purification of ureidoglycollate lyase from rat-liver mitochondria

All procedures were carried out at 0–4 °C. The heavy mitochondrial fraction was prepared from livers (44 g) of 10 rats by the method of de Duve et al. [14] as follows. The homogenate was prepared as described above and centrifuged at 500 *g* for 5 min. The resulting supernatant was then centrifuged at 3300 *g* for 10 min. The resulting pellet (the heavy mitochondrial fraction) was homogenized with 50 ml of glycylglycine, pH 7.4. After sonication for 2 min at 10 Hz, the homogenate was centrifuged at 100000 *g* for 60 min. The resulting supernatant (heavy mitochondrial extract) was applied to a column (4 cm × 20 cm) of DEAE-cellulose, equilibrated with 10 mM Tris/HCl, pH 7.8, containing 5 mM mercaptoethanol and 10% (v/v) glycerol. After washing with 400 ml of the same buffer, the enzyme was eluted with a 1000 ml linear gradient of 0–50 mM KCl in the same buffer. The active fractions (fractions 38–54) were pooled and concentrated to about 10 ml by ultrafiltration. The concentrated enzyme solution was applied to a column (3 cm × 20 cm) of hydroxyapatite, equilibrated with 5 mM potassium phosphate buffer, pH 7.5, containing 5 mM 2-mercaptoethanol and 10% glycerol. After washing the column with 250 ml of the same buffer, the enzyme was eluted with a 600 ml linear gradient of 5–50 mM potassium phosphate buffer, pH 7.5, containing 5 mM 2-mercaptoethanol and 10% glycerol. The active fractions (fractions 22–32) were pooled and concentrated by ultrafiltration. The concentrated enzyme solution was applied to a column (2.5 cm × 90 cm) of Sephacryl S-200, equilibrated with 10 mM triethanolamine/HCl, pH 7.8, containing 5 mM 2-mercaptoethanol, 10% glycerol and 200 mM NaCl. The column was eluted with the same buffer at a flow rate of 20 ml/h. Active fractions (fractions 87–96) were pooled and concentrated to about 5 ml by ultrafiltration. The enzyme solution was applied to a column (2 cm × 20 cm) of DEAE-cellulose, equilibrated with 10 mM Tris/HCl, pH 7.8, containing 5 mM mercaptoethanol and 10% glycerol. After washing with 150 ml of the same buffer, the enzyme was eluted with a 400 ml linear gradient of 5–50 mM KCl in the same buffer. Active fractions (fractions 22–31) were pooled and concentrated by ultrafiltration.

Immunoblotting

For Western blotting [15], slab SDS/polyacrylamide-gel electrophoresis of ureidoglycollate lyase was performed on 12% (w/v) gels by the method of Laemmli [16]. Proteins were electrophoretically transferred to a nitrocellulose membrane and then probed with rabbit antiserum against rat-liver mitochondrial ureidoglycollate lyase. The primary antibody was detected by allowing it to react with goat anti-rabbit IgG–peroxidase conjugate, and was revealed by incubating the nitrocellulose membrane in the presence of the peroxidase substrate.

Other methods

Urea was measured as previously described [17]. Gel filtration and sucrose density gradient centrifugation for the estimation of approximate molecular mass, isoelectric focusing and protein determinations were carried out as described [18].

RESULTS AND DISCUSSION

Rat liver was homogenized with 9 vol. of 0.25 M sucrose in 20 mM glycylglycine, pH 7.4, and the activities of allantoin-degrading enzymes in the homogenate were determined. Only ureidoglycollate lyase activity was detected and the activities of other enzymes (allantoinase, allantoicase and urease) were not detected. Therefore the purification of ureidoglycollate lyase was attempted, to establish the presence of the enzyme in rat liver. The purification procedure of the mitochondrial ureidoglycollate lyase is summarized in Table 1. A 240-fold purification was achieved with a recovery of about 2%.

Figure 1 shows time courses of the formation of reaction products from ureidoglycollate by the purified enzyme preparation. The formation of urea and glyoxylate was linear during a 10 min incubation of the reaction mixture and the concentration of the two products was nearly identical. The results show that ureidoglycollate lyase catalyses the degradation of ureidoglycollate to urea and glyoxylate.

The purified enzyme preparation was stored at −20 °C for at least 3 months without loss of activity. On polyacrylamide slab gel electrophoresis in the absence of SDS, the enzyme preparation migrated to the anode as a single protein band (Figure 2). On polyacrylamide slab gel electrophoresis in the presence of SDS, the enzyme preparation migrated as a single protein band with a molecular mass of about 33 kDa (Figure 2). The molecular mass of the enzyme was estimated to be about 64 kDa by sucrose density gradient centrifugation, suggesting that the enzyme consisted of two identical subunits. The enzyme had a pI of 6.0 and pH optima between 7.8 and 8.2; 125 mM triethanolamine/HCl buffer was used between pH 7.0 and 9.0. The apparent K_m for ureidoglycollate was 17.0 mM.

A representative sedimentation profile in a sucrose density gradient for the post-nuclear homogenate from rat liver is shown in Figure 3. The peroxisomes and mitochondria were separated; the peroxisomes, marked by catalase, were at a density of about 1.25 g/ml, and the mitochondria, marked by glutamate dehydrogenase, at about 1.18 g/ml. Ureidoglycollate lyase activity was recovered both in the mitochondrial fraction and in the soluble top fraction, but not in the peroxisomal fraction. The post-nuclear homogenate was prepared from rat liver pretreated with Triton WR-1339 to ensure separation of lysosomes from mitochondria and peroxisomes [19] and subjected to sucrose density gradient centrifugation, since considerable overlap occurs between the density distributions of the three types of organelle in normal liver. Ureidoglycollate lyase activity showed different distribution profiles from acid phophatase activity as the lysosomal marker (results not shown), showing that ureidoglycollate lyase is not present in the lysosomes. In contrast, the activity ratio of the mitochondrial fraction to the soluble top

Table 1 Purification of mitochondrial ureidoglycollate lyase from rat liver

Details of purification and assay methods are given in the text.

	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Heavy mitochondrial extract	693	554	0.80	1	100
DEAE-cellulose	81.5	265	3.25	4	47.8
Hydroxyapatite	3.2	166	51.4	64	30.0
Sephacryl S-200	0.7	51	74.3	93	9.3
DEAE-cellulose	0.06	11	191	239	2.0

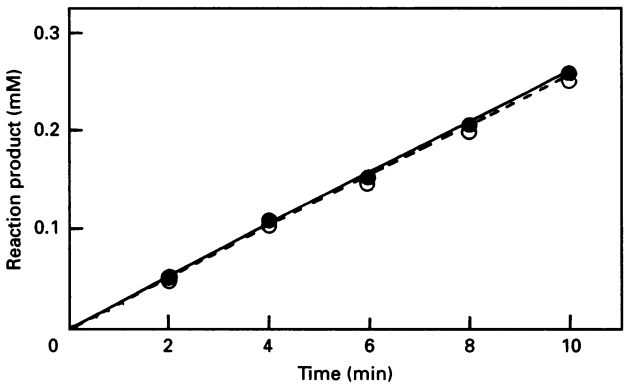

Figure 1 Time cources of the formation of glyoxylate and urea from ureidoglycollate by the purified ureidoglycollate lyase

The reaction mixture contained 50 mM ureidoglycollate, 100 mM triethanolamine/HCl, pH 7.8, and the purified enzyme preparation in a total volume of 25 μl. After incubation, the reaction mixture was buffered by the addition of 0.9 ml of 100 mM triethanolamine/HCl, pH 7.8. Glyoxylate (○) and urea (●) formed were determined as described in the text.

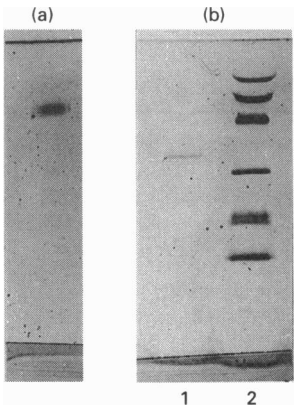

Figure 2 Polyacrylamide slab gel electrophoresis of the purified ureidoglycollate lyase

Electrophoresis was carried out as described in the text. (**a**) The purified enzyme preparation was subjected to electrophoresis in the absence of SDS. (**b**) The purified enzyme preparation was subjected to electrophoresis in the presence of SDS: 1, the purified enzyme preparation; 2, molecular mass markers [phosphorylase (97400 Da), BSA (66200 Da), ovalbumin (45000 Da), carbonic anhydrase (31000 Da), soybean trypsin inhibitor (21500 Da), and lysozyme (14400 Da)].

fraction of ureidoglycollate lyase was much higher than that of glutamate dehydrogenase as the mitochondrial marker, showing that ureidoglycollate lyase activity in the soluble fraction is not from broken mitochondria. These results show that ureidoglycollate lyase is present both in the mitochondria and in the cytosol.

Results of immunoblotting of mitochondrial extracts from bovine, chicken, frog and fish livers with the use of rabbit antiserum against the purified rat-liver mitochondrial ureidoglycollate lyase are shown in Figure 4. Only one protein band was detected, corresponding to a molecular mass of about 33 kDa, from the mitochondrial extracts from rat and bovine livers. However, the antiserum against rat-liver mitochondrial ureidoglycollate lyase did not cross-react with the mitochondrial enzyme from chicken, frog and fish livers.

The distribution of ureidoglycollate lyase activities in various tissues of rat is shown in Table 2. High-level activity was detected in adrenal gland, kidney, liver, brain, submandibular gland and pancreas, Moderate-level activity in heart and lung, and Low-level activity in intestine, spleen and skeletal muscle.

The end products of purine degradation vary from species to species [20]. The degradation of purines to urate is common to all animal species, whereas the degradation of urate is much less complete in higher animals. It is generally accepted that all of the allantoin-degrading enzymes (allantoinase, allantoicase, ureidoglycollate lyase and urease) were lost during mammalian evolution. However, surprisingly, only ureidoglycollate lyase has now been found in mammalian tissues.

In contrast, ureidoglycollate lyase is present in fish and amphibian livers [4]. In those species, the enzyme contributes to the degradation of ureidoglycollate *in vivo*, because the end product of purine degradation is urea [1,2,5]. However, in mammals the enzyme probably does not contribute to purine degradation *in vivo*, because the end product is urate or allantoin [1,2,5]. Rat-liver ureidoglycollate lyase differed from the fish (sardine)-liver enzyme [4] in enzymic, physical and immunological properties. The molecular mass (2×33 kDa) of the rat-liver enzyme differed from that (2×64 kDa) of the sardine-liver enzyme. The apparent K_m (17 mM) of the rat-liver enzyme for ureidoglycollate was much higher than that (0.33 mM) of the sardine-liver enzyme. These data suggest that mammals have lost the function *in vivo* by elevating the K_m for ureidoglycollate during evolution. In sardine liver, ureidoglycollate lyase is located in the peroxisomes with other urate-degrading enzymes (uricase, allantoinase and allantoicase) [4,6]. In contrast, rat-liver ureidoglycollate lyase is located in the mitochondria and is not immunologically cross-reactive with the sardine-liver enzyme. Why is ureidoglycollate lyase the only allantoin-degrading enzyme to have been conserved in rat liver? Why is ureidoglycollate lyase present in various tissues of rats? Both of these interesting questions are so far unanswered. However, ureidoglycollate lyase might play a role in metabolism other than the degradation of purines in mammals. As an example, it might play a role in

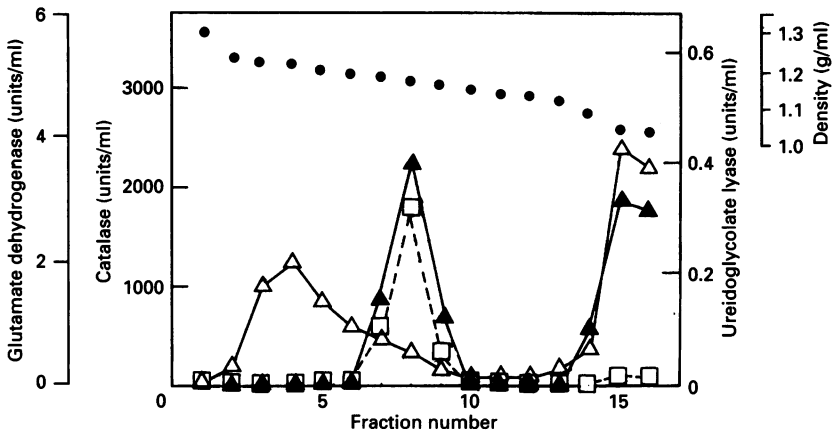

Figure 3 Subcellular distribution of ureidoglycollate lyase in rat liver

The post-nuclear supernatant was prepared from rat liver and subjected to sucrose density gradient centrifugation as described in the text. Fractions of 2.2 ml were collected from the bottom of the tube. □, Glutamate dehydrogenase; △, catalase; ▲, ureidoglycollate lyase.

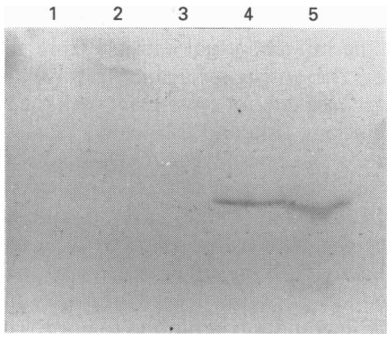

Figure 4 Immunoblotting with the antiserum raised against rat-liver mitochondrial ureidoglycollate lyase

Ureidoglycollate lyase was partly purified from rat, bovine, chicken, frog and mackerel liver as follows. Heavy mitochondrial fraction was prepared from 3 g liver and homogenized with 5 ml of 5 mM glycylglycine, pH 7.5, as described in the text. After sonication for 2 min at 10 kHz, the homogenate was centrifuged at 100000 *g* for 60 min. The resulting supernatant was applied to a column (2.5 cm × 90 cm) of Sephacryl S-200, equilibrated with 10 mM triethanolamine/HCl, pH 7.8, containing 5 mM 2-mercaptoethanol, 10% glycerol and 200 mM NaCl. Active fractions were pooled, concentrated by ultrafiltration and subjected to slab SDS/polyacrylamide gel electrophoresis as described in the text. Lanes 1–5 contain partly purified ureidoglycollate lyase from mackerel (300 munits, lane 1), frog (350 munits, lane 2), chicken (500 munits, lane 3), bovine (200 munits, lane 4) and rat (40 munits, lane 5) liver.

Table 2 Distribution of ureidoglycollate lyase in rat tissues

Each tissue from rats (body weight about 200 g) was minced with scissors and homogenized in 9 vol. of 0.25 M sucrose in 5 mM potassium phosphate buffer, pH 7.5. Each homogenate was sonicated at 10 kHz for 30 s, and used for the enzyme assay. Each value represents the mean ± S.D. for four rats.

Tissue	Ureidoglycollate lyase (munits/mg of protein)
Liver	352 ± 47
Kidney	437 ± 79
Spleen	35 ± 3
Intestine	76 ± 8
Submandibular gland	248 ± 51
Adrenal gland	608 ± 92
Lung	118 ± 17
Heart muscle	108 ± 6
Skeletal muscle	31 ± 23
Pancreas	262 ± 54
Brain	308 ± 47

creatine synthesis, as the structure of ureidoglycollate is similar to that of guanidinoacetate.

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