Barbiturase, a Novel Zinc-containing Amidohydrolase Involved in Oxidative Pyrimidine Metabolism*

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Barbiturase, which catalyzes the reversible amidohydrolisis of barbituric acid to ureidomalonic acid in the second step of oxidative pyrimidine degradation, was purified to homogeneity from Rhodococcus erythropolis JCM 3132. The characteristics and gene organization of barbiturase suggested that it is a novel zinc-containing amidohydrolase that should be grouped into a new family of the amidohydrolases superfamily. The amino acid sequence of barbiturase exhibited 48% identity with that of herbicide atrazine-decomposing cyanuric acid amidohydrolase but exhibited no significant homology to other proteins, indicating that cyanuric acid amidohydrolase may have evolved from barbiturase. A putative uracil phosphoribosyltransferase gene was found upstream of the barbiturase gene, suggesting mutual interaction between pyrimidine biosynthesis and oxidative degradation. Metal analysis with an inductively coupled radiofrequency plasma spectrophotometer revealed that barbiturase contains ∼4.4 mol of zinc per mol of enzyme. The homotetrameric enzyme had \( K_m \) and \( V_{max} \) values of 1.0 mM and 2.5 \( \mu \)mol/min/mg of protein, respectively, for barbituric acid. The enzyme specifically acted on barbituric acid, and dihydro-L-orotate, alloxan, and cyanuric acid competitively inhibited its activity. The full-length gene encoding the barbiturase (\( \text{bar} \)) was cloned and overexpressed in Escherichia coli. The kinetic parameters and physicochemical properties of the cloned enzyme were apparently similar to those of the wild-type.

In a biological system, pyrimidines are metabolized through either a reductive or an oxidative pathway (1, 2). It is well recognized that mammals, plants, and microorganisms utilize the reductive pathway for pyrimidine degradation (3–5), whereas some microorganisms use the oxidative pathway (6–8).

In reductive pyrimidine metabolism, uracil, or thymine is first reduced to its dihydro-derivative, which in turn is hydrolyzed to an N-carbamoyl-\( \beta \)-amino acid and finally decarbamoylated to a \( \beta \)-amino acid. This metabolic route, especially the hydrolysis of dihydro-derivatives catalyzed by dihydropyrimidinase, has attracted much attention, because it is a potential target for drug therapy in the treatment of cancer (9, 10), and it also has been used for the industrial production of optically active amino acids (5, 11, 12). In contrast, oxidative pyrimidine metabolism has been scarcely investigated, and the references available so far are limited to the early studies performed by three groups of scientists (6–8). These reports showed that pyrimidine bases are first oxidized to barbituric acid derivatives, and then the barbituric acid derivatives are further hydrolyzed by barbiturase (EC 3.5.2.1) to urea and malonate derivatives. However, these studies were carried out with crude enzyme preparations, and the results presented were inadequate for confirming the enzymatic conversion of barbituric acid to urea and malonate.

We have elucidated the enzymes involved in the oxidative pathway, and clarified their physiological functions, in a bacterium, Rhodococcus erythropolis JCM 3132, which metabolizes pyrimidine exclusively through the oxidative pathway (13). Our preliminary enzymatic study on the oxidative pathway in this strain revealed that barbiturase catalyzes the amidohydrolisis of barbituric acid to ureidomalonic acid but not to urea and malonate (13). Consequently, we found a novel enzyme, ureidomalonase, which catalyzes the hydrolysis of ureidomalonic acid to urea and malonate, and proposed a detailed metabolic pathway of oxidative pyrimidine metabolism as shown below in Fig. 1A (13).

In the present study, we purified and characterized barbiturase in detail. The full-length gene encoding the barbiturase (\( \text{bar} \)) was also cloned. The enzyme was found to contain zinc, and its amino acid sequence exhibited good homology to that of cyanuric acid amidohydrolase, an enzyme involved in the newly evolved catalytic pathway for herbicide atrazine degradation as shown below in Fig. 1B (14, 15). However, no sequence homology was found with other naturally existing amidohydrolases such as dihydropyrimidinase, dihydro-orotate, allantoinase, cytosine deaminase, and urease, suggesting that barbiturase represents a new family of the amidohydrolase protein superfamily. The putative zinc-binding motif of barbiturase is located in the COOH-terminal region, contrary to in the cases of the zinc-containing amidohydrolases mentioned above, in which it is located in the NH\(_2\)-terminal region, further indicating that barbiturase is a novel zinc-containing amidohydrolase. In addition, adjacent to the barbiturase gene is a putative uracil phosphoribosyltransferase gene, this enzyme being involved in the salvage pathway for uracil in pyrimidine nucleotide biosynthesis, suggested that barbiturase may collaborate with this enzyme in the regulation of pyrimidine metabolism.

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HPLC 16/26 column equilibrated with the buffer containing 0.2 mM NaCl and then eluted with the same buffer. The active fractions were used for characterization.

**Analytical Methods**—The relative molecular weight was determined by HPLC on a Tosoh G3000SW column (7.5 × 300 mm, Tosoh, Japan) containing 0.1 M potassium phosphate, pH 7.0, and 0.2 mM NaCl (pH 7.0) at the flow rate of 0.3 ml/min. The molecular weight of barbiturase was calculated from the mobilities of the standard proteins, glutamate dehydrogenase (290,000), lactate dehydrogenase (142,000), enolase (67,000), adenylyl kinase (32,000), and cytochrome c (12,400).

PAGE was performed in a 12.5% polyacrylamide gel, which was stained with Coomassie Brilliant Blue R-250. The NH2-terminal and internal amino acid sequences of the purified enzyme were determined by automated Edman degradation with a pulsed-liquid-phase protein sequencer (Applied Biosystems 476A). Protein concentrations were determined by the dye binding method of Bradford using a Bio-Rad protein assay kit.

**Preparation of Internal Peptide**—The freeze-dried purified enzyme (22 nmol) was dissolved in 20 mM Tris/HCl containing 8 mM urea (pH 9.0) and incubated at 37 °C for 60 min. It was then digested with 0.11 nmol of lysozyme and 20 mM Tris/HCl containing 4 M urea at 37 °C for 16 h. The mixture was then applied to a SMART micro purification system (Amersham Biosciences, Sweden) equipped with an FRP C2/C18 PC 3.2/3 column and eluted with a linear gradient (6 ml) of acetonitrile (0–80%) in the presence of 0.1% trifluoroacetic acid. The purified peptide solutions were evaporated to dryness using a SpeedVac Plus SC110A (Savant Instruments, Inc., NY) and stored at −20 °C. Amino acid sequencing was performed by Edman degradation as described above.

**Metal Analysis**—All glassware was soaked in 2 M HCl overnight and rinsed thoroughly with ion-exchange distilled (IED) water. Prior to enzyme dialysis, the dialysis tubing was successively treated with hot sodium carbonate (0.1 M), EDTA (12 mM)/sodium acetate (33 mM), and acetate (10 mM) for 30 min each and then washed with IED water. The treated tubing was then soaked in 50% ethanol for 2 h and washed thoroughly with IED water. The highly purified and concentrated enzyme (1.15 mg of protein/ml) was dialyzed extensively against 5 mM Tris/HCl (pH 7.5) containing 10% ethylene glycol (special grade for amino acid analysis from Wako Pure Chemicals, Japan). The dialyzed enzyme was analyzed with an inductively coupled radiofrequency plasma spectrophotometer (ICPS), Shimadzu ICPS-8000 (27,120 MHz). The metal contents of the enzyme sample were determined from the calibration curve for standard solutions, with the dialysis buffer as a control.

**DNA Manipulation and Sequencing**—Total genomic DNA from R. erythropolis JCM 3132 was isolated and purified according to the method of Saito and Miura (16). The protocols used for plasmid isolation, agarose electrophoresis, ligation, and other standard molecular biological techniques were as described by Sambrook et al. (17). DNA labeling with alkaline phosphate for Southern and colony hybridization was performed using the alkaline phosphatase labeling kit (Amersham Biosciences, UK). Sequencing was performed by the dyeoxy chain-termination method using a CEQ DTCS kit dye terminator cycle sequencing kit (Beckman Instruments) with an automated DNA sequencer (CEQ 2000XL DNA Analysis System, Beckman Coulter, Inc.). The GENETYX software system (Software Development Co., Tokyo, Japan) was used for computer analysis of nucleotide sequences and deduced amino acid sequences.

**PCR and Inverse-PCR Cloning**—Two degenerate oligonucleotides (forward primer 5'-GA/A/GG/GA/G/TG/CC/GA/A/G/TGCC/AT (A/C)/T/GT/NT/NC/3' - and reverse primer 5'-TT/TG/A/AT/C/T/TGC (T/G)/G/A/TG/CC/GA/G/TA/I/GC/ATC-3'), which had the motif (A/C)(T/G)(A/G)TGCCA(G/A)TGIC(G/A)(G/T/NT)GC-3', were synthesized based on two internal peptide sequences, EVFAEVPIVW (Ba-1) and DVHWHRQIK (Ba-5), respectively. PCR amplification was performed with ExTaq polymerase (Takara Shuzo, Japan) and the above primers, with the genomic DNA of R. erythropolis as a template. A ThermoBlorb T-Gradient (Biometra, Germany) was programmed for 1 cycle of denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 2 min; and 1 cycle of extension at 72 °C for 5 min. A total of 15 cycles of PCR product was used as a DNA probe in Southern blotting with alkaline phosphatase-labeled 700-bp fragment as a probe. The resultant cloned plasmid, pCR-BAR10, was isolated using a QIAprep Spin Miniprep kit (Qiagen). The cloned 700-bp insert was synthesized with a non-supplemented sample as a control.
sequenced with M13 forward and reverse primers followed by primer-walking with custom oligonucleotides synthesized by GENSET KK, Kyoto, Japan.

Subsequent cloning of the upstream and downstream regions flanking the barbiturase gene was carried out by inverse-PCR (18). Genomic DNA was separately digested with several restriction enzymes, and a NaeI digest of ~2.3 kb was selected on Southern blot analysis as suitable for inverse-PCR. The genomic DNA was then digested with NaeI and self-ligated under dilute conditions (1 μg/ml) that favor intramolecular circularization (19). PCR amplification of this ligated DNA was performed by using two divergent oligonucleotides (S1, 5′-CGTCAGGTTGTTCTCTGAAATGGGAGG-3′, and AS1, 5′-GTCCGGTCTTGGGTGTGACCCGCTGTTGCTGCC-3′, located within the previously obtained insert sequence of pCR-BAR10) as primers. The specific amplified 2.3-kb fragment was purified, ligated with a TA cloning vector and then transformed into E. coli DH5α. Screening for positive colonies was performed by colony hybridization. The resultant cloned plasmid, pCR-BAR59, was isolated and sequenced.

**Expression of Barbiturase in E. coli**—The recombinant form of *R. erythropolis* barbiturase was obtained by PCR amplification of the gene encoding the enzyme and its subsequent cloning into T7 polymerase-driven expression vector pET21-a (Novagen, Milwaukee, WI). For amplification, the forward primer (5′-AAATGCAATGAAGTCTGAGCGGATCC-3′) and the reverse primer (5′-AAGTTTCTTCAAGCTGTTCACTGCT-3′) contained an engineered NdeI site (underlined) and spanned positions −15 to +15 of the coding strand, whereas the reverse primer (5′-AAGTTTCTTCAAGCTGTTCACTGCT-3′) had an engineered EcoRI site (underlined) and corresponded to the sequence ranging from +1106 to +1135 of the non-coding strand. The conditions for PCR amplification with LA Taq polymerase (Takara Shuzo, Japan) were as described above, except that an annealing temperature of 65 °C and an extension temperature of 74 °C for 1 min were employed. The resultant 1.1-kb gene encoding barbiturase was cloned into the NdeI and EcoRI sites in expression plasmid pET21-a, yielding plasmid pET-BAR11N5. This was maintained in *E. coli* DH5α, and the complete sequence of the barbiturase gene was confirmed by sequencing of the genes from three independent colonies harboring the pET-BAR11N5 plasmid. The gene encoding barbiturase was expressed in *E. coli* BL21 (DE3)/pET-BAR11N5 cells grown in LB medium (500 ml in a 2-liter flask) containing a final concentration of 1 mM isopropyl-β-D-thiogalactopyranoside. Cultivation was carried out at 37 °C for 6 h, after inoculation with 10% (50 ml) of an overnight seed culture. Cells were harvested by centrifugation, washed twice with 0.85% NaCl, and then screened for possible stabilizers of the enzyme. Among them, we found ethylene glycol is an effective stabilizer for the barbiturase. In the presence of 10% (v/v) ethylene glycol, the barbiturase could be kept for more than 3 months at 4 °C without detectable loss of activity.

**RESULTS**

**Stabilization of Barbiturase**—During the initial study, we encountered difficulty in purifying the barbiturase, because the enzyme was unstable. The enzyme in a cell-free extract lost ~70% of its initial activity (at 4 °C) within 2 weeks. Therefore, it could not withstand the purification process, and no activity was detected after the first two chromatographic steps. We then screened for possible stabilizers of the enzyme. The enzyme activity was examined after the addition of reducing agents, cofactors, metal ions, protease inhibitors, pyrimidine and purine derivatives, organic acids, detergents, and sugars. Among them, we found ethylene glycol is an effective stabilizer for the barbiturase. In the presence of 10% (v/v) ethylene glycol, the barbiturase could be kept for more than 3 months at 4 °C without detectable loss of activity.

**Purification and Criteria for Purity**—Barbiturase was purified to homogeneity with an overall yield of 8% and a 40-fold increase in specific activity (Table I). The purified enzyme gave a single protein band on SDS-PAGE (Fig. 2). Further evidence of its purity was provided by gel-permeation HPLC on a G3000SW column, there being a quite symmetrical protein absorption peak concomitant with barbituric acid-hydrolyzing activity.

**Molecular Weight and Subunit Structure**—The relative molecular weight of the enzyme was estimated to be 172,000. On SDS-PAGE, the purified enzyme gave a single protein band corresponding to a relative molecular weight of 45,000. Thus, the native enzyme probably consists of four identical subunits.

**Metal Ion Content**—An initial attempt to dialyze the enzyme against 5 mM Tris/HCl buffer (pH 7.5) failed, because the enzyme became aggregated. It was found that the addition of ethylene glycol to the dialysis buffer prevented this enzyme aggregation. Qualitative analysis of metals in the enzyme was performed for 66 elements by ICP-AES. Only zinc gave a significant reading, i.e. ~1.1 ± 0.1 nmol per subunit. Other metals found in detectable amounts were calcium and magnesium.
The purified enzyme showed strict specificity toward barbituric acid. The following compounds were not transformed by the purified enzyme: barbital, cyclobarbital, allobarbituric acid; pyrimidine derivatives such as barbituric acid derivatives such as dihydro-L-orotate, dihydrouracil, dihydrothymine, orotate, uracil, and thymine; and other cyclic-amides such as cyanuric acid, alloxan, parabanic acid, hydantoin, glutarimide, and succinimide.

**Table 1**

| Step total      | Total protein (mg) | Specific activity (units/mg protein) | Activity (% yield) | Fold |
|-----------------|--------------------|-------------------------------------|--------------------|------|
| Cell-free extract| 1650               | 108                                 | 0.0655             | 100  |
| DEAE-Sephaloc (1st) | 90.0             | 57.5                                | 0.839              | 53.2 |
| DEAE-Sephaloc (2nd) | 54.4          | 70.4                                | 1.29               | 65.2 |
| MonoQ HR 5/5     | 22.0               | 40.0                                | 1.82               | 37.0 |
| Phenyl-Superose HR 5/5 | 6.40        | 15.6                                | 2.44               | 14.4 |
| Superdex 200 Hiloade 16/26 | 3.30         | 8.70                                | 2.64               | 8.06 |

**Fig. 2. SDS-PAGE of the wild-type and recombinant barbiturases.** Lane A, marker proteins; lane B, purified recombinant enzyme; lane C, purified wild-type enzyme.

**Fig. 3. Competitive inhibition of barbituric acid hydrolysis by dihydro-L-orotate, alloxan, and cyanuric acid.** A, in the presence of 0 mM (□), 1 mM (●), 3 mM (○), or 5 mM (▲) dihydro-L-orotate; B, in the presence of 0 mM (□), 5 mM (○), 8 mM (△), or 10 mM (▲) alloxan; C, in the presence of 0 mM (◇), 2 mM (▼), 4 mM (+), or 6 mM (▲) cyanuric acid.

The $K_v$ and $V_{max}$ values for barbituric acid were 1.0 mM and 2.5 μmol/min/mg of protein, respectively. Barbituric acid was hydrolyzed to ureidomalonic acid, which showed the same elution profile on HPLC as that in our previous report dealing with product (ureidomalonic acid) identification (13). Urea and malonate were not detected in the reaction mixture of the purified enzyme with barbituric acid as the substrate on analysis by TLC, HPLC, and an enzymatic method using urease. The non-reactive compounds listed above were examined as to inhibition of barbituric acid hydrolysis by the purified barbiturase. It was found that dihydro-L-orotate, alloxan, and cyanuric acid competitively inhibited barbituric acid hydrolysis, the $K_v$ values being 4.5, 1.7, and 0.42 mM, respectively (Fig. 3).

The reaction time courses with various barbituric acid concentrations (2–80 mM) showed time-dependent saturation of the reactions, indicating that the reactions attained equilibrium (data not shown). These results suggested that barbiturase catalyzes the reversible hydrolysis of barbituric acid to ureidomalonic acid. An equilibrium constant ([product]/[substrate]) of $–0.16$ was obtained at pH 8.0.

**Effects of Inhibitors and Metal Ions**—The enzyme activity was assayed under standard conditions in the presence of various compounds (2 mM). The enzyme activity was completely inhibited by sulfhydryl reagents $p$-chloromercuribenzoate and $N$-ethylmaleimide, whereas $N$-bromosuccinimide and 5,5’-dithio-bis-(2-nitrobenzoic acid) showed 60 and 50% inhibition, respectively. Serine protease inhibitor diisopropyl phosphofluoridate strongly inhibited the enzyme activity (90% inhibition). The enzyme was sensitive to metal ion chelators such as o-phenanthroline, 8-hydroxyquinoline, EDTA, and $α,α’$-dipyridyl (80, 60, 30, and 30% inhibition, respectively), suggesting that metal ion ($Zn^{2+}$) is essential for the catalytic activity of the enzyme. The enzyme activity was not enhanced on metal ion addition but partially inhibited by $Ni^{2+}$, $Cd^{2+}$, and $Co^{2+}$ (80, 60, and 50% inhibition, respectively) and completely inhibited by $Hg^{2+}$ and $Cu^{2+}$.

**Effects of pH and Temperature**—The enzyme activity and stability were assayed in MES/NaOH, potassium phosphate, Tris/HCl, HEPES/NaOH, and NaHCO$_3$-Na$_2$CO$_3$ buffer systems (100 mM) at pH 4.0–6.0, 6.5–8.0, 7.0–8.5, 7.0–8.0, and 9.4–10.0, respectively. Under the standard assay conditions, the highest activity was observed at pH 8.0. When the enzyme was incubated at 30 °C for 30 min, more than 80% of the initial activity was retained at pH 6.0–8.0. The initial velocity of the hydrolysis increased with increasing temperature, reaching a maximum at 40–45 °C. No enzymatic activity remained after 30-min incubation at 55 °C or above at pH 8.0.

**Partial Amino Acid Sequence Analysis**—The NH$_2$-terminal and five internal amino acid sequences of barbiturase (Ba-1 to Ba-5) are shown in Fig. 4. These sequences were compared with...
those of proteins stored in protein sequence data bases (Swiss-Prot, GenBank™, EMBL, PIR, and PRF) using BLAST and FASTA software. It was found that the NH₂-terminal and peptide Ba-5 sequences showed good homology to those of the NH₂- and COOH-terminal regions of cyanuric acid amidohydrolase (14), respectively.

Cloning, Sequence, and Putative Zinc-binding Motif of Barbiturase

Degenerative primers derived from the internal peptide sequences of barbiturase specifically amplified a 0.7-kb fragment. The deduced amino acid sequence of the 0.7-kb insert of pCR-BAR10 corresponded precisely to the five internal peptide (Ba-1 to Ba-5) sequence of barbiturase (Fig. 4). Using the inverse-PCR approach, we found the gene-specific non-degenerate 25-mer primers (S1 and AS1, Fig. 4) specifically amplified the upstream (2.0 kb) and downstream (0.3 kb) parts of the locus around the 0.7-kb gene of the NaeI digest, thus allowing us to determine the entire barbiturase structural gene. The enzyme gene was located in a 1.1-kb open reading frame (ORF) starting with an ATG start codon and terminating with a TAG codon and encoded a protein with a molecular weight of 38,997.46 (Fig. 4). A typical Shine-Dalgarno sequence was present 6 bp upstream from the initiation codon. The NH₂-terminal amino acid sequence of the ORF exactly matched the 36-amino acid sequence of the NH₂-terminal of the purified barbiturase determined by Edman degradation except for the first methionine residue, which may have been lost due to post-translational modification.

A homology search of the protein data bases revealed significant homology (48% identity, Fig. 5) with cyanuric acid amidohydrolase, an enzyme that catalyzes the ring-opening reaction of cyanuric acid and is involved in the final step degradation of the S-triazine rings of atrazine herbicides (14). This is likely, because the chemical structure of barbituric acid closely resembles that of cyanuric acid, and both enzymes catalyze amidohydrolytic reactions (Fig. 1). However, cyanuric acid amidohydrolase does not act on barbituric acid but is competitively inhibited by it (14). On the other hand, as mentioned above, barbiturase did not act on cyanuric acid but was inhibited by it.

The putative zinc-binding motif of barbiturase was shown in Fig. 4. This motif, designated as DXH, is part of a conserved sequence pattern suggested to be involved in the metal assem-
Barbiturase

bly center of the amidohydrolases superfamily (23–25). Moreover, site-directed mutagenesis of the conserved histidine residues of this motif showed that they are responsible for zinc binding and essential for the catalytic activity of the enzyme (26, 27). However, surprisingly, the zinc-binding motif of barbiturase is located near the COOH terminus, unlike in all other known amidohydrolases, in which the motif is found near the NH$_2$ terminus.

Interestingly, we found another open reading frame (624 bp) translated in the opposite direction (from −224 to −847) upstream of the barbiturase gene (Fig. 4). The gene encoded a protein of 207 amino acids with a molecular weight of 21,920. A homology search of the deduced amino acid sequence of this ORF revealed very high homology to uracil phosphoribosyltransferase from various sources.

The multiple alignment was performed with the GENETYX-MAC 7.3 program. Gaps denoted by dashes were inserted to obtain maximum homology. Conserved residues are highlighted in white letters on a black background. BBT, barbiturase from R. erythropolis; CAA, cyanuric acid amidohydrolase from Pseudomonas sp. strain MA150; Myctu, cyanuric acid amidohydrolase from Mycobacterium tuberculosis (Swiss-Prot, P03184); Myctu, cyanuric acid amidohydrolase from Mycobacterium tuberculosis; Bacu, Bacillus subtilis (Swiss-Prot, P63643); Lalcl, Lactococcus lactis (Swiss-Prot, P38964); Bacsu, Bacillus subtilis (Swiss-Prot, P38964); Strsl, Streptococcus salivarius (Swiss-Prot, P33273); Neame, Neisseria meningitidis (GenBank™, AE00081); Strel, Streptococcus salivarius (Swiss-Prot, P38964). E. coli Barbiturase was successfully overexpressed as a soluble form in the cytoplasmic fraction of E. coli BL21 cells harboring the pET-BAR11N5 expression plasmid. After isopropyl-β-D-thiogalactopyranoside induction, barbiturase was estimated to constitute −25% of the total crude extract proteins. The specific barbiturase activity in a cell-free extract of the recombinant E. coli (0.654 μmol/min/mg of protein) is 10-fold higher than that of E. coli (0.0655 μmol/min/mg of protein). No barbiturase activity was detected in E. coli BL21 cells harboring the original pET21-a plasmid. The recombinant barbiturase was purified to homogeneity through four chromatographic steps with a yield of about 20% (Fig. 2). A kinetic study of the purified enzyme gave $K_m$ and $V_{max}$ values of 0.67 mM and 2.58 μmol/min/mg of protein, respectively, which are apparently the same as those of the wild-type. The relative molecular weight was estimated to be 167,000 by gel-permeation HPLC. The recombinant enzyme exhibited the same pH and temperature optima and is susceptible to metal ion chelators. The sequence of the NH$_2$-terminal 27 amino acids was PEAIEVRKVLHSDKEVIEGIDYKSG, which is identical to that of the purified wild-type. The loss of the initial methionine residue suggested that the expression of barbiturase in E. coli is also affected by post-translational modification.

DISCUSSION

Over the past several decades, no study has been focused on oxidative pyrimidine metabolism. Before our studies, only a few early works by three groups of scientists had been reported (6–8). Although it is a naturally occurring metabolic pathway, knowledge on the oxidative pathway and its biological importance is very limited. We have studied the reductive pathway from the physiological and application perspectives (31–37). At the same time, we initiated a study on oxidative pyrimidine

Fig. 6. Comparison of the deduced amino acid sequence of an open reading frame upstream of the barbiturase gene with those of uracil phosphoribosyltransferases from various sources. The multiple alignment was performed with the GENETYX-MAC 7.3 program. Gaps denoted by dashes were inserted to obtain maximum homology. Conserved residues are highlighted in white letters on a black background. Rhoer, R. erythropolis (this study); Myctu, Mycobacterium tuberculosis (Swiss-Prot, P84928); Bacu, Bacillus subtilis (Swiss-Prot, P33273); Lalcl, Lactococcus lactis (Swiss-Prot, P509926); Metth, Methanobacterium thermoautotrophicum (EMBL, AE00081); Strel, Streptococcus salivarius (Swiss-Prot, P38964); Neame, Neisseria meningitidis (GenBank™, AL162754).
Barbiturase metabolism by screening for microorganisms that utilize pyrimidine through the oxidative pathway, and found an active strain, R. erythropolis JCM 3132. Enzymatic study revealed that the catalytic action of barbiturase is different from that previously reported, and a novel enzyme, ureidomalonase, is proposed to be involved in the oxidative pathway (Fig. 1A). In this report, we report the detailed physicochemical properties of barbiturase and molecular cloning of the enzyme.

Barbiturase consists of four identical subunits and is very specific toward barbituric acid. The enzyme catalyzes the conversion of barbituric acid to ureidomalonic acid, and the equilibrium of the reaction is inclined toward barbituric acid formation. However, the existence of ureidomalonase supports the successive hydrolysis of ureidomalic acid, which would pull the reaction toward barbituric acid decomposition. The enzyme activity was competitively inhibited by dihydro-L-orotate, which is an intermediate in the pyrimidine biosynthesis pathway. Likewise, it has been reported that barbituric acid inhibits several enzymes that participate in de novo pyrimidine biosynthesis (38–40). These results suggest that there are some mutual interaction between pyrimidine anabolism and oxidative catabolism. This proposition is supported by the fact that, adjoining to the barbiturase gene is the putative uracil phosphoribosyltransferase gene, this enzyme being involved in the salvage pathway for uracil in pyrimidine nucleotide biosynthesis. Uracil phosphoribosyltransferase is also involved in the control of the rate of transcription of messenger RNA of the pyrimidine nucleotide biosynthetic operon (30). Barbiturase may collaborate with uracil phosphoribosyltransferase in catalytic, and probably genetic, regulation of pyrimidine metabolism, especially in organisms that degrade pyrimidine through the oxidative pathway.

Barbiturase is a zinc enzyme (containing four atoms of zinc per molecule of enzyme), as directly determined by ICPS. In this respect, barbiturase is similar to dihydropyrimidinase/hydantoinase and dihydro-ortotate, which are involved in reductive and biosynthesis pyrimidine metabolism, respectively. The dihydropyrimidinase purified from rat and bovine liver contains four atoms of zinc per mol of enzyme (20, 21). The dihydro-ortotate from hamster was suggested to contain two atoms of zinc per mol of enzyme (26), whereas recently the E. coli enzyme was revealed to have four atom of zinc per mol of enzyme (24). This enzyme by three-dimensional structure analysis (41). A recently purified and cloned human guanine deaminase (which also possesses the metal binding motif DXHXX) was determined to contain two atoms of zinc per mol of enzyme (42). The zinc-binding site of barbiturase is located at amino acids 320 (DVHWH, Fig. 4). It is found in the COOH-terminal region, contrary to the cases of other known amidohydrolases, in which the zinc-binding site is located near the NH2-terminal region. This enzyme has been identified as a novel zinc-containing amidohydrolase. The primary structure of barbiturase is very similar to that of cyanuric acid amidohydrolase. Cyanuric acid has been identified as a central intermediate in the pathway of atrazine degradation (43), and the catalytic function of cyanuric acid amidohydrolase is to completely mineralize the S-triazine ring (14) (Fig. 1B). This enzyme is a homotetramer, and its physicochemical properties, such as metal ion effects, and pH and temperature optima, are similar to those of barbiturase.

**Fig. 7. Phylogenetic tree for amidohydrolases.** This tree was constructed by the UPGMA procedure using the GENETYX-MAC 7.5 program. The tree was divided into several amidohydrolase families, as presented earlier (23, 25). There are mainly six families from the evolutionary standpoint, as shown in boxes: dihydropyrimidinase/hydantoinase (DHP/HYN), alantoinase (ALN), dihydro-ortotate (DHO), adenine deaminase (ADN), urease/cytosine deaminase (URE/CYN), and the newly proposed barbiturase/cyanuric acid amidohydrolase (BBT/CAA). All sequences are retrieved from Swiss-Prot data base, and the accession numbers for each sequence are given in parentheses: DHP, human (P14117); DHP, rat (Q62150); HYN, Pseudomonas putida (Q59689); HYN, Bacillus stearothermophilus (Q45515); HYN, Agrobacterium radiobacter (Q41848); HYN, Arthrobacter aurescens (P81006); ALN, E. coli (P77671); ALN, B. subtilis (302137); ALN, frog (P40757); ALN, Saccharomyces cerevisiae (P32375); DHO, Methanobacterium thermoautotrophicum (027199); DHO, Pyrococcus hortaea (057740); ADN, human (P32778); DHO, Drosophila melanogaster (P05990); B. subtilis (P25995); DHO, Thermus aquaticus (P29995); ADN, human (P00813); ADN, mouse (P03958); ADN, E. coli (P22333); URE, Hemophilus influenzae (P44391); URE, Klebsiella aerogenes (P15314); URE, B. subtilis (P77837); URE, Mycobacterium tuberculosis (P50042); URE, Lactococcus lactis (P26929); URE, Clostridium perfringens (P94699); CYN, Candida albicans (P75984); CYN, S. cerevisiae (Q12178); BBT, R. erythropolis (this study); CAA, Pseudomonas sp. strain NRRLB-12227 (GenBank™, AF086815).
ever, extensive dialysis of the enzyme against EDTA did not result in a decrease in enzyme activity, indicating that no metal ion binds to the active site of the enzyme. Moreover, amino acid sequence alignment of cyanuric acid amidohydrolase with barbiturase showed no corresponding DXHXH motif, further suggesting that the enzyme has no metal binding site (Fig. 5). Until this report, the evolutionary origin of cyanuric acid amidohydrolase could not be predicted, because no sequence similarity was found with known proteins in the protein data bases (14). Based on the strong sequence similarity, we propose that cyanuric acid amidohydrolase may have originally evolved from barbiturase, because the latter participates in a naturally existing metabolic pathway, whereas the former gene was reported to be located on a transposable element (44).

The phylogenetic relationship among amidohydrolases from microorganisms and mammals determined by the UPGMA (Unweighted Pair-Group Method with Arithmetic Mean) procedure with the GENETYX-MAC 7.3 program is shown in Fig. 7. Barbiturase and cyanuric acid amidohydrolase are categorized into the same group, but they are distinctly different from other amidohydrolase families. Although barbiturase and cyanuric acid amidohydrolase did not exhibit sequence similarity with other amidohydrolases, the dendrogram revealed that both enzymes are evolutionary closer to cytosine deaminase and urease, and are far away from dihydropyrimidinase/ hydratedlase, allantoinase, and dihydro-ornatase on the evolutionary tree. This phenomenon implies that the amidohydrolase (barbiturase) of the oxidative pathway and the amidohydrolase (dihydropyrimidinase) of the reductive pathway have developed in different evolutionary directions. In summary, we propose that barbiturase and cyanuric acid amidohydrolase belong to a new family of the amidohydrolase superfamily.

Barbituric acid is considered to be the non-anesthetic compound. However, substitution by alkyl or aryl chains at the C5 position of barbituric acid leads to a compound affecting the arousal reaction. Hypnotic and convulsant reagents such as 5-benzylbarbituric acid, barbital, phenobarbital, and 5,5-disubstituted barbiturates have been reported to be useful for the therapy for cancer and other pathological and physiological disorders (45, 46). Complete degradation of these drugs may likely involve the ring cleavage of the core structure of barbituric acid. Although so far there is no direct evidence of barbiturase activity or the existence of oxidative pyrimidine metabolism in mammals, some early articles reported that radioactive $^{15}$N- or $^{14}$C-urea, a possible product of oxidative but not reductive metabolism, was detected in the urine when $^{15}$N- or $^{14}$C-labeled uracil was intravenously administered to rats (47, 48). However, further investigation has to be carried out to verify whether barbiturase or the oxidative pathway is functioning in mammals. Nevertheless, together with that on the reductive pathway, knowledge on the oxidative pathway will provide some insights as to its physiological importance in nucleic acid metabolism.

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