Biochemical and Biophysical Research Communications **286,** 222–226 (2001)
doi:10.1006/bbrc.2001.5356, available online at http://www.idealibrary.com on IDEAL®

Novel Amidohydrolytic Reactions in Oxidative Pyrimidine Metabolism: Analysis of the Barbiturase Reaction and Discovery of a Novel Enzyme, Ureidomalonase

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Received June 29, 2001

Amidohydrolytic reactions in oxidative pyrimidine metabolism were investigated in detail. Barbiturase has been reported to catalyze the amidohydrolysis of barbituric acid to urea and malonic acid. However, purification of the enzyme revealed that it catalyzes the ring-opening of barbituric acid to ureidomalonic acid. The existence of a consecutive enzyme named ureidomalonase, which hydrolyzes ureidomalonic acid to urea and malonic acid, was also discovered during the purification of barbiturase.

Key Words: **barbiturase; ureidomalonase; amidohydrolysis; barbituric acid; ureidomalonic acid; oxidative pyrimidine metabolism.**

Pyrimidine is metabolized through a reductive or/and oxidative pathway (1) (Fig. 1). It is commonly known that mammals, plants and microorganisms utilize the reductive pathway for pyrimidine degradation (1–3), while some microorganisms use the oxidative pathway (4–6). In reductive pyrimidine metabolism, uracil or thymine is first reduced to a dihydro-derivative, which in turn is hydrolyzed to an *N*-carbamoyl-β-amino acid and finally decarbamoylated to β-amino acid (Fig. 1). This metabolic route, especially the hydrolysis of dihydro-derivatives catalyzed by dihydropyrimidinase, has been studied extensively because of the biotechnological attraction for the industrial production of optically active amino acids (3, 7, 8).

The oxidative pathway of pyrimidine degradation was first reported in 1952, almost concurrently by three groups of scientists, in bacteria, *Mycobacterium*, *Corynebacterium*, and *Norcardia*, isolated from soil (4–6, 9). In this pathway, uracil or thymine is first oxidized to barbituric acid or 5-methylbarbituric acid, respectively. It was proposed that barbiturase (EC 3.5.2.1) catalyzes the amidohydrolysis of barbituric acid to urea and malonic acid (Fig. 1). However, as these authors pointed out, the precise way in which barbituric acid is converted to urea and malonic acid has not been established (4). Since then, no follow-up article has appeared on the oxidative pathway.

We initiated a study on the enzymes involved in pyrimidine oxidative degradation in a bacterium, *Rhodococcus erythropolis* JCM 3132, which was found as an active pyrimidine-degrading strain. Here, we report new findings for the enzymatic reaction catalyzed by barbiturase and the occurrence of a novel enzyme, ureidomalonase, functioning in a successive reaction in oxidative pyrimidine metabolism.

MATERIALS AND METHODS

Microorganism and cultivation medium. *Rhodococcus erythropolis* JCM 3132 was used for all experiments. The minimum medium comprised 1 g KH_2PO_4, 1 g K_2HPO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 2 g NH_4Cl, 1 mg thiamine hydrochloride, 2 mg riboflavin, 2 mg nicotinic acid, 2 mg pantothenic acid, 2 mg pyridoxine hydrochloride, 0.1 mg biotin, 1 mg *p*-aminobenzoate, 0.1 mg folic acid, 1 mg $FeSO_4 \cdot 7H_2O$, 1 mg $Na_2MoO_4 \cdot 2H_2O$, 1 mg $MnSO_4$, 1 mg $CuSO_4$, and 1 mg $ZnCl_2$ in 1 liter of deionized water, pH 7.0. The uracil-rich medium comprised 2 g uracil, 0.2 g yeast extract, 0.2 g tryptone, 1 g KH_2PO_4, 1 g K_2HPO_4, 0.5 g sea salts (Sigma, U.S.A.), 1 mg $FeSO_4 \cdot 7H_2O$, 1 mg $Na_2MoO_4 \cdot 2H_2O$, 1 mg $MnSO_4$, 1 mg $CuSO_4$, and 1 mg $ZnCl_2$ in 1 liter of tap water, pH 7.0.

Evaluation of pyrimidine metabolism. *R. erythropolis* JCM 3132 was cultivated in 5 ml minimum medium supplemented with 0.15% (w/v) uracil at 28°C for 3 days with shaking. Cells were harvested by centrifugation (10,000*g*, 10 min), washed twice with 0.85% (w/v) of NaCl, and then suspended in 0.5 ml of 20 mM potassium phosphate buffer (pH 8.0). The cell suspension was ultrasonicated at 4°C for 10 min, and then centrifuged at 14,000*g* for 30 min. The resultant supernatant was used for analysis of the enzymatic activities of oxidative and reductive pyrimidine metabolism as described below.

To investigate the induction of barbiturase production, the enzyme activities of cells grown in the minimum medium supplemented with 1% (v/v) glycerol and 0.15% (w/v) each pyrimidine-related compound were examined (Table 1). Cultivation and preparation of a cell-free

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0006-291X/01 $35.00

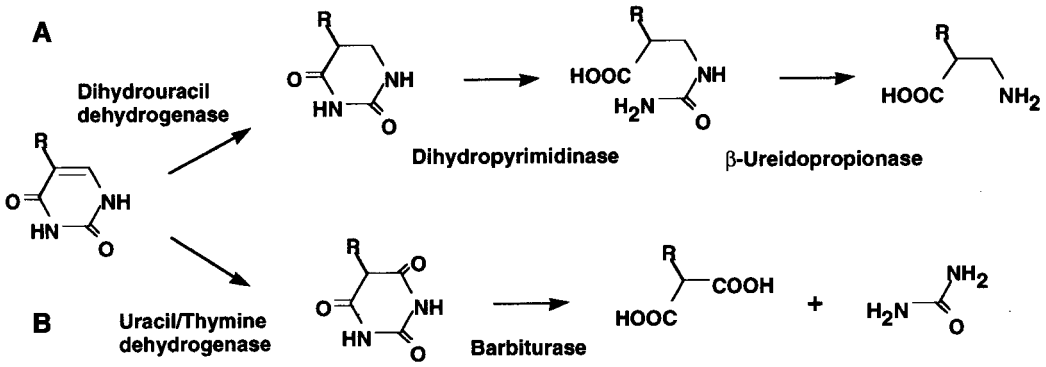

FIG. 1. Reductive (A) and oxidative (B) pyrimidine metabolism.

extract were carried out as described above, and the barbiturase activity was assayed as described below.

Purification of barbiturase. The bacterium was cultivated in 10 liters of uracil-rich medium at 28°C for 4 days with shaking. Cells (7 g wet weight) harvested by centrifugation (10,000*g* at 4°C) were used for purification. All purification procedures were carried out at 0 to 5°C. The buffer used was 20 mM potassium phosphate (pH 7.0) containing 10% ethylene glycol and 0.2 mM dithiothreitol.

The cells were suspended in 20 ml of buffer and then disrupted with glass beads in a Dyno-Mill KDL (W. A. Bachofen, Switzerland) for 25 min. The disrupted cell suspension was centrifuged at 14,000*g* for 60 min, and the resultant supernatant was used as the cell-free extract. The cell-free extract was dialyzed against 10 liters of buffer for 12 h. The dialyzed sample was then applied to a DEAE-Sephacel column (2.5 × 40 cm) previously equilibrated with the buffer. After the column has been washed with 1 liter of buffer, the enzyme was eluted with a linear gradient of 0 to 1 M NaCl in 1 liter of buffer. Barbiturase eluted with approximately 0.4 M NaCl was mixed with solid NaCl to obtain a concentration of 4 M and then applied to a phenyl-Sepharose CL-4B column (2.5 × 20 cm). After the column has been washed with buffer containing 4 M NaCl, the enzyme was eluted with a decreasing salt gradient (4 to 0 M NaCl) in 500 ml of buffer. Barbiturase eluted with approximately 1.0 M NaCl was concentrated by ultrafiltration with a 10,000 cutoff membrane. The concentrated enzyme was applied to a Sephacryl S-200 HR column (2.0 × 80 cm) equilibrated with buffer containing 0.2 M NaCl, and then eluted with the same buffer. The active fractions were used for further experiments.

Assaying of enzyme activities involved in oxidative and reductive pyrimidine metabolism. The enzyme activities in the oxidative and reductive pathways (barbiturase, dihydropyrimidinase, uracil/thymine dehydrogenase, dihydropyrimidine dehydrogenase, and ureidomalonase) were assayed as described below.

Barbiturase was assayed in a reaction mixture comprising 100 mM potassium phosphate (pH 8.0), 10 mM barbituric acid, and an appropriate amount of enzyme/cell-free extract, in a total volume of 100 μl. Two units of urease (Sigma, U.S.A.) was optionally added when monitoring urea as a reaction product.

Dihydropyrimidinase was assayed by the same method as for the barbiturase assay except that dihydrouracil or dihydrothymine was used as the substrate.

Uracil/thymine dehydrogenase was assayed in a reaction mixture comprising 100 mM Tris/HCl (pH 8.5), 10 mM uracil or thymine, 1 mM methylene blue and an appropriate amount of cell-free extract, in a total volume of 100 μl.

Dihydropyrimidine dehydrogenase was assayed in a reaction mixture comprising 100 mM Tris/HCl (pH 8.5), 10 mM uracil or thymine, 2 mM NADH/NADPH and an appropriate amount of cell-free extract, in a total volume of 100 μl.

Ureidomalonase was assayed by means of a coupling reaction with barbiturase. The reaction mixture comprised 200 mM potassium phosphate (pH 8.0), 20 mM barbituric acid, 0.1 unit of ureidomalonase-free barbiturase, 2 units of urease and an appropriate amount of ureidomalonase, in a total volume of 50 μl.

All reactions were carried out at 30°C for 30 to 60 min and stopped with 10 μl of 15% (v/v) perchloric acid, followed by neutralization with 90 μl of 500 mM potassium phosphate, pH 7.0. The reaction mixtures were centrifuged at 10,000*g* for 10 min, and the supernatants were analyzed as to the decrease in each substrate by high-performance liquid chromatography (HPLC) as described below. Urea production was also monitored as to barbiturase and ureidomalonase activity as described below.

One unit (U) of enzyme was defined as the amount of enzyme that catalyzed the consumption of the substrate or the formation of the product at a rate of 1 μmol/min under the assay conditions described above.

Analytical methods. The analysis of pyrimidine metabolites were carried out with a HPLC at 210 nm, fitted with a reverse phase Cosmosil $5C_{18}$ AR-II-packed column (4.6 × 250 mm; Nacalai Tesque, Japan), at a flow rate of 1.0 ml/min, with 100 mM KH_2PO_4 (pH 2.5) as the eluent. Analysis was also carried out with a HPLC at 210 nm, fitted with an ion-exchange QAE-2SW TSK-gel column (4.6 × 250 mm; Tosoh, Japan), at a flow rate of 1.0 ml/min, with 150 mM potassium phosphate (pH 6.4) as the eluent.

TABLE 1

Effects of Pyrimidine-Related Compounds on the Barbiturase Production by *R. erythropolis* JCM 3132

Supplements	Barbiturase activity (U/mg protein)	Relative activity (%)
Glycerol	0.12	100
Glycerol + barbituric acid	0.53	442
Glycerol + uracil	0.66	550
Glycerol + thymine	0.93	775
Glycerol + dihydrouracil	0.084	70
Glycerol + dihydrothymine	0.11	92
Glycerol + dihydro-L-orotate	0.041	34

Note. The activity was the average of three separate determinations that were reproducible within ±10%.

TABLE 2

Purification of Barbiturase from *R. erythropolis* JCM 3132

Step	Total protein (mg)	Barbituric acid decomposition		Urea production	
		Total activity (U)	Specific activity (U/mg)	Total activity (U)	Specific activity (U/mg)
DEAE-Sephacel	115	20.0	0.17	24.7	0.21
Phenyl-Sepharose CL-4B	38	14.3	0.38	16.5	0.43
Sephacryl S-200 HR	22	10.0	0.45	n.d.	n.d.

Note. n.d., not detected.

Urea production was monitored based on the amount of ammonia produced by urease. The amount of ammonia was colorimetrically determined with an assay kit for ammonia (Wako Pure Chemicals, Japan).

RESULTS

Evaluation of Pyrimidine Metabolism in R. erythropolis

R. erythropolis JCM 3132 grew well in the synthetic minimum medium with 0.15% (w/v) uracil as the sole carbon source. In the cell-free extract of uracil-grown *R. erythropolis,* only the oxidative pyrimidine-metabolizing activities were detected. Uracil and thymine were oxidized by uracil/thymine dehydrogenase at the same rates (0.40 U/mg protein), and barbituric acid was hydrolyzed by barbiturase at a higher rate (0.80 U/mg protein). The uracil/thymine dehydrogenase activity in the cell-free extract was unstable (the activity totally disappeared within 3 days), while the barbiturase activity was stable in the presence of 10% ethylene glycol (data not shown).

The following activities of reductive pyrimidine metabolism were not detected: dihydropyrimidine dehydrogenase activity with NADH or/and NADPH for uracil or thymine as the substrate, and dihydropyrimidinase activity for dihydrouracil or dihydrothymine as the substrate.

Induction of Barbiturase Production

Cells were cultivated in the minimum medium supplemented with 1% (v/v) glycerol as an energy source, and 0.15% (w/v) of various pyrimidine-related compound (Table 1) as potential inducer of barbiturase production. The enzyme activity was determined in the cell-free extract. The results showed that the cells grown with the addition of uracil, thymine or barbituric acid exhibited higher barbiturase activity than ones grown with the addition of dihydrouracil, dihydrothymine or dihydroorotate (Table 1). These results suggested that the production of barbiturase is enhanced by oxidative pyrimidine metabolites to support the degradation of pyrimidine through the oxidative pathway.

Purification of Barbiturase and Existence of a Consecutive Enzyme

Barbiturase was purified by DEAE-Sephacel, phenyl-Sepharose CL-4B and Sephacryl S-200 HR column chromatographies (Table 2). In the initial two steps, barbituric acid-decomposing activity was found together with urea-producing activity. However, after Sephacryl S-200 column chromatography, the barbituric acid-decomposing activity was not associated with the urea-producing activity (Table 2). An unknown product (which did not correspond to urea or malonic acid) was detected on HPLC analysis (Fig. 2). Interestingly, urea was produced from barbituric acid when the following downstream fractions were added to the barbituric acid-decomposing fractions (Fig. 3). The downstream fraction itself catalyzed neither barbituric acid decomposition nor urea production directly from barbituric acid.

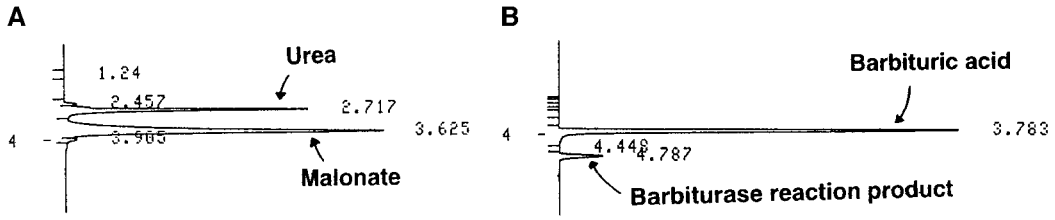

FIG. 2. HPLC analysis of the barbiturase reaction product on a reverse-phase Cosmosil $5C_{18}$ AR-packed column. (A) Standards, urea, and malonic acid; (B) barbiturase reaction mixture. Analysis was carried out under the conditions given under Materials and Methods.

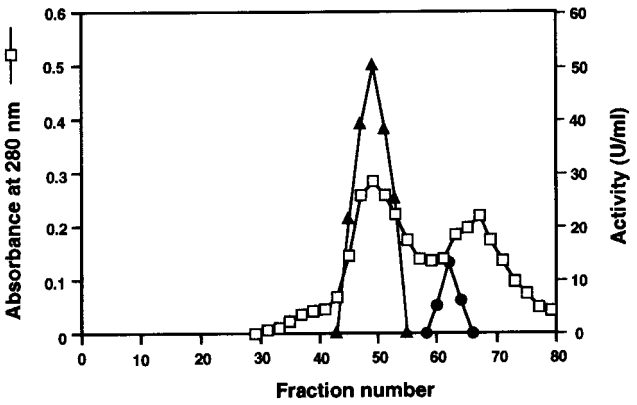

FIG. 3. Purification of barbiturase and the consecutive enzyme by Sephacryl S-200 HR column chromatography. Barbituric acid-decomposing activity, (▲); urea-producing activity, with the addition of barbituric acid-decomposing fraction (●).

These results suggest that the conversion of barbituric acid to urea involves two reaction steps, i.e., barbituric acid transformation to an unknown compound and subsequent transformation of the unknown compound to urea. The first reaction is catalyzed by barbiturase and the latter one by another consecutive enzyme.

Isolation of the Barbiturase Reaction Product and Identification of the Consecutive Enzyme as Ureidomalonase

Isolation of the barbiturase reaction product was carried out by HPLC on an ion-exchange column. The reaction product was collected as a single peak clearly separated from barbituric acid. However, reconfirmation of its purity by HPLC showed the presence of barbituric acid due to spontaneous reversion of the isolated product into barbituric acid. The spontaneous formation of barbituric acid cannot be avoided by changing the pH conditions or by immediate extraction into organic solvents. However, the product of the barbiturase reaction was predicted to be ureidomalonic acid based on several observations: (1) it was spontaneously cyclized to barbituric acid; (2) it gave no absorption peak around 250 nm, therefore it was considered to be a non-cyclic compound; (3) it was transformed to urea by the consecutive enzyme; and (4) malonic acid was coproduced with urea from it by the consecutive enzyme (Fig. 4).

These results suggest that the barbiturase reaction product is ureidomalonic acid, and that the consecutive enzyme catalyzes the hydrolysis of ureidomalonic acid to urea and malonic acid (Fig. 5). The consecutive enzyme is a novel enzyme and should be named ureidomalonase.

DISCUSSION

R. erythropolis metabolized pyrimidine through an oxidative pathway. The production of barbiturase, which is involved in the pathway, was enhanced by the addition of uracil, thymine or barbituric acid, indicating that barbiturase was produced for their assimilation.

The purification of barbiturase revealed that it catalyzes the ring-opening of barbituric acid to ureidomalonic acid, but it did not further hydrolyze ureidomalonic acid to urea and malonic acid. Ureidomalonic acid produced by barbiturase is further hydrolyzed by a novel enzyme, ureidomalonase, to urea and malonic acid (Fig. 5). Ureidomalonase therefore sequentially functions with barbiturase in oxidative pyrimidine metabolism.

This is the first detailed analysis of the amidohydrolytic reaction in oxidative pyrimidine metabolism. Further studies on the enzymes in the oxidative pathway and their regulation will be interesting in comparison with the well-known reductive pyrimidine metabolism. Purification and detailed characterization of barbiturase, ureidomalonase and uracil/thymine dehydrogenase are currently in progress.

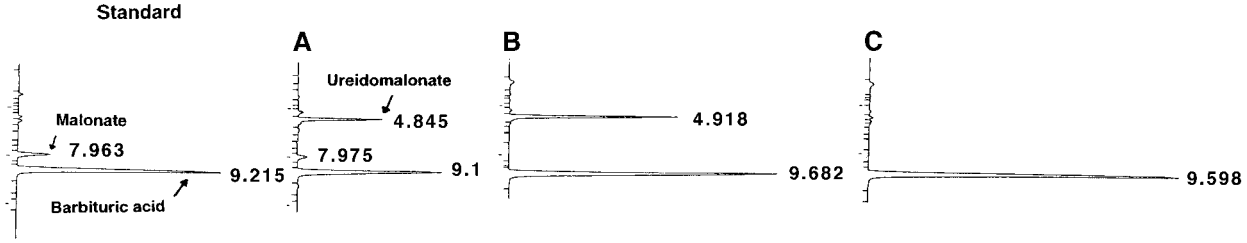

FIG. 4. HPLC analysis of barbiturase and the consecutive enzyme reaction with barbituric acid on an ion-exchange QAE-2SW TSK-gel column. Analysis was carried out under the conditions given under Materials and Methods. Standards, barbituric acid and malonic acid; (A) reaction mixture with barbiturase and the consecutive enzyme; (B) reaction mixture with only barbiturase; (C) reaction mixture with only the consecutive enzyme.

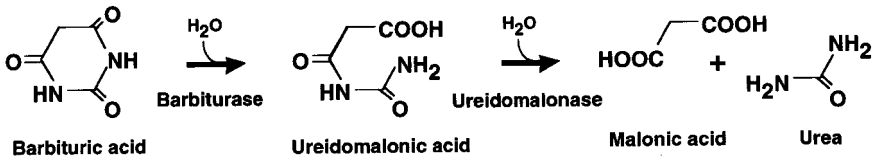

FIG. 5. Proposed metabolism of barbituric acid in *Rhodococcus erythropolis* JCM 3132.

ACKNOWLEDGMENTS

This work is supported in part by Japan Society for the Promotion of Science (JSPS). C. L. Soong is a postdoctoral fellow supported by JSPS.

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