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D-Arginase of *Arthrobacter* sp. KUJ 8602: Characterization and Its Identity with Zn^{2+}-Guanidinobutyrase

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D-Arginase activity was found in the cells of an isolate, *Arthrobacter* sp. KUJ 8602, grown in the L-arginine medium, and the enzyme was purified and characterized. Its molecular weight was estimated to be about 232,000 by gel filtration, and that of the subunit was approximately 40,000 by SDS-PAGE, suggesting that the enzyme is a homohexamer. The enzyme acted on not only D-arginine but also 4-guanidinobutyrate, 3-guanidinopropionate and even L-arginine. The V_{max}/K_m values for 4-guanidinobutyrate and D-arginine were determined to be 87 and 0.81 μmol/min/mg/mM, respectively. Accordingly, the enzyme is regarded as a kind of guanidinobutyrase [EC 3.5.3.7]. The pH optima for 4-guanidinobutyrate and D-arginine were 9.0 and 9.5, respectively. The enzyme was inhibited competitively by 5-aminovalerate, and thiol carboxylates such as mercaptoacetate served as strong mixed-type inhibitors. The enzyme contained about 1 g-atom of firmly bound Zn^{2+} per mol of subunit, and removal of the metal ions by incubation with 1,10-phenanthroline resulted in loss of activity. The inactivated enzyme was reactivated markedly by incubation with either Zn^{2+} or Co^{2+}, and slightly by incubation with Mn^{2+}. The nucleotide sequence of enzyme contains an open reading frame that encodes a polypeptide of 353 amino acid residues (M_r: 37,933). The predicted amino acid sequence contains sequences involved in the binding of metal ions and the guanidino group of the substrate, which show a high homology with corresponding sequences of Mn^{2+}-dependent amidinohydrolases such as agmatinase from *Escherichia coli* and L-arginase from rat liver, though the homology of their entire sequences is relatively low (24–43%).

Key words: amidinohydrolase, *Arthrobacter*, D-arginine, guanidinobutyrase, zinc.

Abbreviations: 4-GB, 4-guanidinobutyrate; GBase, guanidinobutyrase; CHES, *N*-cyclohexyl-2-amino-ethanesulfonic acid.

Several amidinohydrolases have recently been purified and characterized. Most are Mn^{2+}-dependent and catalyze the hydrolysis of guanidino compounds to the corresponding ω-amino compounds and urea. For example, L-arginase (L-arginine amidinohydrolase, EC 3.5.3.1) catalyzes the hydrolysis of L-arginine to L-ornithine and urea. L-Arginase is the final enzyme of the urea cycle in ureotelic animals, but it also plays an important role in regulation of arginine catabolism, affecting the metabolism of creatine, polyamines, proline and nitric oxide in various organisms. Agmatinase (agmatine amidinohydrolase, agmatine ureohydrolase, EC 3.5.3.11) catalyzes the hydrolysis of agmatine, a decarboxylation product from L-arginine, to putrescine and urea, and is found in bacteria (*1–3*), fungi (*4*), human liver (*5*), and rat brain (*6*). These amidinohydrolases are regarded as members of the arginase-family proteins: several important residues are highly conserved in their amino acid sequences. They diverged from an ancestral protein to show their particular substrate specificities (*7–10*). L-Arginases of rat liver (*11*) and *Bacillus caldovelox* (*12*) were crystallized, and their three-dimensional structures revealed the amino acid residues participating in binding of Mn^{2+} and substrate. Their catalytic mechanisms were also shown (*13*).

Guanidinobutyrase (GBase, 4-guanidinobutyrate amidinohydrolase, EC 3.5.3.7), which catalyzes the hydrolysis of 4-guanidinobutyrate (4-GB), a metabolite of L-arginine, was found in *Pseudomonas* strains (*14–16*), *Brevibacterium helvolum* (*17*), *Streptomyces grius* (*18*), tench liver (*19*), lizard liver (*20*), and chicken liver and kidney (*20*). The enzyme was highly purified from cells of *Pseudomonas* strains (*14–16*) and *Brevibacterium helvolum* (*17*) and enzymologically characterized, though its structure has not been studied.

The existence of D-arginase (D-arginine amidinohydrolase, EC 3.5.3.10) remains a matter of controversy. Kotake and Nakayama (*21*) and Mabuchi (*22*) showed that the extract of intestinal mucosa of rabbit hydrolyzed D-arginine more rapidly than the L-enantiomer, while Sano (*23*) succeeded in isolating the enzyme responsible for the hydrolysis of D-arginine, which they named "hetero-arginase" to distinguish it from L-arginase. Nadai

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(*24*) observed that the liver homogenates of human, rat and mouse had a high D-arginase activity but hydrolyzed L-arginine twice as rapidly as they did the D-enantiomer. Mora *et al.* (*20*) reported that the partially purified preparation of GBase from chicken liver also hydrolyzed D-arginine but was inert toward L-arginine. These mammalian amidinohydrolases acting on D-arginine, however, have not been so highly purified as to be characterized well, while microbial D-arginase has not been demonstrated.

We here describe the occurrence, purification, characterization and structure of an amidinohydrolase acting on D-arginine produced by an isolate, *Arthrobacter* sp. KUJ 8602, which utilizes D-arginine as a sole carbon and nitrogen source, and compare the enzyme with other amidinohydrolases.

MATERIALS AND METHODS

Materials—D-Homoarginine, 6-guanidinocaproate, and 5-guanidinovalerate were synthesized from the corresponding amino compounds by amidination with *S*-methylisothiourea sulfate according to Kimmel (*25*). D-Arginine monohydrochloride and L-homoarginine were the products of Watanabe Chemical Industry (Osaka). 4-GB, 3-guanidinopropionate and L-2-amino-3-guanidinopropionate were from Sigma Chemical (St. Louis, USA). All other reagents were from Nakalai Tesque (Kyoto) and Wako Pure Chemical Industries (Osaka).

Organism and Culture Conditions—*Arthrobacter* sp. KUJ 8602 was isolated from soil with a medium (pH 7.2) containing 0.01% ammonium sulfate, 0.01% yeast extract, 0.2% K_2HPO_4, 0.2% NaCl, 0.1% $MgCl_2 \cdot 6H_2O$, and 0.1% D-arginine hydrochloride, and identified taxonomically and by 16S rRNA analysis. After incubation with shaking at 30°C for 20 h, bacterial growth was followed by measuring turbidity at 660 nm. The enzymatic production of D-ornithine from D-arginine was determined by HPLC on a Crownpak CR (+) chiral column (Daicel Chem. Ind., Tokyo). For purification of the enzyme, the cells were grown in the medium (pH 6.0) consisting of 0.5% polypeptone, 0.05% yeast extract, 0.2% K_2HPO_4, 0.1% NaCl, 0.01% $MgCl_2 \cdot 6H_2O$, and 2.5% L-arginine hydrochloride, which was used as an inducer of enzyme production. The enzyme was inducibly produced by either L- or D-arginine. The cells were cultivated at 30°C for 18 h in 7 liters of medium in a 14-liter jar fermentor (Marubishi BioEng., Tokyo) with aeration and agitation. *Escherichia coli* (Nova Blue) was obtained from Novagen and grown aerobically at 37°C in Lusia-Bertani (LB) medium supplemented ampicillin (100 μg/ml). Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM.

Enzyme Assay—The standard assay mixture contained 60 mM CHES-NaOH buffer (pH 9.0), 20 mM 4-GB and enzyme in a final volume of 0.5 ml. The reaction mixture was preincubated at 37°C for 5 min without the substrate, and the reaction was initiated by addition of the substrate. After incubation for 15 min, 0.5 ml of 12.5% trichloroacetic acid was added to the solution to terminate the reaction. The mixture was centrifuged, and urea formed was colorimetrically determined (*26*). One unit of the enzyme is defined as the amount of enzyme that catalyzes the formation of 1 μmol of urea per min.

Protein Determination—Protein was determined by the method of Bradford (*27*) with bovine serum albumin as a standard.

Purification of Enzyme—Purification was carried out at about 4°C, unless otherwise stated.

1. Preparation of cell extract: Cells were washed twice with 10 mM potassium phosphate buffer (pH 6.2), and about 200 g (wet weight) of cells was suspended in 500 ml of 10 mM potassium phosphate buffer (pH 6.2). The suspension was divided into 50-ml portions, and each portion was subjected to ultrasonication for 20 min with ultrasonic disintegrator followed by ultracentrifugation (180,000 ×*g* for 1.5 h) to remove intact cells and cell debris. The unbroken cells were resuspended in the same buffer and sonicated for another 30 min.

2. Anion-exchange chromatography (1st): The cell extract was dialyzed against 10 mM potassium phosphate buffer (pH 6.2) containing 200 mM KCl and put on a column of a DEAE-Toyopearl 650M (2.5 cm × 25 cm) equilibrated with the same buffer. The column was then washed with the same buffer, then the absorbed proteins were eluted at a flow rate of 1.0 ml/min with the buffer containing 250 mM KCl and the buffer containing 1.0 M KCl, and 5-ml fractions were collected.

3. Anion-exchange chromatography (2nd): The active fractions were pooled and diluted with 10 mM potassium phosphate buffer (pH 6.2). The enzyme solution was put on a DEAE-Toyopearl 650M column (2.5 cm × 10 cm), and the column was washed with the buffer containing 200 mM KCl. The enzyme was eluted at a flow rate of 0.6 ml/min with a linear gradient of 200–250 mM KCl dissolved in 400 ml of the buffer, and 3-ml fractions were collected.

4. Hydroxyapatite chromatography: The enzyme solution was pooled and dialyzed against 10 mM potassium phosphate buffer (pH 6.0). The enzyme was put on a hydroxyapatite column (1.0 cm × 15 cm) equilibrated with the same buffer. The enzyme was eluted at a flow rate of 0.3 ml/min with a linear gradient of 400 ml of 10–400 mM potassium phosphate buffer (pH 6.0), and 2-ml fractions were collected.

5. Gel filtration chromatography: The active fractions were pooled and concentrated by ultrafiltration. The enzyme was put on a column of Superdex 200 HiLoad (16/60) with a PSLC system (Amersham Pharmacia Biotech.) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl. The enzyme was eluted with the same buffer, and 1-ml fractions were collected at a flow rate of 1.6 ml/min. The active fractions were pooled and dialyzed against 20 mM Tris-HCl buffer (pH 8.0).

Preparation of 1,10-Phenanthroline–Inactivated Enzyme—The enzyme (18 μg) was treated with 20 mM potassium phosphate buffer (pH 6.0) containing 15 mM 1,10-phenanthroline. After incubation at 37°C for 70 min, the mixture was dialyzed twice against 1 liter of 20 mM Tris-HCl buffer (pH 8.0) containing 10 μM EDTA to prevent a spontaneous reactivation at 4°C. The enzyme obtained was used as the 1,10-phenanthroline-inactivated enzyme.

Preparation of Enzyme Reconstituted with Zn^{2+}—The Zn^{2+}-reconstituted enzyme was prepared by incubation of the 1,10-phenanthroline-inactivated enzyme at 37°C for 30 min in 20 mM Tris-HCl buffer (pH 8.0) con-

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Table 1. **Purification of enzyme.**

Step	Total activity[a] (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell extract	222	2,890	0.077	100	1
DEAE-Toyopearl (1st)	135	42.3	3.19	61	40
DEAE-Toyopearl (2nd)	112	24.6	4.56	50	59
Hydroxyapatite	92.5	13.3	6.95	42	90
Superdex 200	73.4	8.43	8.71	33	113

[a]The enzyme activity was measured by using 20 mM D-arginine as the substrate.

taining 20 μM $ZnCl_2$, and the mixture was dialyzed against 20 mM Tris-HCl buffer (pH 8.0). The concentration of EDTA derived from the inactivated-enzyme preparation was 0.5 μM.

Determination of Metals Bound to Enzyme—The enzyme-bound metals were determined with an atomic absorption spectrophotometer, Hitachi Z 5000, by using of 24 μg of the enzyme dialyzed at 4°C for 12 h against two changes of 1,000 volumes of 20 mM Tris-HCl buffer (pH 8.0) passed through a column of Bio-Rad Chelex 100 in the $Tris^+$ form. The dialysate was used as a blank. Zn^{2+}, Co^{2+}, Mn^{2+}, and Ni^{2+} were spectrophotometry determined at wavelengths of 307.6, 252.1, 403.1, and 325.5 nm, respectively.

N-Terminal and Internal Amino Acid Sequence Analysis—The N-terminal and internal sequences of the enzyme were determined by Edman degradation with an automated sequencer (model 477A: Applied Biosystems). For N-terminal sequence analysis, approximately 20 μg of the enzyme dialyzed against water was used. To obtain the internal peptide, 20 mg of the enzyme was incubated with 5 M urea at 60°C for 60 min, and the denatured protein was incubated with 100 mM Tris-HCl buffer (pH 8.0), 4 M urea, 2.8 μg of trypsin, and 10 mM $CaCl_2$ at 37°C for 18 h. The internal peptides were separated by reverse-phase HPLC with a column Wakosil-II 5C18 AR (Wako), and the amino acid sequences of peptides were determined.

Isolation and Sequencing of Enzyme Gene—Chromosomal DNA from *Arthrobacter* sp. was isolated with DNeasy™ Tissue kit (Qiagen). Degenerated oligonucleotides, oligo 1, 2, 3, and 4, were designed on the basis of N-terminal MEELRIEA (residues 1–8), internal amino acid sequences VGVPFD (residues 45–50, on the internal peptide IP-1), NRPLYI (residues 239–244, on IP-2) and DIDVLD (residues 246–251, on IP-2), respectively. Oligo 1, 5′-ATG GAR GAR YTI AAY ATH GAR GC-3; oligo 2, 5′-GTI GGI GTI CCI TTY GAY-3′; oligo 3, 5′-DAT RTA IAR IGG ICK RTT-3′; and oligo 4, 5′-RTC DAT IAC RTC DAT RT-3′ (R = A or G; Y = C or T; H = A or C or T, I = inosine).

A 30-cycle PCR was performed with oligos 1 and 4 at an annealing temperature of 45°C (first cycle 40°C) for 3 min, followed by extension at 72°C for 1 min in the presence of 2.5 mM $MgCl_2$ with the *Arthrobacter* sp. chromosomal DNA as a template (1st PCR). With the 1st PCR products as a template, the 30-cycle PCR was performed with oligos 2 and 3 at an annealing temperature of 52°C (first cycle 40°C) for 3 min (2nd PCR). Other conditions were the same as those of the 1st PCR. The PCR was carried out with LA Taq polymerase (Takara) in Gene Amp PCR (PE Applied Biosystems). The 2nd PCR 600-bp product was extracted with Geneclean II kit (BIO 101 INC) and ligated into the pT7 Blue T-vector (Novagen) with a ligation kit (Takara). The resulting plasmid was transfected into competent cells of *E. coli* Novablue (Novagen). White Ap^r clone cells were picked up and grown in 5 ml of Luria-Bertani's broth (LB) containing ampicillin. The plasmid was purified and used as the template for sequencing.

Based on the partial 600-bp DNA sequence determined, four oligos were designed: oligo 5, 5′-TTT CGA TGG CCT CGT TGA TG-3′; oligo 6, 5′-CAA GGC GTT CTG CTG GAT GG-3′; oligo 7, 5′-CGC TTC GGT TTC GGA ATC GT-3′; and oligo 8, 5′-GAC GTC TAC TAC CAG GGC G-3′. Genome-walking PCR was performed with LA PCR™ *in vitro* cloning kit (Takara) according to the manufacturer's instructions. Chromosomal DNA from *Arthrobacter* sp. was digested with *Hin*dIII or *Eco*RI and ligated to a *Hin*dIII or *Eco*RI cassette, respectively. The resulting DNA fragments were used as the template for amplification by PCR. The conditions of PCRs were used in a same strategy, with annealing at 55°C for 30 s and extension at 72°C for 1 min. The PCR fragments were sequenced, and the entire enzyme gene sequence was determined.

RESULTS

Purification of Enzyme—Bacteria were screened for D-arginase production with a medium containing D-arginine as the sole carbon and nitrogen source, and *Arthrobacter* sp. KUJ 8602 isolated from soil was found to produce the enzyme. The enzyme was purified from the cells grown in a polypeptone-yeast extract medium containing 2.5% L-arginine as an inducer. D-Arginine can replace the L-enantiomer. A typical result of the purification is summarized in Table 1. The enzyme was purified to homogeneity with a yield of 33%.

Molecular Weight and Subunit Structure—The enzyme showed a single band upon SDS-PAGE, and its molecular weight was determined to be about 40,000. The enzyme migrated on the Superdex 200 column showing a molecular weight of about 232,000. Accordingly, the enzyme is composed of six subunits with identical molecular weight.

Substrate Specificity and Kinetic Parameters for Substrate—The enzyme showed considerably high substrate specificity (Table 2). The best substrate was 4-GB, followed by D-arginine and 3-guanidinopropionate: accordingly the enzyme is regarded as a kind of guanidinobutyrase (GBase, EC 3.5.3.7). It showed very low activity toward 3-guanidinopropionate and 5-guanidinovalerate, lower and higher homologues of 4-GB, and was inert toward guanidinoacetate, L-2-amino-3-guanidinopropionate and creatine (less than 0.1% that observed for 4-GB). 4-GB was

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Table 2. **Substrate specificities and kinetic parameters for substrate.**

Substrate	Relative activity[a] (%)	K_m (mM)	V_{max} (μmol/min/mg)	V_{max}/K_m (μmol/min/mg/mM)
D-Homoarginine	0.6	33.0	2.66	0.081
6-Guanidinocaproate	0.32	–[b]	–	–
L-Homoarginine	0.26	22.3	0.817	0.036
D-Arginine	5.4	22.0	17.8	0.81
5-Guanidinovalerate	1.4	1.92	2.43	1.3
L-Arginine	0.92	20.7	3.81	0.18
4-Guanidinobutyrate	100	2.10	182	87
3-Guanidinopropionate	2.1	12.0	5.22	0.44
L-2-Amino-3-guanidinopropionate	0	–	–	–
Guanidinoacetate	0	–	–	–
L-Canavanine	0.57	–	–	–
Agmatine	0.70	–	–	–
Taurocyamine	0.48	–	–	–
Creatine	0	–	–	–

The enzyme activity was measured by using various guanidino compounds (20 mM) as the substrate under the standard assay conditions. The amount of enzyme used were 0.024 unit for 4-GB and 0.096 unit for other compounds. [a]Values below 0.1 are expressed as 0. [b]–, not determined.

also outstandingly reactive on the basis of the catalytic efficiency. The ratio of activity for D-arginine (D-arginase activity) to that for L-arginine (L-arginase activity) of the cell free extract was approximately the same as that of the purified enzyme.

Effect of pH on Enzyme Activity—The effect of pH on the activity is shown in Fig. 1. The maximum activity was observed in the wide range of pH 7.5–9.5 for the reaction with 4-GB, but the pH optima for the reactions with D- and L-arginine were in narrow pH ranges of around 9.5 and 10.0, respectively.

Inhibitor—The effect of various compounds on the enzyme activity was examined under the standard assay conditions (Table 3). ω-Amino acids and short chain fatty acids inhibited the enzyme. 5-Aminovalerate and *n*-valerate competitively inhibited the enzyme, and the apparent K_i values were 0.31 and 3.5 mM, respectively. Thiol compounds such as mercaptoacetate, 3-mercaptopropionate, and 2-mercaptoethanol strongly inhibited the enzyme. Mercaptoacetate (0.2 mM) and mercaptopropionate (5 mM) inhibited about 97% of the activity, and the inhibition found to be a mixed type. α-Amino acids (*e.g.*,

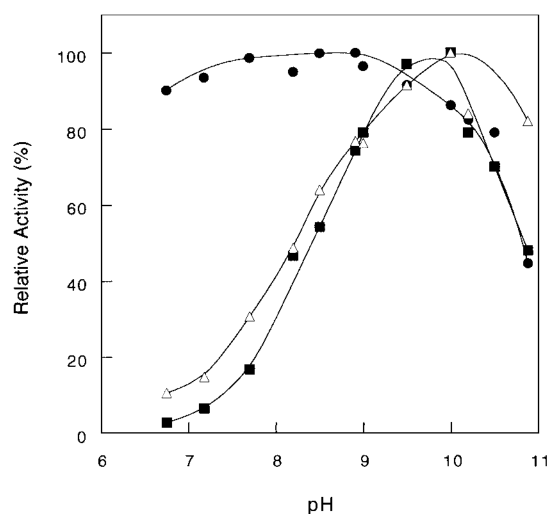

Fig. 1. **Effect of pH on enzyme activity.** The enzyme activity was measured in the reaction mixture containing a buffer at a concentration of 60 mM. The buffers were Tris-HCl, pH 6.8–8.9; CHES-NaOH, pH 9.0–10.0; and sodium carbonate, pH 10.0–10.9. All buffers were prepared at 37°C. The activity for 4-guanidinobutyrate (solid circle) was measured with 0.048 unit (0.3 μg) of the enzyme, and those for D-arginine (solid square) and L-arginine (open triangle) were measured with 0.096 unit (0.6 μg) of the enzyme.

Table 3. **Effect of various compounds on enzyme activity.**

Compound	Relative activity (%)
None	100
6-Aminocaproate	82
5-Aminovalerate	46
4-Aminobutyrate	80
β-Alanine	94
Glycine	92
Taurine	94
D-Lysine	93
L-Lysine	85
D-Ornithine	93
L-Ornithine	87
Putrescine	93
1,3-Propanediamine	95
n-Caproate	95
n-Valerate	73
n-Butyrate	64
Propionate	82
Acetate	95
3-Mercaptopropionate	3.0
D-Cysteine	80
L-Cysteine	83
2-Mercaptopropionate	60
Mercaptoacetate	1.3
2-Mercaptoethanol	15
Cysteamine	29

The compounds were added to the standard assay mixture at the final concentrations of 5.0 mM.

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Table 4. **Effect of metal chelating reagent on enzyme.**

Metal chelating reagent	Buffer	Residual activity (%)
None	Potassium phosphate (pH 6.0)	86
	Tris-HCl (pH 8.0)	93
	CHES-NaOH (pH 10.0)	100
EDTA	Potassium phosphate (pH 6.0)	57
	Tris-HCl (pH 8.0)	84
	CHES-NaOH (pH 10.0)	90
1,10-Phenanthroline	Potassium phosphate (pH 6.0)	22
	Tris-HCl (pH 8.0)	63
	CHES-NaOH (pH 10.0)	70
EGTA	Tris-HCl (pH 8.0)	98
2,2′-Dipyridyl	Tris-HCl (pH 8.0)	88

The enzyme (0.9 μg) was incubated with various 20 mM buffers containing 25 mM metal chelating reagents at 37°C for 40 min. After the incubation, 0.05-ml portions of the mixtures were diluted five times with cold 20 mM Tris-HCl buffer (pH 8.0), then the enzyme activity was determined. The initial activity was taken as 100%.

cysteine, ornithine, and lysine) hardly inhibited the enzyme.

Inactivation by Metal Chelating Reagents and Reactivation by Metal Ions—The effect of metal chelating reagents on the enzyme activity was examined (Table 4). Among various chelating reagents tested, 1,10-phenanthroline most effectively inhibited the enzyme in Tris-HCl buffer (pH 8.0): more than 35% of the original activity was lost. Other reagents such as 2,2′-dipyridyl, EGTA and EDTA were less inhibitory. 1,10-Phenanthroline inhibited the enzyme effectively in potassium phosphate buffer (pH 6.0). The enzyme was fully inactivated by incubation with 15 mM 1,10-phenanthroline in 20 mM potassium phosphate buffer (pH 6.0) at 37°C for 70 min.

The enzyme was showed almost the full activity without addition of any metal ions (at the concentration of 0.1 and 0.5 mM). The effect of divalent metal ions on the 1,10-phenanthroline-inactivated enzyme was examined by incubation with various metal chlorides (Fig. 2). The inactivated enzyme was markedly reactivated by addition of either Zn^{2+} or Co^{2+}. The addition of Mn^{2+} and Ni^{2+} (100 μM) was less effective: about 32 and 15% of initial activity was recovered, respectively. Fe^{2+}, Mg^{2+}, Ca^{2+}, and Cu^{2+} were inactive. The inactivated enzyme showed the maximum activity on addition of 20 μM Zn^{2+}, though higher concentrations of Zn^{2+} were inhibitory.

Contents of Zn^{2+}, Co^{2+}, Mn^{2+}, and Ni^{2+} were determined by atomic absorption analysis. The native enzyme contained 0.82 g-atom of Zn^{2+} and a small amount of contaminating Mn^{2+}, while Co^{2+} and Ni^{2+} were not detected. The 1,10-phenanthroline-inactivated enzyme contained substantially no Zn^{2+} and Mn^{2+}. The Zn^{2+}-reconstituted enzyme contained 1.03 g-atom Zn^{2+} per subunit and showed the same K_m and V_{max} values as the native enzyme (Table 5). These results indicated that Zn^{2+} was bound specifically to the enzyme and was essential for catalysis. Accordingly, the 1,10-phenanthroline-inactivated enzyme is an apoform.

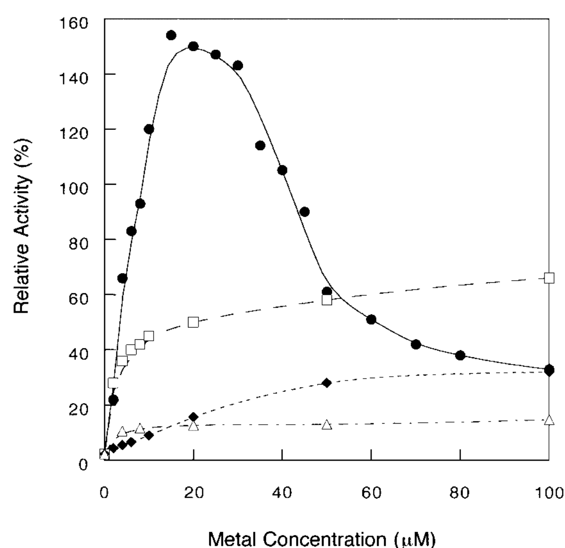

Fig. 2. **Effect of divalent metal ions on the 1,10-phenanthroline-inactivated enzyme.** The inactivated enzyme (0.45 μg) was incubated at 37°C for 20 min with various concentrations of $ZnCl_2$ (solid circle), $CoCl_2$ (open square), $MnCl_2$ (solid diamond), and $NiCl_2$ (open triangle) in 20 mM Tris-HCl buffer (pH 8.0), then the enzyme activity was measured.

Sequencing of N-Terminal Region and Internal Region—The N-terminal amino acid sequence of the enzyme is MEELRIEAXGXLG, the identity of the 9th and 11th residues being uncertain. This shows no similarity to the sequence of the N-terminal region of several amidinohy-

Table 5. **Contents of metal ions bound to enzyme.**

Enzyme	Metal content[a] (g-atom/subunit)		Specific activity (U/mg)		K_m (mM)		V_{max} (μmol/min/mg)	
	Zn^{2+}	Mn^{2+}	4-GB	D-Arg	4-GB	D-Arg	4-GB	D-Arg
Native	0.82	0.048	160	8.6	2.1	22	182	17.8
1,10-Phenanthroline-inactivated	0.0062	<0.001	<0.1	<0.01	–[b]	–	–	–
Zn^{2+}-reconstituted	1.03	<0.001	140	6.6	2.3	22	166	16.7

[a]Metal content was determined by atomic absorption spectroscopy as outlined under "MATERIALS AND METHODS". [b]–, not determined.

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-150 TTTTTATGGAATCTTTACGCCTGCTGTGAGCGGCGTCTCATCCTTTTGGAAGGTTGTAAA  -91

 -90 CAAGTGATGCATATTAGACAACTTTGAGTGTATGATGGGTGTCACAGTCCGCAGAATCAA  -31

 -30 TCCCCCTATTCACTCAAGGAGTGGCGTACTTTGGAAGAGCTCCGCATCGAGGCCAACGGC   30
   1                                M  E  E  L  R  I  E  A  N  G   10

  31 AACCTGGGCCCCATCGATTCATCCCGTATCCCGCGCTACGCCGGTGCCGCCACGTATGCC   90
  11  N  L  G  P  I  D  S  S  R  I  P  R  Y  A  G  A  A  T  Y  A   30

  91 CGCCTGCCCCGCCTGGACCAGGtCTCCAAGGCCGACGTCACCGTGGTCGGCGTTCCCTTC  150
  31  R  L  P  R  L  D  Q  V  S  K  A  D  V  T  V  V  G  V  P  F   50
                                 IP-1
 151 GACTCCGGCGTTTCGTACCGCCCCGGAGCCCGCTTCGGCGCCAACCACGTGCGTGAGGCC  210
  51  D  S  G  V  S  Y  R  P  G  A  R  F  G  A  N  H  V  R  E  A   70

 211 AGCCGTCTGCTCCGCCCGTACAACCCGGCCTGGGACGTCAGCCCGTTCGAGAACATCCAG  270
  71  S  R  L  L  R  P  Y  N  P  A  W  D  V  S  P  F  E  N  I  Q   90

 271 GTAGCCGACGCCGGCGACATGGCAGTGAACCCGTTCAACATCAACGAGGCCATCGAAACC  330
  91  V  A  D  A  G  D  M  A  V  N  P  F  N  I  N  E  A  I  E  T  110

 331 ATCCAGCAGAACGCCTTGGACCTCACGGCCAACGGCAGCAAGCTGGTCACCCTCGGCGGC  390
 111  I  Q  Q  N  A  L  D  L  T  A  N  G  S  K  L  V  T  L  G  G  130

 391 GACCACACCATCGCCCTCCCGCTGCTCCGGGCAGCAGCGGAACGCGCAGGCGAACCCATC  450
 131  D  H  T  I  A  L  P  L  L  R  A  A  A  E  R  A  G  E  P  I  150

 451 GCCATGCTGCACTTTGATGCCCACCTGGATACCTGGGACACCTACTTTGGCGCCGAATAC  510
 151  A  M  L  H  F  D  A  H  L  D  T  W  D  T  Y  F  G  A  E  Y  170

 511 ACGCACGGTACGCCGTTCCGCCGGGCCGTCGAAGAAGGCATCCTGGACACGGAGGCCATC  570
 171  T  H  G  T  P  F  R  R  A  V  E  E  G  I  L  D  T  E  A  I  190

 571 AGCCACGTCGGTACCCGCGGTCCGCTGTACGGCAAGAAGGACCTCGACGACGACCACCGC  630
 191  S  H  V  G  T  R  G  P  L  Y  G  K  K  D  L  D  D  D  H  R  210

 631 TTCGGTTTCGGAATCGTCACCTCCGCCGACGTCTACTACCAGGGCGTCCTGGAGACGGTG  690
 211  F  G  F  G  I  V  T  S  A  D  V  Y  Y  Q  G  V  L  E  T  V  230

 691 GCAAAGATCCGCGACCGCATCGGCAACCGCCCGCtGTACATCTCGGTGGACATCGATGTC  750
 231  A  K  I  R  D  R  I  G  N  R  P  L  Y  I  S  V  D  I  D  V  250
                 IP-2
 751 CTTGATCCGGCCCACGCCCCCGGCACCGGCACCCCCGAAGCAGGCGGCATCACCAGCCGC  810
 251  L  D  P  A  H  A  P  G  T  G  T  P  E  A  G  G  I  T  S  R  270

 811 GAACTGCTGGAGATCATCCGCGGCTTCCGCGGCATGAACCTCGTTGGCGCGGACGTGGTG  870
 271  E  L  L  E  I  I  R  G  F  R  G  M  N  L  V  G  A  D  V  V  290

 871 GAAGTCGCCCCGGCCTACGACCACGCAGAAATCACCGGAGTCGCCGGCAGCCACGTTGCC  930
 291  E  V  A  P  A  Y  D  H  A  E  I  T  G  V  A  G  S  H  V  A  310

 931 TATGAACTGGTGACCCTCATGGCAGACAACGCCGTTGAAGGTGACCGCCACGGTGCCCCG  990
 311  Y  E  L  V  T  L  M  A  D  N  A  V  E  G  D  R  H  G  A  P  330
        IP-3
 991 AACGGcTACGCGCAGCAGGCCCTCGGCGCCCGCATCCAGGAAGTCGCACAGGCCATCGGA 1050
 331  N  G  Y  A  Q  Q  A  L  G  A  R  I  Q  E  V  A  Q  A  I  G  350

1051 GGGCAGCGATGATCGATTTCGATCCTGGCACAGCAGCTCCCACCAAGGGGACCAACCAGC 1110
 351  G  Q  R  *                                                   353

1111 GCAACGGTGGGGACCTCGTCGTCGAGACCCTTGAAGCGCTGGGTGCCAAGACTGTTTTTG 1170

1171 GTATCCCGGGCCAGCACGCCCTGGGTCTCTTTGATGCCATGGGCCGCGGCAACCTGCACT 1230
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Fig. 3. **Nucleotide and deduced amino acid sequences of the guanidinobutyrase gene of *Arthrobacter* sp. KUJ8602.** The deduced amino acid sequence is shown below the corresponding nucleotide sequence. The amino acids sequenced by Edman degradation of N-terminal and internal peptides (IP-1, 2 and 3) obtained after trypsin-digestion of the enzyme are boxed. The potential –35 and –10 promoter regions are single-underlined. The probable Shine-Dalgarno sequence is double-underlined. The initial and terminal codons are highlighted in bold type. The arrows indicate inverted repeat sequences.

drolases. Amino acid sequences of internal peptides (IP-1, -2, and -3) formed by trypsin-digestion were ADVTVVGVPFDXGV (IP-1), IGNPLYISVDIDVLD (IP-2), and ELVTLMADNAVXGDXY (IP-3). Internal peptides IP-1 and -2 showed similarity to those of some L-arginases and agmatinases (Figs. 3 and 4).

Molecular Cloning of the Enzyme Gene—PCR primers were designed from the sequences of the N-terminal region and IP-1 and -2 peptides. The amplified DNA fragment of about 600 bp was cloned into a pT7 Blue T-vector and nucleotides were sequenced. The results showed an open reading frame lacking initiation and terminal codons. The overlapping segments of about 600 bp for the upstream region and 740 bp for the downstream region were amplified by a genome-walking PCR method, and nucleotides were sequenced to obtain the entire gene.

The nucleotide sequence and the deduced amino acid sequence are shown in Fig. 3. The enzyme gene consists of an open reading frame of 1,059 bp encoding a protein of 353 amino acid residues. The upstream region contained no ATG initial codon, but it did include a TTG codon and an AG-rich region at nucleotide –15 to –10 (AAGGAG), which could be considered to be a Shine-Dalgarno sequence. The molecular weight of the enzyme was estimated to be 40,000 by SDS-PAGE, and calculated to be 37,933 from the amino acid sequence data. The deduced N-terminal 13 amino acid sequence corresponded exactly to that of the enzyme protein as analyzed from the initial methionine by use of a peptide sequencer. Therefore, the initiation codon of the gene was estimated to be an unusual codon, TTG. All the internal peptide fragments were also contained in the deduced amino acid sequence. The –35 and –10 regions were located at nucleotides –81 to –76 (CATATT) and –60 to –55 (TATGAT), respectively. Inverted repeat sequences were found at positions 1056–1079 and 1125–1136 downstream of the stop codon, TGA. This inverted repeat sequence is presumably involved in regulating the termination of translation.

Comparison of Amino Acid Sequences of the Enzyme and Amidinohydrolases—The deduced amino acid sequence of the *Arthrobacter* GBase was aligned with those of several other amidinohydrolases (Fig. 4). The alignment was performed by using the program CLUSTAL W (*28*) and by manually editing the regions containing many non-contiguous gaps. The enzyme contained similar amino acid sequences to those of several amidinohydrolases. Sequence identities with GBase from *Pseudomonas aeruginosa*, guanidinoproclavaminate amidinohydrolase from *Streptomyces clavuligerus*, agmatinases from human and *E. coli*, agmatinase from *B. subtilis*, and L-arginases from human liver, rat liver, *B. caldovelox,* and *B. subtilis* were 41, 43, 40, 36, and 24–29%, respectively. No cysteinyl residues were found in the *Arthrobacter* enzyme.

DISCUSSION

The extract of *Arthrobacter* sp. cells grown in the L-arginine medium showed not only D-arginase activity but also L-arginase and GBase activities, and extracts from the cells grown in the D-arginine and 4-GB media exhibited closely similar activities (data not shown). We determined arginine racemase activities of the extracts of the cells grown in the D- or L-arginine medium according to Yorifuji *et al.* (*38*), except for addition of 1 mM mercaptoacetate to inhibit the D-arginase activity, but found neither racemase activity. Accordingly, both enantiomers of arginine and 4-GB serve as an inducer.

The purified enzyme exhibited a relatively high substrate specificity. It catalyzes the hydrolysis of guanidino compounds with 2–4 carbon chains. The enzyme acts on 4-GB, D-arginine, 3-guanidinopropionate, 5-guanidino-

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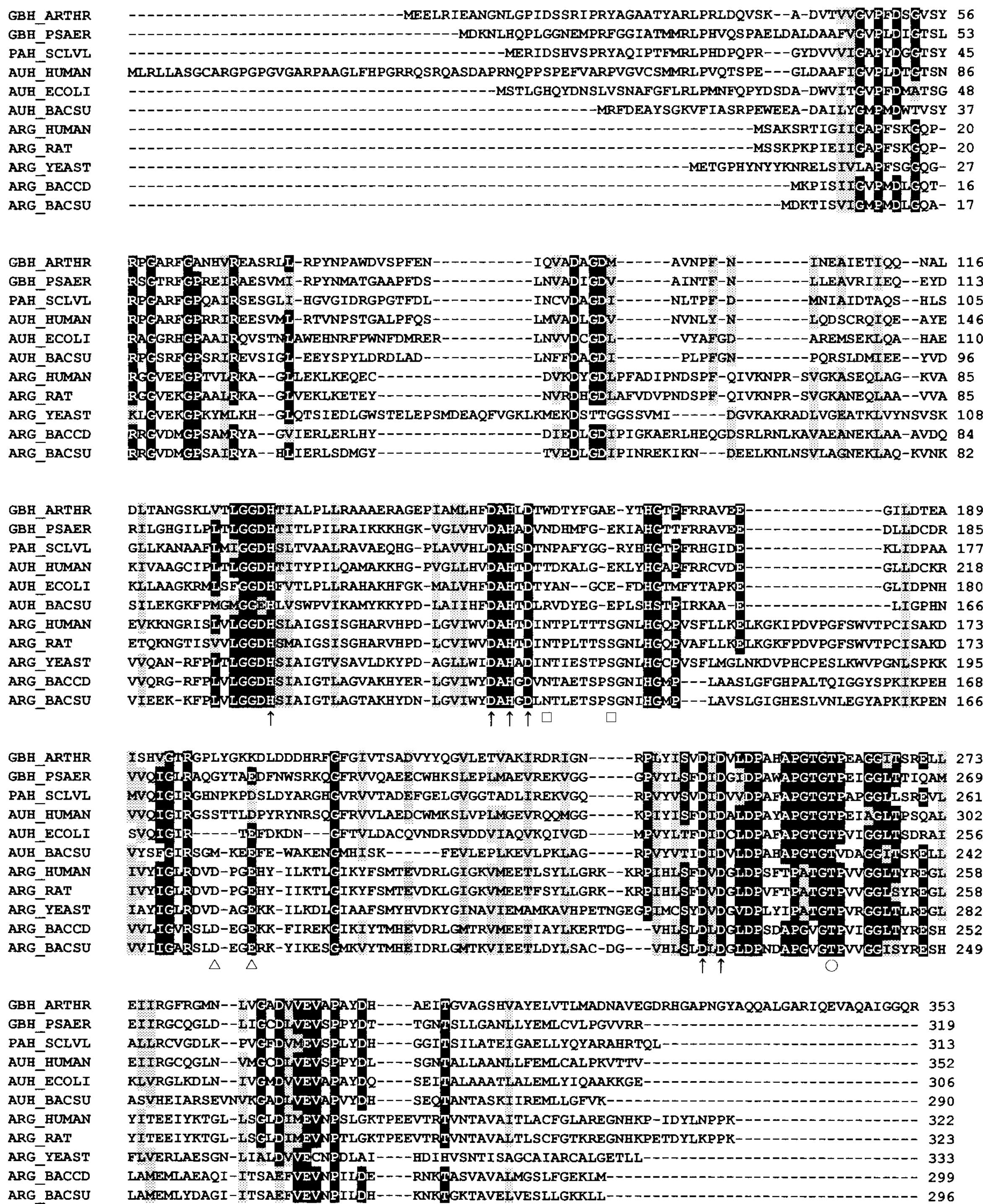

Fig. 4. **Multiple alignment of amidinohydrolases.** Individual sequences are designated as follows: GBH_ARTHRO, *Arthrobacter* sp. GBase; GBH_PSAER, *Pseudomonas aeruginosa* GBase (*29*, *16*); PAH_SCLVL, *Streptomyces clavuligeus* guanidinoproclavaminate amidinohydrolase (*30*); AUH_HUMAN, human liver agmatinase (*5*); AUH_ECOLI, *Escherichia coli* agmatinase (*31*); AUH_BACSU, *Bacillus subtilis* agmatinase (*32*); ARG_HUMAN, human liver arginase (*33*), ARG_RAT; Rat liver arginase (*34*); ARG_YEAST, *Saccharomyces cerevisiae* arginase (*35*); ARG_BACCD, *Bacillus caldovelox* arginase (*36*); ARG_BACSU, *Bacillus subtilis* arginase (*37*). Identical and similar residues in more than 70% of the sequences are drawn on black and gray, respectively. The arrows, circles, triangles and squares indicate the residues of the rat liver and *B. caldovelox* arginases involved in the binding of metal ions, guanidino group, α-amino group and α-carboxyl group, respectively, of substrate.

varelate, and L-arginine in that order, and K_m and V_{max} values for these compounds suggest that the natural substrate is 4-GB. Accordingly, the enzyme is regarded as a kind of amidinohydrolase, namely, a guanidinobutyrase (GBase, EC 3.5.3.7). Almost all of these enzymes catalyze reactions with only one of an enantiomeric or diastereo-

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meric pair of chiral compounds; exceptions are racemases and epimerases [EC class 5.1], which usually equally act on both isomers. Glutaminase [EC 3.5.1.2] from *Pseudomonas aeruginosa* (*39*) and glutaminase-asparaginase [EC 3.5.1.38] from *Achromobacteraceae* (*40*) also catalyze the hydrolysis of both enantiomers of glutamine, and glutamine and asparagine, respectively, though the L-enantiomers are much more rapidly hydrolyzed (the ratio of activities for the L-form and D-form: 100/26–90). Glutamine and asparagine are enzymatically hydrolyzed at an amide moiety at a distance from a chiral α-carbon, and arginine is similar in this regard. Accordingly, the substrates of *Arthrobacter* enzyme are probably bound loosely to the active center, resulting in the low stereospecificity of the enzyme. Furthermore, D-arginine and D-homoarginine are much more reactive than their L-enantiomers, and also 4-guanidinovalerate and 5-guanidinocaproate, respectively. This suggests that a negatively charged group is located at a position favorable to binding to an amino group in D-configuration. Moreover, agmatine and taurocyamine are more slowly hydrolyzed than 5-guanidinovarelate and 3-guanidinopropionate, respectively. *n*-Butyrate and *n*-valerate as well as 5-aminovalerate inhibit the enzyme competitively, and putrescine little inhibits the enzyme. These results suggested that an α-carboxyl group and a short alkyl chain in the substrate are essential for binding to the active center of enzyme. This is supported by the fact that 3-mercaptopropionate acted more strongly as a mixed-type inhibitor than 2-mercaptoethanol and cysteamine. We have to await elucidation of the three-dimensional structure of the enzyme to show the relationship between the substrate structure and the reactivity.

The *Arthrobacter* enzyme shows a pH optimum between pH 9.0 and 10.0 as reported for several kinds of L-arginases (*41–43*), some other GBases (*14–17*, *19*) and glycocyaminase (EC 3.5.3.2, guanidinoacetate amidinohydrolase) (*44*). The lower activity for D- and L-arginine below pH 9.0 is probably due to the positive charge of the α-amino group of the substrates. The *Arthrobacter* enzyme is unique in showing activity for 4-GB over a broad pH range (6.8–9.5), the other amidinohydrolases mentioned above being active only in a narrow range.

The enzyme showed practically full activity without addition of any metal ions, though various amidinohydrolases such as L-arginases (*41–43*, *45*, *46*), GBase (*14–16*) and glycocyaminase (*44*) from *Pseudomonas* strains require Mn^{2+} for the full activity. The *Arthrobacter* enzyme probably contains more tightly bound metal ions. It contained about 1 atom of Zn^{2+} per subunit which is essential for catalysis, and removal of the metal ions from the enzyme protein by incubation with 1,10-phenanthroline resulted in loss of activity. The inactivated enzyme was reactivated by binding of 1 atom of Zn^{2+} added per subunit. Mn^{2+} is less effective for the reactivation than Zn^{2+}. Mn^{2+}-dependent amidinohydrolases such as L-arginases from *Bacillus subtilis* (*47*), *Bacillus anthracis* (*48*), *Rhodobacter capsulatus* (*49*), and human liver (*46*), and agmatinase from *E. coli* (*50*) are inhibited by Zn^{2+}, though the rat liver arginase (*51*), and GBase and glycocyaminase from *Pseudomonas* strains (*15*, *44*) are only slightly susceptible. Zn^{2+} has not been reported as a specific activator of these Mn^{2+}-enzymes. In contrast, a similar enzyme, GBase containing tightly bound Zn^{2+}, has been purified from *Brevibacterium helvolum* (*17*). Both *Arthrobacter* and *Brevibacterium* are members of the group of coryneform bacteria, which are phylogenetically close to the actinomycete group and remote from the fluorescent *Pseudomonas* group. The *Arthrobacter* and *Brevibacterium* GBases hydrolyze D- and L-arginine, and 3-guanidinopropionate, whereas the Mn^{2+}-GBases from *Pseudomonas* strains do not act on these compounds: they exhibit high substrate specificities. The *Arthrobacter* and *Brevibacterium* GBases are composed of subunits with molecular weight of 40,000 and 45,000, respectively, which are larger than those of the *Pseudomonas* GBases (about 35,000) and other Mn^{2+}-enzymes including the *E. coli* agmatinase (33,500) and various L-arginases (31,000–35,000). The Zn^{2+}-enzymes are different from Mn^{2+}-enzymes in properties and distribution.

The *Arthrobacter* GBase is the first Zn^{2+}-type amidinohydrolase for which the entire amino acid sequence has been determined. The sequence is most highly homologous with those of GBase from *Ps. aeruginosa* and guanidinoproclavaminate amidinohydrolase from *S. clavuligerus*, and similar to those of various agmatinases. The sequence similarity of the enzyme with L-arginases was relatively low. The most highly conserved sequences are -LGGDH-, -DAHXD-, and -DIDXLDP-, which are well conserved in most of the amidinohydrolases. It is suggested that four aspartyl and two histidyl residues participate in binding of two atoms of Mn^{2+} with the protein based on the three-dimensional structure of arginases from rat liver (*11*) and *B. caldovelox* (*12*). Interestingly, these sequences are found in the *Arthrobacter* enzyme containing 1 atom Zn^{2+} per subunit. L-Arginase from *Saccharomyces cerevisiae* contains 1 atom of loosely bound Mn^{2+} per subunit participating in catalysis and 1 atom of tightly bound Zn^{2+} that plays an important role in structural stabilization, and it is suggested that the latter Zn^{2+} may bind to a zinc finger-like element containing two cysteinyl residues (*52*). In contrast, no such an element is found in the *Arthrobacter* enzyme. It was reported that two Mn^{2+} ions bind with the rat liver arginase to form a Mn-water-Mn bridge, and one Mn^{2+} activates a bound water molecule by ionizing it to a hydroxide anion (*13*). The hydroxide anion attacks nucleophilically the carbon of the guanidino group and cleaves the C^{ε}-N^{δ} bond (*13*). This mechanism observed for L-arginase is similar to those of leucine aminopeptidase [EC 3.4.11.1] (*54*) and β-lactamase [EC 3.5.2.6] (*55*). The active sites of these enzymes potentially bind two Zn^{2+} ions. The activated water bound to one Zn^{2+} attacks the carbonyl carbon of a peptide bond or β-lactam of each substrate, and the C-N bond is finally hydrolyzed. Moreover, the *Arthrobacter* enzyme conserves the amino acid residues of the metal (Mn)-binding site of L-arginase. These facts suggest that the conserved amino acid residues of *Arthrobacter* enzyme probably participate in Zn-binding, and the reaction mechanism of these metalloenzymes are probably similar, though there is no clear relationship between the primary structure and the metal-binding ability.

Most amidinohydrolases, including the *Arthrobacter* enzyme contain a histidyl residue corresponding to His141 of the rat liver arginases. Site-directed mutagen-

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esis suggests that this residue (His172 of the *Arthrobacter* enzyme) probably acts as a proton shuttle in catalysis (*53*).

A study of the three-dimensional structure of the *B. caldovelox* arginase bound with L-arginine revealed that some residues participate in binding of L-arginine (*12*). Glu271 and Thr260 in the *B. caldovelox* arginase bind to the guanidino group of L-arginine, and both residues are conserved as Glu291 and Thr261 in the *Arthrobacter* enzyme. In the *B. caldovelox* arginase, Ser135 and Asn128 form hydrogen bonds with the α-carboxylate oxygen of L-arginine, Glu181 and Asp178 bind the α-amino group of L-arginine. Sequence reveals that these amino acid residues are conserved in all arginases, but are either not found, or replaced by other amino acid residues in the *Arthrobacter* enzyme and other enzymes except L-arginases.

Thus, the amino acid residues participating in binding of guanidino group of the substrate, metal binding and activation of a water molecule are conserved in the *Arthrobacter* enzyme, though the homology between the *Arthrobacter* enzyme and L-arginases so far studied is not high. Therefore, the Zn^{2+}-GBase of *Arthrobacter* sp. probably diverged from a common ancestral protein to L-arginases. We are now studying the enzyme by site-directed mutagenesis and crystallography to further clarify the functions of these conserved amino acid residues.

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REFERENCES

1. Satishchandran, C. and Boyle, S.M. (1986) Purification and properties of agmatine ureohydrolyase, a putrescine biosynthetic enzyme in *Escherichia coli. J. Bacteriol.* **165**, 843–848
2. Tricot, C., Pierard, A., and Stalon, V. (1990) Comparative studies on the degradation of guanidino and ureido compounds by *Pseudomonas*. *J. Gen. Microbiol.* **136**, 2307–2313
3. Sekowska, A., Bertin, P., and Danchin, A. (1998) Characterization of polyamine synthesis pathway in *Bacillus subtilis* 168. *Mol. Microbiol.* **29**, 851–858
4. Boldt, A., Miersch, J., and Reinbothe, H. (1971) Metabolism of agmatine in fruit-bodies of the fungus *Panus tigrinus* (Bull. ex Fr.) Sing. *Phytochemistry* **10**, 731–738
5. Mistry, S.K., Burwell, T.J., Chambers, R.M., Rudolph-Owen, L., Spaltmann, F., Cook, W.J., and Morris, S.M. Jr. (2002) Cloning of human agmatinase. An alternate path for polyamine synthesis induced in liver by hepatitis B virus. *Am. J. Physiol. Gastrointest. Liver Physiol.* **282**, G375–381
6. Sastre, M., Regunathan, S., Galea, E., and Reis, D.J. (1996) Agmatinase activity in rat brain: a metabolic pathway for the degradation of agmatine. *J. Neurochem.* **67**, 1761–1765
7. Jenkinson, C.P., Grody, W.W., and Cederbaum, S.D. (1996) Comparative properties of arginases. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **114**, 107–132
8. Ouzounis, C.A. and Kyrpides, N.C. (1994) On the evolution of arginases and related enzymes. *J. Mol. Evol.* **39**, 101–104
9. Perozich, J., Hempel, J., and Morris, S.M. Jr. (1998) Roles of conserved residues in the arginase family. *Biochim. Biophys. Acta* **1382**, 23–37
10. Sekowska, A., Danchin, A., and Risler, J.L. (2000) Phylogeny of related functions: the case of polyamine biosynthetic enzymes. *Microbiology* **146**, 1815–1828
11. Kanyo, Z.F., Scolnick, L.R., Ash, D.E., and Christianson, D.W. (1996) Structure of a unique binuclear manganese cluster in arginase. *Nature* **383**, 554–557
12. Bewley, M.C., Jeffrey, P.D., Patchett, M.L., Kanyo, Z.F., and Baker, E.N. (1999) Crystal structures of *Bacillus caldovelox* arginase in complex with substrate and inhibitors reveal new insights into activation, inhibition and catalysis in the arginase superfamily. *Structure* **7**, 435–448
13. Khangulov, S.V., Sossong, T.M. Jr., Ash, D.E., and Dismukes, G.C. (1998) L-Arginine binding to liver arginase requires proton transfer to gateway residue His141 and coordination of the guanidium group to dimanganese (II, II). *Biochemistry* **37**, 8539–8550
14. Chou, C.S. and Rodwell, V.W. (1972) Metabolism of basic amino acids in *Pseudomonas putida*. γ-Guanidinobutyrate amidinohydrolase. *J. Biol. Chem.* **247**, 4486–4490
15. Yorifuji, T., Kato, M., Kobayashi, T., Ozaki, S., and Ueno, S. (1980) 4-Guanidinobutyrate amidinohydrolase from *Pseudomonas* sp. ATCC 14676: Purification to homogeneity and properties. *Agric. Biol. Chem.* **44**, 1127–1134
16. Nakada, Y. and Itoh, Y. (2002) Characterization and regulation of the gbuA gene, encoding guanidinobutyrase in the arginine dehydrogenase pathway of *Pseudomonas aeruginosa* PAO1. *J. Bacteriol*. **184**, 3377–3384
17. Yorifuji, T., Shimizu, E., Hirata, H., Imada, K., Katsumi, T., and Sawamura, S. (1992) Guanidinobutyrase for L-arginine degradation in *Brevibacterium helvolum*. *Biosci. Biotechnol. Biochem.* **56**, 773–777
18. Thoai, N.V., Thome-beau, F., and Olomucki, A. (1966) Induction et spécificité des enzymes de la nouvelle voie catabolique de L'arginine. *Biochim. Biophys. Acta* **115**, 73–80
19. Campo, M.L., Fuentes, J.M., and Soler, G. (1992) An arginine regulated γ-guanidinobutyrate ureahydrolase from tench liver (*Tinca tinca* L.). *Arch. Int. Physiol. Biochim. Biophys.* **100**, 55–60
20. Mora, J., Tarrab, R., Martuscelli, J., and Soberon, G. (1965) Characteristics of arginases from ureotelic and non-ureotelic animals. *Biochem. J*. **96**, 588–594
21. Kotake, Y. and Nakayama, Y. (1937) Chounennmaku arginase no kenkyuu (in Japanese). *Osaka Igakkai Zasshi* **37**, 543–544
22. Mabuchi, M. (1939) Ieusagi arginase sayou no kougakutokuisei ni tsuite (in Japanese). *Osaka Igakkai Zasshi* **38**, 1421–1433
23. Sano, M. (1941) Studienüber die Arginase. *J. Biochem.* **33**, 467–484
24. Nadai, Y. (1958) Studies on arginase. II. Distribution and properties of D-arginase. *J. Biochem.* **45**, 1011–1020
25. Kimmel, J.R. (1967) Guanidination of protein. *Methods Enzymol.* **11**, 584–589
26. Geyer, J.W. and Dabich, D. (1971) Rapid method for determination of arginase activity in tissue homogenates. *Anal. Biochem.* **39**, 412–417
27. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
28. Higgins, D.G., Thompson, J.D., and Gibson, T.J. (1996) Using CLUSTAL for multiple sequence alignment. *Methods Enzymol.* **266**, 383–402
29. Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrener, P., Hickey, M.J., Brinkman, F.S.L., Hufnagle, W.O., Kowalik, D.J., Lagrou, M., Garber, R.L., Goltry, L., Tolentino, E.,

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Westbrook-Wadman, S., Yuan, Y., Brody, L.L., Coulter, S.N., Folger, K.R., Kas, A., Larbig, K., Lim, R.M., Smith, K.A., Spencer, D.H., Wong, G.K.S., Wu, Z., Paulsen, I.T., Reizer, J., Saier, M.H., Hancock, R.E.W., Lory, S., and Olson, M.V. (2000) Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature* **406**, 959–964

30. Wu, T.K., Busby, R.W., Houston, T.A., McIlwaine, D.B., Egan, L.A., and Townsend, C.A. (1995) Identification, cloning, sequencing, and overexpression of the gene encoding proclavaminate amidino hydrolase and characterization of protein function in clavulanic acid biosynthesis. *J. Bacteriol.* **177**, 3714–3720
31. Szumanski, M.B. and Boyle, S.M. (1990) Analysis and sequence of the speB gene encoding agmatine ureohydrolase, a putrescine biosynthetic enzyme in *Escherichia coli. J. Bacteriol.* **172**, 538–547
32. Sekowska, A., Bertin, P., and Danchin, A. (1998) Characterization of polyamine synthesis pathway in *Bacillus subtilis* 168. *Mol. Microbiol.* **29**, 851–858
33. Haraguchi, Y., Takiguchi, M., Amaya, Y., Kawamoto, S., Matsuda, I., and Mori, M. (1987) Molecular cloning and nucleotide sequence of cDNA for human liver arginase. *Proc. Natl Acad. Sci. USA* **84**, 412–415
34. Kawamoto, S., Amaya, Y., Murakami, K., Tokunaga, F., Iwanaga, S., Kobayashi, K., Saheki, T., Kimura, S., and Mori, M. (1987) Complete nucleotide sequence of cDNA and deduced amino acid sequence of rat liver arginase. *J. Biol. Chem.* **262**, 6280–6283
35. Sumrada, R.A. and Cooper, T.G. (1984) Nucleotide sequence of the *Saccharomyces cerevisiae* arginase gene (CAR1) and its transcription under various physiological conditions. *J. Bacteriol*. **160**, 1078–1087
36. Bewley, M.C., Lott, J.S., Baker, E.N., and Patchett, M.L. (1996) The cloning, expression and crystallisation of a thermostable arginase. *FEBS Lett.* **386**, 215–218
37. Gardan, R., Rapoport, G., and Debarbouille, M. (1995) Expression of the rocDEF operon involved in arginine catabolism in *Bacillus subtilis*. *J. Mol. Biol.* **249**, 843–856
38. Yorifuji, T., Ogata, K., and Soda, K. (1971) Arginine racemase of *Pseudomonas graveolens*. *J. Biol. Chem.* **246**, 5085–5092
39. Soda, K., Oshima, M., and Yamamoto, T. (1972) Purification and properties of isozymes of glutaminase from *Pseudomonas aeruginosa*. *Biochem. Biophys. Res. Commun.* **46**, 1278–1284
40. Robberts, J.R., Holcenberg, J.S., and Dolowy, W.C. (1972) Isolation, crystallization, and properties of *Achromobacteraceae* glutaminase-asparaginase with antitumor activity. *J. Biol. Chem.* **247**, 84–90
41. Patchett, M.L., Daniel, R.M., and Morgan, H.W. (1991) Characterisation of arginase from the extreme thermophile '*Bacillus caldovelox*'. *Biochim. Biophys. Acta* **1077**, 291–298
42. Nakamura, N., Fujita, M., and Kimura, K. (1973) Purification and properties of L-arginase from *Bacillus subtilis. Agric. Biol. Chem.* **37**, 2827–2833
43. Yamanaka, K., Hara, T., and Gino, M. (1976) Purification and properties of L-arginase from *Corynebacterium* sp. NaCl-tolerant bacterium. *J. Ferment. Technol*. **54**, 838–849
44. Yorifuji, T., Tamai, H., and Usami, H. (1977) Purification, crystallization and properties of Mn^{2+} dependent guanidinoacetate amidinohydrolase from a *Pseudomonas. Agric. Biol. Chem.* **41**, 959–966
45. Green, S.M., Eisenstein, E., McPhie, P., and Hensley, P. (1990) The purification and characterization of arginase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **265**, 1601–1607
46. Carvajal, N., Torres, C., Uribe, E., and Salas, M. (1995) Interaction of arginase with metal ions: studies of the enzyme from human liver and comparison with other arginases. *Comp. Biochem. Physiol.* **112B**, 153–159
47. Issaly, I.M. and Issaly, A.S. (1974) Control of ornithine carbamoyltransferase activity by arginase in *Bacillus subtilis*. *Eur. J. Biochem.* **49**, 485–495
48. Soru, E. (1983) Chemical and immunological properties of *B. anthracis* arginase and its metabolic involvement. *Mol. Cell. Biochem.* **50**, 173–183
49. Moreno-Vivian, C., Soler, G., and Castillo, F. (1992) Arginine catabolism in the phototrophic bacterium *Rhodobacter capsulatus* E1F1. Purification and properties of arginase. *Eur. J. Biochem.* **204**, 531–537
50. Carvajal, N., Lopez, V., Salas, M., Uribe, E., Herrera, P., and Cerpa, J. (1999) Manganese is essential for catalytic activity of *Escherichia coli* agmatinase. *Biochem. Biophys. Res. Commun.* **258**, 808–811
51. Bond, J.S., Failla, M.L., and Unger, D.F. (1983) Elevated manganese concentration and arginase activity in livers of streptozotocin-induced diabetic rats. *J. Biol. Chem.* **258**, 8004–8009
52. Green, S.M., Ginsburg, A., Lewis, M.S., and Hensley, P. (1991) Roles of metal ions in the maintenance of the tertiary and quaternary structure of arginase from *Saccharomyces cerevisiae. J. Biol. Chem.* **266**, 21474–21481
53. Cavalli, R.C., Burke, C.J., Kawamoto, S., Soprano, D.R., and Ash, D.E. (1994) Mutagenesis of rat liver arginase expressed in *Escherichia coli*: role of conserved histidines. *Biochemistry* **33**, 10652–10657
54. Kim, H. and Lipscomb, W.N. (1993) X-ray crystallographic determination of the structure of bovine lens leucine aminopeptidase complexed with amastatin: formulation of a catalytic mechanism featuring a gem-diolate transition state. *Biochemistry* **32**, 8465–8478
55. Diaz, N., Suarez, D., and Merz, K.M. Jr. (2001) Molecular dynamics simulations of the mononuclear zinc-beta-lactamase from *Bacillus cereus* complexed with benzylpenicillin and a quantum chemical study of the reaction mechanism. *J. Am. Chem. Soc.* **123**, 9867–9879

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