A novel amidase involved in bacterial cyclic imide metabolism was purified from Blastobacter sp. strain A17p-4. The enzyme physiologically functions in the second step of cyclic imide degradation, i.e., the hydrolysis of monoamidated dicarboxylates (half-amides) to dicarboxylates and ammonia. Enzyme production was enhanced by cyclic imides such as succinimide and glutarimide but not by amide compounds which are conventional substrates and inducers of known amidases. The purified amidase showed high catalytic efficiency toward half-amides such as succinic anhydride ($K_m = 6.2 \text{ mM}$; $k_{cat} = 5.76 \text{ s}^{-1}$) and glutaric anhydride ($K_m = 2.8 \text{ mM}$; $k_{cat} = 2.23 \text{ s}^{-1}$). However, the substrates of known amidases such as short-chain ($C_2$ to $C_4$) aliphatic amidase, long-chain ($C_6$) aliphatic amidase, amino acid amidase, aliphatic diamides, $\alpha$-keto acid amidases, N-carbamoyl amino acids, and aliphatic ureides were not substrates for the enzyme. Based on its high specificity toward half-amides, the enzyme was named half-amidase. This half-amidase exists as a monomer with an $M_r$ of 48,000 and was strongly inhibited by heavy metal ions and sulfhydryl reagents.

A variety of cyclic amide-metabolizing systems occur in nature and play important roles in pyrimidine and purine metabolism, amino acid metabolism (histidine degradation), antibiotic metabolism ($\beta$-lactam decomposition), creatinine degradation, etc.

Cyclic imide is a kind of cyclic amide, and the metabolism of cyclic imides has been studied in relation to the detoxification of the antiepileptic agents ethosuximide and phenytoin in mammals (3, 36). During the course of a study on cyclic amide transformation for hydantoin from the practical viewpoint of industrial t-amino acid production, we recently found that microorganisms also transform cyclic imides (21, 24–27). Microbial transformation of cyclic imides found in the bacterium Blastobacter sp. strain A17p-4 (22) involves ring opening of cyclic imide to monoamidated dicarboxylate (half-amide) catalyzed by amidase (23), half-amide hydrolysis to dicarboxylate catalyzed by amidase, and subsequent trichloroacetic acid (TCA) cycle-like reactions (Fig. 1). The reactions and enzymes (amidase and amidase) involved in the metabolism have practical potential for production of organic acids from cyclic imides or their metabolites and for fine enzymatic synthesis of useful compounds. For example, pyruvate, an effective precursor in the synthesis of various drugs and agrochemicals, was produced from succinimide or its metabolites (especially fumarate, a cheap material) through cyclic imide-transforming pathway (Fig. 1A and B) (28). Imidase was applied for the regiospecific synthesis of useful half-amide (3-carbamoyl-$\alpha$-picolinic acid, an intermediate for herbicide) from a cyclic imide (2,3-pyridinedicarbonyl) (J. Ogawa, M. Ito, T. Segawa, C.-L. Soong, and S. Shimizu, Abstr. Annu. Meet. ‘99 Soc. Biosci. Bioeng., abstr. 182, 1999 [in Japanese]). Amidase also has a potential for the chiral resolution of dicarboxylates through the stereoselective hydrolysis of half-amides (Fig. 1C).

We report here an amidase catalyzing the second step of cyclic imide transformation. This amidase, named half-amidase, was distinct from known amidases especially in substrate specificity. We also confirmed the physiological role of imidase and half-amidase in cyclic imide transformation by investigating their induction/expression profiles.

MATERIALS AND METHODS

Chemicals. Glutamic acid and adipinamic acid were chemically synthesized as described previously (6). $\alpha$-Ketoglutaramic acid and $\alpha$-ketosuccinamic acid were prepared by t-amino oxidase from t-glutamate and t-asparagine, respectively (16).

Microorganisms and medium for cultivation. Blastobacter sp. strain A17p-4 (AKU 990; Faculty of Agriculture, Kyoto University, Kyoto, Japan) was used as the enzyme source and for all experiments. Synthetic minimum liquid medium comprised 1 g of KH$_2$PO$_4$, 1 g of K$_2$HPO$_4$, 0.3 g of MgSO$_4$$\cdot$7H$_2$O, 2 g of NH$_4$Cl, 1 g of thiamine hydrochloride, 2 mg of riboflavin, 2 mg of nicotinic acid, 2 mg of pantethinic acid, 2 mg of pyridoxine hydrochloride, 0.1 mg of biotin, 1 mg of $p$-amino benzoic acid, 0.5 mg of folic acid, and 10 mg of each metal ion (CaCl$_2$$\cdot$2H$_2$O, MnCl$_2$$\cdot$2H$_2$O, MnCl$_2$$\cdot$4H$_2$O, CoCl$_2$$\cdot$6H$_2$O, ZnSO$_4$$\cdot$7H$_2$O, CdCl$_2$$\cdot$2H$_2$O, CuSO$_4$$\cdot$5H$_2$O, FeSO$_4$$\cdot$7H$_2$O, BeSO$_4$$\cdot$4H$_2$O, and RhCl$_3$) in 1 liter of deionized water, pH 7.0.

Production of half-amidase. Minimum liquid medium supplemented with 20 mM sucrose as a carbon source and each test compound at 0.15% (wt/vol) was used for experiment 1; minimum liquid medium supplemented with each test compound at 0.15% (wt/vol) as a sole source of carbon was used for experiment 2. All cultivations were carried out at 28°C for 4 days. Cells from 5 ml of culture were harvested by centrifugation at 10,000 $\times$ g for 10 min, then washed twice with 0.85% (wt/vol) NaCl, and suspended in 0.5 ml of 20 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM dithiothreitol. The cell suspension was ultrasonicated at 4°C for 15 min and centrifuged at 14,000 $\times$ g for 30 min. The resultant supernatant was used for assay of half-amidase activity as described below.

Expression of half-amidase and imidase. Four sets of cultivations (A through D) were carried out. All cultures in 2-liter flasks contained minimum liquid medium (200 ml) supplemented with 20 mM sucrose. Cultivations were carried out at 28°C with shaking. At the end of exponential growth (107 h), cultures A and C were supplemented with 20 mM glutarimide and 20 mM succrose, respectively, while cultures B and D were supplemented with actinomycin D to a final concentration of 1 µg/ml together with addition of 20 mM glutarimide and 20 mM succrose, respectively. At various intervals of time, a 5-ml aliquot of each culture was sampled and cell growth was measured at an absorbance of 610 nm. Half-amidase and imidase activities were then assayed from the cell extracts of each culture prepared as described above.

Enzyme assays. The standard half-amidase assay mixture comprised, in 100 µl, 10 µmol of potassium phosphate (pH 7.0), 2 µmol of succinamic acid, and an appropriate amount of enzyme. After incubation at 30°C for 30 to 60 min, the reaction was stopped with 10 µl of 15% (by volume) perchloric acid, followed by neutralization with 90 µl of 500 mM potassium phosphate (pH 7.0). The reaction mixture was centrifuged at 14,000 $\times$ g for 10 min, and the supernatant was analyzed for decreases in the concentration of succinamic acid and increase in that of succinic acid. Analysis was performed using a Shimadzu LC-6A high-performance liquid chromatography (HPLC) apparatus at 210 nm on a Cosmosil

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SC18 AR-packed column (4.6 by 250 mm; Nacalai Tesque, Kyoto, Japan) at a flow rate of 1.0 ml/min, with 250 mM KH₂PO₄ (pH 4.4) as the eluent. Imidase activity was assayed as described previously (23).

Enzyme purification. All steps were carried out at 0 to 5°C; the buffer used was 20 mM potassium phosphate (pH 7.0) containing 0.1 mM dithiothreitol throughout the purification process unless otherwise stated.

(i) Step 1. Blastobacter sp. was cultured in minimum liquid medium supplemented with 1% succinimide at 28°C with shaking. Cells (20 g [wet weight] from a 10-liter culture) were harvested by centrifugation (10,000 × g) and suspended in 20 ml of buffer. The cell suspension was disrupted with glass beads (0.25 by 0.50 mm; Dyno-Mill; W. A. Bachofen, Basel, Switzerland) for 30 min. The disrupted cell suspension was centrifuged at 14,000 × g for 60 min, and the resultant supernatant was used as the cell extract (230 ml).

(ii) Step 2. The cell extract was dialyzed against buffer and then applied to a DEAE-Sephacel column (2.5 by 10 cm) preequilibrated with buffer. After the column was washed with 250 ml of buffer, the enzyme was eluted with a linear gradient of 0 to 0.5 M NaCl in 500 ml of buffer. The active fractions were combined (90 ml) and concentrated by ultrafiltration with a 10,000-kDa-cutoff membrane.

(iii) Step 3. The enzyme solution was mixed with solid NaCl to obtain a concentration of 4 M and then applied to a phenyl-Sepharose CL-4B column (2.5 by 10 cm) preequilibrated with buffer. After the column was washed with 250 ml of buffer, the enzyme was eluted with a linear gradient of 0 to 0.5 M NaCl in 500 ml of buffer. The active fractions were combined (90 ml) and concentrated by ultrafiltration with a 10,000-kDa-cutoff membrane.

(iv) Step 4. The concentrated enzyme was applied to a Sephacryl S-200 column (1.5 by 10 cm) preequilibrated with buffer containing 0.2 M NaCl and eluted with the same buffer. The active fractions were pooled (40 ml) and dialyzed against buffer.

(v) Step 5. The dialyzed enzyme was applied to a Mono Q HR 5/5 column equilibrated with buffer and then eluted with an increasing salt concentration (from 0 to 0.5 M NaCl). The active fractions were combined (2 ml), dialyzed against buffer, and further purified on a SMART system (Pharmacia LKB, Uppsala, Sweden).

(vi) Step 6. The dialyzed active fraction was applied to a Mono Q PC 1.6/5 column equilibrated with buffer and then eluted with an increasing salt concentration of 0 to 0.3 M NaCl. The active fractions (0.2 ml) were used for characterization.

Analytical methods for half-amidase. The relative molecular mass was determined by HPLC on a GS-520 column (7.6 by 500 mm; Asahi Kasei, Tokyo, Japan) and a G-3000 SW column (7.5 by 600 mm; Tosoh, Tokyo, Japan) at 0.3 ml/min with an elution buffer of 20 mM potassium phosphate (pH 7.0) containing 0.2 M NaCl and 0.1 mM dithiothreitol. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12.5% polyacrylamide gel and protein concentration determination were performed as described previously (31). NH₂-terminal amino acid sequence analysis was performed as described previously (30).

RESULTS

Production of half-amidase. The production of half-amidase was significantly enhanced by the addition of cyclic imides such as succinimide and glutarimide (Table 1, experiment 1). The intermediates of cyclic imide metabolism of succinic acid also showed stimulatory effects. Blastobacter sp. was able to grow in minimum medium with succinimide or glutarimide as the sole source of carbon (Table 1, experiment 2). With these cyclic imides as sole sources of carbon, the half-amidase activity
TABLE 1. Effects of various compounds on the production of half-amidase\textsuperscript{a}

| Compound                        | Mean ± SD | Relative activity (%) |
|---------------------------------|-----------|-----------------------|
|                                | Optical density at 610 nm | Sp act (μmol/min/mg of protein) |
| None                            | 0.335 ± 0.012 | 0.176 ± 0.018 | 100 |
| Succinimide                     | 0.250 ± 0.012 | 0.325 ± 0.010 | 185 |
| Glutarimide                     | 0.392 ± 0.011 | 0.476 ± 0.008 | 270 |
| Succinamic acid                 | 0.314 ± 0.011 | 0.163 ± 0.016 | 93  |
| Adipimamic acid                 | 0.323 ± 0.014 | 0.150 ± 0.008 | 85  |
| Succinic acid                   | 0.414 ± 0.005 | 0.300 ± 0.015 | 170 |
| Adipic acid                     | 0.355 ± 0.018 | 0.188 ± 0.008 | 107 |
| Lactamide                       | 0.223 ± 0.011 | 0.176 ± 0.015 | 100 |
| n-Valeramide                    | 0.267 ± 0.006 | 0.213 ± 0.012 | 121 |
| Acetamide                       | 0.222 ± 0.013 | 0.178 ± 0.005 | 99  |
| Benzamide                       | 0.140 ± 0.010 | Trace\textsuperscript{b} | ND\textsuperscript{c} |
| Dihydouracil                    | 0.330 ± 0.019 | 0.213 ± 0.010 | 121 |
| Dihydrotrothymine               | 0.202 ± 0.004 | 0.159 ± 0.013 | 90  |
| Ammonium chloride               | 0.236 ± 0.010 | 0.201 ± 0.005 | 114 |
| l-Glutamine                     | 1.100 ± 0.016 | 0.240 ± 0.004 | 135 |

Expt 2

| Additives as sole carbon sources | Mean ± SD | Relative activity (%) |
|---------------------------------|-----------|-----------------------|
| Succinimide                     | 0.233 ± 0.010 | 0.582 ± 0.013 | 331 |
| Glutarimide                     | 0.101 ± 0.006 | 0.960 ± 0.016 | 545 |

\textsuperscript{a} The standard deviations were obtained from four separate determinations. The methods for experiments 1 and 2 are described in Materials and Methods.

\textsuperscript{b} Less than the specific activity of 10\textsuperscript{-3} μmol/min/mg of protein.

\textsuperscript{c} ND, not determined.

increased approximately three to five times compared to that found with sucrose grown cells, and succinimide supported good growth.

Amide compounds which act as substrates and inducers of known amidase (acetamide and lactamide for the amidase of Pseudomonas aeruginosa [11, 13]; benzamide for that of Aspergillus nidulans [9]; cyclic ureides for dihydropyrimidinase; ammonium chloride and l-glutamine for ω-amidase) were less effective or somewhat repressive on the production of half-amidase (Table 1).

Expression of half-amidase and amidase in the presence of cyclic imide. Half-amidase activity increased with cell growth and decreased at the mid-logarithmic phase of growth, but upon the addition of cyclic imide (glutamine), the production of half-amidase increased rapidly (Fig. 2, glutamimide addition culture, without actinomycin D). However, when cyclic imide and actinomycin D were added together, no enhancement of half-amidase production was observed due to the inhibition of RNA synthesis by actinomycin D (Fig. 2, glutamimide addition culture, with actinomycin D). Similar results were observed with amidase (Fig. 2, glutamimide addition culture). In control experiments, the addition of sucrose did not enhance half-amidase production but increased slightly amidase production (Fig. 2, sucrose addition culture). These results suggested that cyclic imides induce the expression of amidase and specifically half-amidase at gene level.

Purification of half-amidase and criteria for purity. Typical results of purification are presented in Table 2. Half-amidase was purified approximately 700-fold from the soluble cell extract of Blastobacter sp. The active fractions from the Mono Q PC 1.6/5 column were ascertained to be pure from the appearance of a single protein band on SDS-PAGE.

Substrate specificity and kinetic properties. Normal hyperbolic kinetics were observed with all compounds hydrolyzed by the enzyme. The $K_m$, $k_{cat}$, and $k_{cat}/K_m$ values for preferred substrates, calculated from double-reciprocal Lineweaver-Burk plots, are shown in Table 3. The enzyme showed the highest catalytic efficiency ($k_{cat}/K_m$) toward half-amides such as succinamic acid and glutamic acid but not hydrolyzed diamides such as succinimide, fumaramide, and adipamide. The enzyme also showed activity toward lactamide, the middle-chain amides (n-valeramide, n-caproamide, and lauramide), an unsaturated aliphatic amide (crotoamide), and aromatic amides (benzamide and 2-phenylpropionamide), but with catalytic efficiency very low compared to half-amides.

The amide compounds judged to be inactive as substrates are some short- and long-chain amides, aromatic amides, amino acid amides, α-keto acid amides, urea, asparagine, glutamine, β-ureidopropionate, and the N-carbamoyl amino acids, which are common substrates of acyl amidase (2, 19, 20), aryl amidase (8, 10), aminopeptidase (1, 5), ω-amidase (4, 17), urease (18), asparaginase (29), glutaminase (29), β-ureidopropionase (21), and N-carbamoyl amino acid amidohydrolases (24, 25, 27), respectively.

Half-amidase did not show amidase transferase activity, as determined by acylhydroxamate formation (12) from hydroxylamino and several amidases listed in Table 3 as acyl donors. The enzyme did not show esterase and protease activities with methyl or ethyl ester acid and with amino acid amide or dipeptide as substrates, respectively.

Relative molecular mass, subunit structure, and NH\textsubscript{2}-terminal amino acid sequence. The half-amidase was determined to be a monomer. Based on the results of HPLC gel filtration and SDS-PAGE analysis, the relative molecular weight of the native enzyme was estimated to be 48,000 and 46,000, respectively. The NH\textsubscript{2}-terminal amino acid sequence of the enzyme was determined to be Met-Leu-Leu-Ala-Asn-Asp-Pro-Val-His-Ala-Phe-Val-Asp-Tyr-Pro-Ala-Glu-Val-Pro. A BLAST search for similarity with entries in the protein sequence databases (Swissprot, GenBank, and EMBL) found no apparent homology with other reported amidases (12, 15).
Effects of inhibitors. Enzyme activity, assayed under standard conditions in the presence of various compounds (2 mM), was inhibited by sulfhydryl reagents such as sodium arsenate, 5,5'-dithiobis(2-nitrobenzoate), and N-ethyl maleimide with 98, 79, and 26% inhibition, respectively. Serine protease inhibitors such as phenylmethanesulfonyl fluoride, tosyl phenylalanyln chloromethyl ketone, and diisopropylphosphofluoridate caused 44, 29, and 27% inhibition, respectively. Metal ion chelators did not show any inhibition. Heavy metal ions such as Hg²⁺, Cu²⁺, Zn²⁺, Ag⁺, and Cd²⁺ inactivated enzyme activity by 99, 90, 88, 76, and 79%, respectively. This susceptibility of the half-amidase to inhibitors is similar to findings for other amidases from \textit{Rhodococcus} sp. (8, 12, 20) and \textit{Pseudomonas} sp. (2, 10).

Effects of imides, amides, ammonia, and organic acids. The activity of half-amidase toward succinamic acid was examined in the presence of various substrates of known amidases such as L-glutamine, L-asparagine, and \textit{n}-butylamide. None of these compounds showed significant inhibitory effects. The half-amidase activity was not inhibited by cyclic imide metabolism intermediates (succinimide, fumarate, L-malate, pyruvate), the products (succinate, ammonium chloride), product-like compounds.

Table 2. Purification of half-amidase from \textit{Blastobacter} sp. strain A17p-4

| Step                  | Vol (ml) | Total protein (mg) | Total activity (µmol/min) | Sp act (µmol/min/mg) | Recovery (%) | Fold |
|-----------------------|----------|--------------------|---------------------------|---------------------|--------------|------|
| Cell extract          | 230      | 1,890              | 12.4                      | 0.00656             | 100          | 1.00 |
| DEAE-Sephacel         | 90       | 114                | 11.0                      | 0.0965              | 88.7         | 14.7 |
| Phenyl-Sepharose      | 65       | 52.7               | 3.10                      | 0.0588              | 25.0         | 8.96 |
| Sephacryl S-200       | 40       | 7.00               | 1.01                      | 0.144               | 8.15         | 22.0 |
| Mono Q HR 5/5         | 2.0      | 0.103              | 0.209                     | 2.03                | 1.69         | 309  |
| Mono Q PC 1.6/5       | 0.2      | 0.0221             | 0.111                     | 5.02                | 0.895        | 765  |

FIG. 2. Effect of cyclic imide and the RNA synthesis inhibitor actinomycin (act.) D on the expression of half-amidase and imidase. The experimental conditions (sets A through D) are described in Materials and Methods. Symbols: set A (glutarimide addition without actinomycin D), ■ (half-amidase activity), □ (imidase activity), and ○ (cell growth); set B (glutarimide addition with actinomycin D), ● (half-amidase activity), ● (imidase activity), and ▲ (cell growth); set C (sucrose addition without actinomycin D), ○ (half-amidase activity), △ (imidase activity), and ◇ (cell growth). Addition of glutarimide or sucrose without or with actinomycin D is indicated by a thick arrow. The values of half-amidase activity, imidase activity, and optical density (OD) are the averages of three separate determinations that were reproducible within ±5%.
TABLE 3. Substrate specificity of half-amidase from Blastobacter sp. strain A17p-4

| Substrate        | $K_m$ (mM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ M$^{-1}$) |
|------------------|------------|----------------------|----------------------------------|
| Succinamic acid  | 6.2 ± 0.4  | 5.76 ± 0.30          | 0.93 ± 0.02                      |
| Glutaric acid    | 2.8 ± 0.1  | 2.23 ± 0.10          | 1.62 ± 0.06                      |
| Adipic acid      | 8.0 ± 1.4  | 0.36 ± 0.05          | 0.046 ± 0.002                    |
| Lactamide        | 3.7 ± 0.3  | 0.11 ± 0.01          | 0.033 ± 0.002                    |
| n-Valeramide     | 5.1 ± 0.2  | 0.18 ± 0.01          | 0.037 ± 0.006                    |
| n-Caproamide     | 17.0 ± 2.7 | 0.18 ± 0.04          | 0.011 ± 0.001                    |
| Crotoamide       | 8.8 ± 0.4  | 0.10 ± 0.01          | 0.012 ± 0.001                    |
| Benzamide        | 6.5 ± 0.1  | 0.13 ± 0.01          | 0.020 ± 0.001                    |
| 2-Phenylnpropioamide | 3.1 ± 0.1 | 0.078 ± 0.002        | 0.026 ± 0.001                    |

* Reactions were carried out under standard assay conditions described in Materials and Methods with the variation of substrates, and the amount of ammonia produced was colorimetrically estimated by indophenol method. As for the ester substrates, the reduction of ester or the production of acid was determined by HPLC as described in Materials and Methods. The kinetic constant values are averages ± standard deviations of three separate determinations. The half-amidase is a monomer, and its $NH_2$-terminal amino acid sequence showed no homology with other known amidases. The half-amidase exhibited narrow substrate specificity toward half-amides, suggesting that the enzyme functions specifically in cyclic imide metabolism and is distinct from known amid-, amino acid amide-, or cyclic amide-transforming enzymes.

The amidase catalyzing the first step of the cyclic imide metabolism showed high activity and affinity toward cyclic imides (23), and the half-amidase showed the highest activity and catalytic efficiency toward the ring-opened products of cyclic imides, i.e., half-amides, suggesting that the half-amidase acts cooperatively with the amidase in the degradation of cyclic imides. The production of both enzymes was enhanced by cyclic imides (Fig. 2; Table 1) (22), indicating that these enzymes were expressed for cyclic imide utilization. In cyclic imide metabolism, half-amidase is positively regulated at the gene level by cyclic imide. Regulation of the amidase gene by cyclic imide is not understood, while it is specifically regulated at the catalytic level by succinate, the product of half-amidase reaction and a key intermediate of cyclic imide metabolism in relation to the TCA cycle (22, 23).

In mammals, cyclic imides were reported to be metabolized by the combined action of dihydroxyiminidase (14) and $\omega$-amidase (7), which are involved in pyrimidine and amino acid metabolism, respectively. Rat liver $\omega$-amidase, which is considered to be a mammalian counterpart of the bacterial half-amidase, has been reported to hydrolyze succinic acid and glutaric acid, as well as $\omega$-ketoglutaric acid and $\omega$-ketosuccinic acid, which are not substrates of the half-amidase. Thus, in contrast to the bacterial half-amidase, the rat $\omega$-amidase possessed different substrate specificity and physiological roles, namely, metabolism of glutamine and asparagine (7, 17).

Above all, half-amidase and amidase, which are responsible for bacterial cyclic imide transformation, are unique enzymes with characteristics distinct from those of other cyclic amide-transforming enzymes. The application of these enzymes are now under investigation and will be reported elsewhere.

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