Three novel malonamidases (E1a, E1b, and E2) occurring constitutively in *Bradyrhizobium japonicum* were purified to electrophoretic homogeneity. They were found to catalyze one or more of the following three types of reactions: malonyl transfer to hydroxylamine (reaction 1), hydroxylaminolysis of malonamate (reaction 2), and hydrolysis of malonamate (reaction 3). The molecular sizes of E1a, E1b, and E2 were 126, 107, and 103 kDa, respectively, and they were each composed of two identical subunits. The PIs of E1a and E1b, 5.5 and 5.0 respectively, were similar, but that of E2 was 7.2. Optimum pH values varied with the type of reactions catalyzed, but among the enzymes they were found to be similar. The affinity of E2 for malonamate was about 30- and 70-fold higher than that of E1a and E1b, respectively. Acetate and propionate inhibited E1a activity competitively, whereas malonate inhibited E2 activity noncompetitively. The amino acid composition and N-terminal amino acid sequence of the three enzymes were found to be different. These enzymes were also immunologically different. E1a was found to form a malonyl-enzyme intermediate during the catalysis through the isolation of [14C]malonyl enzyme with gel filtration and through isotope exchange experiments with [14O]malonate. These malonamidases may play a role for the self-protection against malonate toxicity in nodule bacteroids and may also be involved in the transport of fixed nitrogen to the plant cell.

An enzymatic activity catalyzing the formation of malonylhydroxamate from malonate and hydroxylamine was detected during the course of an investigation of enzymes utilizing malonate as a substrate in *Bradyrhizobium japonicum* bacteroids (1). The class of dicarboxylate ω-amidase (EC 3.5.1.3), corresponding to this enzymatic activity, shows a high specificity for malonate and malonamate as its substrates. ω-Amidase was first reported by Meister et al. (2) in studies with rat liver extracts. The rat liver enzyme was purified to homogeneity by Hersh (3). Microbial ω-amidases from *Bacillus subtilis*, *Thermus aquaticus*, *Neurospera crassa*, and *Saccharomyces cerevisiae* (4–6) were also studied. Dicarboxylate ω-amidase in rat liver has been proposed to play a role as a key enzyme in the ω-amidase pathway which is an alternative route for glutamine metabolism (3). However, malonamidase has never been isolated except for the determination of the enzyme activity in *B. japonicum* bacteroids (1, 7). This enzyme has been proposed to be involved in the fixed nitrogen transport from bacteroids to the plant (1).

Malonate is found in large amounts in the tissues of various plants, especially in many legumes (8). In nodules of 33-day-old soybeans, malonate is the most predominant acid. Furthermore, the malonate concentration in roots and nodule is depressed after the addition of either nitrate or ammonium sulfate (9). Recently, the proposed role of malonate as a precursor of oxaloacetate/malate from the tricarboxylic acid cycle and amino acid biosynthesis has also been reviewed (10). Although the question of symbiosis between plants and bacteria has been extensively investigated, little consideration has been given to malonate.

In this paper we present the isolation and purification to homogeneity of the malonamidases from *B. japonicum* and the characterization of some of its physical and biochemical properties. The characterization of the enzyme is of particular interest with regard to their proposed role in the nitrogen flow by malonamidase in *B. japonicum*-soybean symbiosis.

**EXPERIMENTAL PROCEDURES**

**Materials**—*B. japonicum* USDA 110 was obtained from the Korea Genetic Engineering Research Institute, Daejon, Korea. Sodium malonate, Tris(2-carboxyethyl)phosphine, Ac clip, and Actinomycin D were from Sigma; Alfa gel Blue, hydroxyapatite, and Coomassie Brilliant Blue R-250 were from Bio-Rad; DETAPAC, Sephadex G-100, and 1,1,1,3,3,3-hexamethyldisilazane, and Meldrum's acid were from Aldrich. [1-14C]Malonate (9.5 mCi/mmol) and [2-14C]malonate (19.9 mCi/mmol) were from Du Pont-New England Nuclear; Pansorbin cells were from Calbiochem Behring. Immobilon-P polyvinylidene difluoride transfer membrane and Immobilon NC nitrocellulose transfer membrane were purchased from Millipore Co. Silica gel 60-F (0.2 mm) thin layer chromatography plate was obtained from Merck. All other reagents were of analytical grade.

**Malonamidase Assays**—Malonamidases catalyzed more than one of the three different reactions (Reactions 1–3) listed below:

\[
\text{HOOCCH}_2\text{COOH} + \text{NH}_2\text{OH} \rightarrow \text{HOOCCH}_2\text{CONH} + \text{H}_2\text{O} \\
\text{Reaction 1}
\]

\[
\text{HOOCCH}_2\text{CONH}_2 + \text{NH}_2\text{OH} \rightarrow \text{HOOCCH}_2\text{CONH} + \text{H}_2\text{O} \\
\text{Reaction 2}
\]

\[
\text{HOOCCH}_2\text{CONH}_2 + \text{H}_2\text{O} \rightarrow \text{HOOCCH}_2\text{COOH} + \text{NH}_3 \\
\text{Reaction 3}
\]

The first assay method was based on the malonyl transfer to hydroxylamine (Reaction 1). Reaction mixtures containing (in μmol) MOPS buffer, pH 6.8, 50; sodium malonate, 20; neutralized NH₂OH, 100; and an enzyme and water in a total volume of 0.5 ml were incubated for 30
min at 37 °C. After the reaction, the malonohydrate formed was determined by the same method described previously for malonyl-CoA synthetase (11). The second method was based on hydroxyminosynthesis of malonate (Reaction 2). Reaction mixtures containing (in μmol) Tris-HCl, pH 6.5, 50; malonate 5; neutralized NH₄OH 100; an enzyme and water in a total volume of 0.5 ml were incubated for 30 min at 37 °C. After washing the column with ~50 ml of buffer D, E1a was eluted by the application of a 200-ml linear gradient of 0-2.5 M NaCl in buffer D containing 15% glycerol. The gel was soaked in 0.1 M EDTA. E2 was eluted by the application of a 200-ml linear gradient (2.0-0 M) of NaCl in buffer D containing 2 mM ZnSO₄. The fractions containing the enzyme activity were pooled, dialyzed twice against 2 liters of buffer B, and concentrated. The E1b solution was loaded onto a DEAE-Sephacel (2.8 x 25 cm) column pre-equilibrated with buffer B. After washing the column with the same buffer, E1b was eluted by the application of a 200-ml linear gradient (0.5-0 M) of NaCl in buffer B. E2 was eluted at 0.2-0.25 M NaCl. The fractions containing the E2 activity from the previous step were pooled and applied to Red-120 agarose column (1.6 cm) pre-equilibrated with buffer B. As soon as the loading was completed, the undesorbed proteins were eluted by the application of a 300-ml linear descending gradient (2.0-0 M) of NaCl in buffer B containing 2 mM EDTA. Fractions containing the enzyme activity were pooled, dialyzed against buffer B, and concentrated. This E2 solution was stored at 4 °C and used for all subsequent experiments.

**Purification of Malonamidase E2—ZnSO₄ was added to the enzyme solution containing E2 and the solution was adjusted to make a final concentration of 0.5 mM. This solution was applied to phenyl-Sepharose CL-4B (2.8 x 4 cm) equilibrated with buffer B containing 2 mM NaCl and 0.5 mM ZnSO₄. After loading was completed, the unadsorbed materials were washed with 100 ml of buffer B containing 2 mM NaCl and 2 mM ZnSO₄, followed with 50 ml of buffer B containing 2 mM NaCl and 2 mM EDTA. E2 was eluted by the application of a 300-ml linear descending gradient (2.0-0 M) of NaCl in buffer B containing 2 mM EDTA. Fractions containing the enzyme activity were pooled and dialyzed twice against 2 liters of buffer B. The dialyzed solution containing E2 activity was loaded onto a DEAE-Sephacel (2.8 x 25 cm) column pre-equilibrated with buffer B. After washing the column with the same buffer, E2 was eluted by the application of a 200-ml linear gradient (0.5-0 M) of NaCl in buffer B. E2 was eluted at 0.2-0.25 M NaCl. The fractions containing the E2 activity from the previous step were pooled and applied to Red-120 agarose (1.8 x 3.5 cm) pre-equilibrated with buffer B. As soon as the loading was completed, the adsorbed proteins were eluted by the application of a linear gradient of NaCl (0-2.0 M) in buffer B. E2 was eluted at 1.7-1.9 M NaCl. Fractions containing the enzyme activity were pooled, dialyzed against buffer B, and concentrated. This E2 solution was stored at 4 °C and used for all subsequent experiments.

**Gel Electrophoresis—SDS-PAGE was performed using the method of Laemmli (15). Markers proteins were myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase b, 97.4 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa. Native pore-gradient (5-30%) PAGE was performed to determine the molecular weight of the purified proteins. Markers proteins were thyroglobulin, 609 kDa; ferritin, 440 kDa; catalase, 232 kDa; lactate dehydrogenase, 140 kDa; bovine serum albumin, 66 kDa. Isoelectric focusing was performed on a pre-formed gel, pH 3-10, by Phast System (Pharmacia). Reference proteins (pI) were lentil lectin (basic 8.66), middle (8.45), and acidic (8.15), horse myoglobin (basic 7.86), acidic (6.85), human carbonic anhydrase B (6.9), bovine carbonic anhydrase B (5.86), β-lactoglobulin A (5.50), soybean trypsin inhibitor (4.58), and amyloglucosidase (3.50). The gels were stained with Coomassie Blue.

**Activity Staining—Native PAGE was performed as described above. The gel was soaked in 0.1 M MOPS, pH 6.8, buffer for 10 min with gentle agitation. The gel was transferred to a reaction mixture containing (μmol)/MOPPS buffer, pH 6.8, 50; sodium malonate, 60 (in the case of E2, malonamate, 5); neutralized NH₄OH, 100; and water in a total volume of 10 ml. After incubation for 1 h at 37 °C, the gel was fixed in 10% trichloroacetic acid solution for 10 min. After discarding the trichloroacetic acid solution and rinsing, the gel was air-dried for 2 h and destained in the assay procedure above. The dark-brown colored band, which appeared within 10–20 min as a result of malonamidase reaction, remained for about 30 min, and diffused out. The developed gel was photographed immediately and stained with Coomassie Blue for the demonstration of a corresponding protein band.

**Protein Determination—The protein content of the extract and the fractions from all steps of purification were determined using the Sigma bicinchoninic acid protein assay kit with bovine serum albumin as standard.

**Preparation of Substrates—α-Ketoglutaramate and α-ketosuccinamate were prepared by the oxidation of the corresponding amino acids with l-amino acid oxidase according to the method of Meister et al. (16). Malonamic acid was prepared by the method of Rigo et al. (17) with a slight modification. 1.1,1,3,3,3-Hexamethyldisilazane (25 g, 154.9 mmol) was added via a syringe through a septum cap to a solution of Maldum acid (122.3 g, 154.9 mmol) in 200 ml of anhydrous CH₂Cl₂, with stirring under nitrogen at room temperature. After 2 h, absolute methanol (20 ml) was slowly added to the reaction mixture. The mixture was allowed to stand in a 20 °C freezer overnight. The resulting acid precipitate was filtered and washed with CH₂Cl₂, then with ether. The obtained precipitate was redisolved in 0.5 M malonamic acid, the Dowex 1-x8 anion-exchange chromatography was performed using a linear gradient of 0.5–5 M formic acid. The fractions containing malonamic acid were identified with E2, pooled, and stored in a lyophilized state. The purity and structure of synthetic malonamic acid was confirmed by GC-MS as described below. 1H and 13C NMR spectra were synthesized by the reaction of malonamide with H₂, H₂O, H₂O, D₂O, and malonyl dichloride (1.3 ml) were mixed in a round bottom flask (molar ratio 2:1). After 1 min, malonic acid remained as a solid product.
It was dissolved in 20 ml of ethyl acetate and decolorized with Norit. 20 ml of benzene was added, and the mixture was kept at −20 °C for 12 h. [140]Malic acid was crystallized and dried under reduced pressure. [140]Malic acid was methylated with ethereal diazomethane and analyzed by GC-MS to be [140]malonic acid containing 50% 14O.

The initial enzyme reaction was identified by thin layer chromatography with 1-butanol/ethanol/water (2:2:1) as a developing solvent. The thin layer chromatography was performed by the ascending method, using Silica Gel 60-F precoated plates. The location of hydroxamate on thin layer chromatography plate was monitored by spraying 5% FeCl3 solution in 95% alcoholic 0.1 M HCl.

Amino Acid Composition and N-terminal Amino Acid Sequence—Amino acid analysis of the malonamidases was performed in a Waters Pico Tag system amino acid analyzer. Samples for analysis were hydrolyzed with 200 µl of 6 N HCl at 110 °C for 24 h in an evacuated sealed tube. Half-cystine was determined as cysteic acid after performic acid oxidation. The total amount of tryptophan was estimated by methanesulfonic acid hydrolysis (18). N-terminal amino acid sequence analysis was performed in a model 477A Pulse-Liquid Protein Sequencer (Applied Biosystems, Inc.) with 1 nmol of the protein which was electroblotted onto a polyvinylidene difluoride membrane according to the method of Matsudaira (19).

Isotope Exchange Experiment—The reaction mixture contained 160 µM [140]malonate, 0.1 M MOPS buffer, pH 6.8, and 32 units of purified Ela in a final volume of 0.5 ml. In the control reaction mixture, the enzyme was omitted. The reaction mixtures were incubated for 13 days at 37 °C. During the incubation, the reaction mixtures were centrifuged, and 32 units of fresh enzyme were added to them every 2 days. After the incubation, the reaction mixtures were loaded onto 500 µl of Dowex 1-X8 resin in a 1.5-ml Eppendorf tube and were washed with 1 ml of water five times. The bound compounds were eluted with 1 ml of 5 M formic acid twice. The combined eluents were freeze-dried. The compounds were methylated with ethereal diazomethane and analyzed by GC-MS as described below.

GC-MS Spectrometry—Mass spectrometry was carried out with a Hewlett-Packard GC-MS model 5988 fitted with a HP-5 column (25 m × 0.25 mm i.d., Hewlett-Packard Co.). Helium flow was 1 ml min⁻¹, and electron energy was 70 eV. Samples of 1-3 µl in methanol were introduced by direct injection through a septum. A solvent control was run between each sample to ensure that the injection port and column were free of the previous sample. Malonic acid dimethyl ester was passed through the column with a retention time of 0.2 min under the following program: 10 min at 70 °C; 10 °C min⁻¹ to 250 °C; 3 min at 250 °C.

Formation of Malonyl-Ela—The reaction mixture prepared for the isolation of malonyl-Ela consisted of 5 µmol of MOPS buffer, pH 6.8, 0.8 µmol of sodium malonate, 0.2 µmol of [14C]malonate, and 0.14 nmol of purified Ela in a final volume of 100 µl. After the mixture was incubated at 37 °C for 1 h, it was chilled and immediately passed over a Sephadex G-25 column (1.0 × 35 cm), equilibrated with 20 mM Tris-HCl buffer, pH 7.4, containing 20 mM malonate and 15% glycerol at 4 °C. The reaction mixtures prepared for bond characterization of malonyl-Ela consisted of 50 µmol of MOPS buffer, pH 6.8, 0.1 µmol of [14C]malonate, and 0.47 nmol of purified Ela in a final volume of 100 µl. The control sample contained the denatured enzyme at the same condition. After the mixtures were incubated for 2 days at 37 °C, the enzymes from the reaction mixtures were recovered by filtration through a Centricon-30 membrane filter. The remaining [14C]malonate was removed by washing five times with 500 µl of 20 mM Tris-HCl buffer, pH 7.4. The isolated [14C]malonate was subsequently characterized.

Immunoblot Analysis—Antiserum to the purified Ela was prepared in New Zealand White rabbit. The purified Ela (300 µg in 1 ml of 20 mM Tris-HCl, pH 7.4) was emulsified with an equal volume of complete Freund’s adjuvant and was injected subcutaneously at multiple sites over the back of rabbits. After the initial dose, a booster injection of the purified enzyme (200 µg of protein) in 20 mM Tris-HCl buffer, pH 7.4 (1 ml) and incomplete adjuvant (1 ml) was given twice subcutaneously at 2-week intervals in a similar manner. The formation of antibodies was confirmed by electroimmunoaffinity according to Ouchterlony and Nilsson (20). The rabbit was bled 12 days after the last injection. IgG fraction was purified by ammonium sulfate precipitation (40% saturation) and DEAE-Sepharose chromatography (21), dialyzed sufficiently against Tris-buffered saline (TBS), and used for subsequent experiments. The antibody concentration was determined according to Sambrook et al. (22). Immunodetection was carried out with the antibodies prepared for purified El2 as the first antibody (1:50 dilution), alkaline phosphate-conjugated goat anti-rabbit antibody as the second antibody, and p-nitroblue tetrazolium and 5-bromo-3-indolyl phosphate as the substrate, according to protoblot immunoscreening system protocol.

RESULTS

Occurrence of Malonamidase Activities in Free-living B. japonicum Cells and Their Nodule Bacteroids

Novel enzymatic activity which catalyzes the formation of malonohydroxamate from malonate and hydroxylamine was discovered in the cell-free extract of free-living B. japonicum. This enzyme was expressed constitutively in cells grown on Brown's minimal medium (23) supplemented with a variety of carbon sources, such as glucose, arabinose, glycerol, acetate, succinate, malonate, histidine, YM, and GYP (the specific activities (in unit/mg dry cell weight) of the enzyme in the extract of cells were 0.024, 0.02, 0.017, 0.016, 0.02, 0.021, 0.014, 0.017, and 0.022, respectively). In addition, the cell-free extract of B. japonicum contained another enzyme activity which catalyzes the formation of malonohydroxamate from malonate, synthetic amide derivative of malonate, and hydroxylamine. This activity was also expressed constitutively in YM and GYP medium. The enzymes were fractionated by Affi-Gel blue chromatography at pH 7.4. The breakthrough fractions contained enzyme activity with malonate or malonamide. The enzyme corresponding to this activity was designated as malonamidase El. However, the fractions obtained from 2 mM NaCl stepwise elution showed activity only with malonamide. The enzyme corresponding to this activity was designated as malonamidase El2. El2 was divided into two types of enzyme which were eluted separately from DEAE-Sepharose chromatography. The enzymes designated as malonate and NaCl elution were designated as Ela and Elb, respectively. The malonamidase activities of these were measured in the plant cytosol and bacteroids of soybean nodule infected with B. japonicum and the physiological roles of these enzymes were proposed (1).

Purification of Malonamidases

The crude extract prepared from cells grown in GYP medium was subjected to batch treatment with Affi-Gel blue at pH 7.4. El1 did not bind to Affi-Gel blue in this condition, whereas El2 did. El1 in the breakthrough fraction was precipitated by ammonium sulfate between 40–75% saturation. El1 was further purified by the combination of gel filtration with Sephadex G-100 and ion exchange with DEAE-Sepharose. El1 was divided into Ela and Elb types through DEAE-Sepharose chromatography. Although Ela and Elb were separately fractionated with a linear gradient of NaCl (0–0.5 M) (data not shown), they were separated more effectively by the differential elution with malonate and NaCl. After chromatography with DEAE-Sepharose, 95% of El1 activity was recovered in Ela, while 15% of that was recovered in Elb. El1 obtained from the previous step was purified by the combination of blue and red dye affinity chromatography at pH 6.5. El1 was eluted from blue gel under the buffer D containing 7 mM ATP, but was bound to red gel in the same condition. The El1 from red gel was purified ~450-fold to a specific activity of 10.4 unit·mg⁻¹ with a recovery of about 14% (Table 1). The Elb from the DEAE-Sepharose ion-exchange chromatographic step was passed through the hydroxyapatite column, whereas most of the non-enzyme proteins were retained in the column. The Elb from hydroxyapatite was purified about 30-fold to a spe-
E2 was purified about 900-fold to a specific activity of 405 pmol/min/mg by DEAE-Sephacel. The E2 which bound to phenyl-Sepharose preparations resulting from the final purification weight standards; lane G blue at pH 7.4 was eluted with 2 M NaCl stepwise elution. Specific activity of 1.9 pmol/min/mg in comparison to the enzyme (pg). Proteins were stained with Coomassie Blue.

The purity of the malonamidases was electrophoretically homogeneous under the presence of 2 mM EDTA. This step was definitely attributed to the purification of E2. The purity of the malonamidase preparations resulting from the final purification step of each enzyme was examined by SDS-PAGE. All of the isolated malonamidases were electrophoretically homogeneous (Fig. 1). PAGE of the purified enzyme showed a protein band which was coincident with the band from activity staining (data not shown).

**Reactions Catalyzed by Malonamidases**

Malonamidases catalyze all or some of the three different reactions described under “Experimental Procedures.” The relative rate of the reactions (1, 2, and 3) for each enzyme varied: 2 > 3 > 1 for E1a, 3 > 2 > 1 for E1b, and 2 >> 3 for E2. E2 did not show any activity with malonate. All of the above characteristics distinguish the three malonamidases. Meister et al. (2) had reported that dicarboxylate ω-amidase catalyzed reactions 1, 2, and 3 with broad specificity, but that the order of the reaction rates was 2 > 3 > 1. E1a showed a similar activity pattern with that of dicarboxylate ω-amidase.

**Characterization of Malonamidases**

**Molecular Weight and pH**—The molecular weights of E1a, E1b, and E2 were determined by native gradient PAGE to be 126,000, 107,000, and 103,000, respectively. SDS-PAGE of E1a, E1b, and E2 revealed only one protein band. The enzyme had molecular weights of 58,900, 51,600, and 47,800, respectively. SDS-PAGE of E1, E1b, and E2 were determined by a gel isoelectric focusing (pI 3–9) system (Pharmacia).

**Optimal pH**

| Reaction | pH | E1a | E1b | E2 |
|----------|----|-----|-----|----|
| Reaction 1 | 6.8 | ND | ND | ND |
| Reaction 2 | 7.5–8.5 | ND | ND | ND |
| Reaction 3 | 8.0 | ND | ND | ND |

**Specific activity (μmol/min/mg)**

| Reaction | E1a | E1b | E2 |
|----------|-----|-----|----|
| For reaction 1 | 11.3 | 4.8 | 7.2 |
| For reaction 2 | 29.7 | 8.8 | 269 |
| For reaction 3 | 16 | 20.6 | 12.6 |

**Summary of E1a and E1b activity.**

**The values determined into disregard the E1b activity.**

**ND, not determined.**

**The values included the activity by malonamidase E1.**

---

**Table I**

| Purification | Volume | Total protein | Total unit | Specific activity | Recovery | Fold |
|--------------|--------|---------------|------------|-------------------|----------|------|
| E1 Crude extract | 170 | 1,381 | 31.8* | 0.02 | 100 | 1 |
| 1st Affi-Gel blue | 440 | 1,128 | 30.8* | 0.03 | 97 | 1.2 |
| Sephadex G-100 | 81 | 118 | 24.1* | 0.06 | 76 | 2.4 |

**ND, not determined.**

**Table II**

| Protein molecular weight | 126,000 | 107,000 | 103,000 |
| Subunit molecular weight | 58,900 | 51,600 | 47,800 |
| Subunit structure | Homodimer | Homodimer | Homodimer |
| pI | 5.5 | 5.0 | 7.2 |
| Optimum pH | 6.8 | ND | ND |
| Reaction 1 | 7.5–8.5 | ND | ND |
| Reaction 2 | 8.0 | ND | ND |
| Reaction 3 | 2.10 x 10⁵ | ND | 1.5 x 10⁵ |

**The tyrosine and tryptophan content of each subunit is shown in Table V. These extinction coefficients were calculated based on these results.**

**The amount of ammonium ion produced was determined by nesslerization as described under “Experimental Procedures.”**

**The values determined into disregard the E1b activity.**

**ND, not determined.**

**The values included the activity by malonamidase E1.**

---

**Fig. 1. SDS-PAGE of purified malonamidases.** S, molecular weight standards; lane 1, E1a (1 pg); lane 2, E1b (1.4 pg); lane 3, E2 (0.9 pg). Proteins were stained with Coomassie Blue.
**B. japonicum Malonamidase**

**TABLE III**

| Reaction | Substrate Specificity of Malonamidases |
|----------|----------------------------------------|
| E1a      | Activity | Relative rate | Activity | Relative rate | Activity | Relative rate |
| Reaction 1 | Malonate | 10.9 | 100 | 8.12 | 100 | ND | 0 |
|          | 2-Methylmalonate | 5.9 | 55 | 10.0 | 123 | ND | 0 |
|          | Acetate | ND | ND | ND | ND | ND | 0 |
|          | Succinate | 0.23 | 2 | 0.6 | 7 | ND | 0 |
|          | Oxalate | ND | ND | ND | ND | ND | 0 |
|          | Propionate | ND | ND | ND | ND | ND | 0 |
| Reaction 2 | Malonamate | 21.2 | 100 | 13.6 | 100 | 48.1 | 100 |
|          | Acetamide | ND | ND | ND | ND | ND | 0 |
|          | Succinate | ND | ND | ND | ND | ND | 0 |
|          | Malonamide | ND | ND | ND | ND | ND | 0 |
| Reaction 3 | Malonamate | 8.5 | 100 | 44 | 100 | ND | 0 |
|          | Acetamide | 0.27 | 3 | 0.16 | 0.4 | ND | 0 |
|          | Succinate | 0.4 | 5 | 0.5 | 1 | ND | 0 |
|          | α-Ketosuccinate | ND | ND | ND | ND | ND | 0 |
|          | α-Ketoglutamate | ND | ND | ND | ND | ND | 0 |

* ND, not detectable.

*This reaction was assayed using phenol-hypochlorite method as described under "Experimental Procedures."

**Enzyme Stability and Optimum pH**—The purified E1 and E2 in buffer A and B, respectively, showed no detectable loss of enzyme activity at 4 °C for 1 month, and their activities were maintained after repeated freezing (-20 °C) and thawing. However, in phosphate buffer, E2 showed complete loss of its activity within 1 day. The optimum pH for an enzyme varied depending on the reaction it catalyzed (Table II). E1a activity for Reaction 1 depended on the buffer used; MOPS buffer produced the highest activity. Considering the concentration of unionized form (R-COOH) of malonic acid (<15%) above pH 6.8, the sharp pH optimum of E1a activity for Reaction 1 indicates that the unionized form of malonic acid may be the true substrate. This may be the reason for the high substrate concentration required for Reaction 1. The above result corresponds to those of previous studies done with other bacterial α-amidase (4).

**Substrate Specificity**—The substrate specificity of malonamidases was examined for three different reactions using a variety of carboxylic acids and their amide derivatives as substrates (Table III). When methyl-malonate was used as a substrate instead of malonate in the malonyl transfer to hydroxylamine (Reaction 1), methyl-malonohydroxamate was synthesized at a rate of 55 and 123% by E1a and E1b, respectively. However, no other carboxylic acids examined (acetate, succinate, oxalate, propionate, citrate, isocitrate, glutarate, and malate) were converted into hydroxamic acid. In addition, in hydroxaminolysis of malonamate (Reaction 2) and hydrolysis of malonamate (Reaction 3), all malonamidases were highly specific for malonate. Meister et al. (2) had reported that dicarboxylate α-amidase uses α-ketoglutaramate, glutamate, succinate, α-ketosuccinate, succinate, or glutarate, but not malonate or malonamidase as their substrates. These results indicate that novel malonamidases are clearly distinct from dicarboxylate α-amidase.

**Product Identification**—The formation of malonohydroxamate from malonate (or malonamate) and hydroxylamine by malonamidases was confirmed by the isolation of these products using silica gel thin layer chromatography (Rf = 0.27). Malonamate was hydrolyzed by malonamidase into malonate and ammonia. An attempt to detect the formation of malonate from malonate and ammonium chloride with E1a or E1b failed in contrast to the ability of α-amidase to catalyze amide formation (3). However, malonamate formation by E1a or E1b may be possible in bacteroids which keep a high concentration of ammonia.

**Kinetic Parameters**—With increasing concentration of substrates, the rate of malonoxyhydrxamate formation and malonamate hydrolysis increased, and typical Michaelis-Menten saturation kinetics were obtained. The double-reciprocal plots were linear, and from these plots the kinetic parameters in the three different reactions were determined for malonamidases (Table IV). However, the kinetic parameters for E1b and E2 in malonamate hydrolysis could not be determined because E1b showed a saturation pattern below the detection range of the assay and because E2 catalyzed the hydrolytic reaction of malonate too slowly. From K_{cat}K_{m}, it was found that the substrate affinities of E1a and E1b for malonate were similar and that the substrate affinity of E2 for malonate was about 30- and 70-fold higher than those of E1a and E1b, respectively.

**Amino Acid Compositions and N-terminal Amino Acid Sequences**—The amino acid compositions and the N-terminal amino acid sequences of malonamidases are presented in Table V. There about 554, 422, and 440 residues/monomer of E1a, E1b, and E2, respectively. The amino acid compositions of the three malonamidases are different. Since E1b has an acidic pI (5.0) and contains 29 basic residues (His, Arg, and Lys), the major fraction of the 37 Asx and Glx residues must represent aspartic and glutamic acids rather than the amide derivatives. On the other hand, since E1a contains 33 basic residues, the major fraction of the 117 Asx and Glx residues must represent the amide derivatives. In addition, since E1a and E2 contain a number of

$$\text{H}_2\text{O}$$

$$E + \text{HO}^+\text{OC CH}_2\text{CO}^\ominus\text{OH} \rightarrow [E-X-\text{OC CH}_2\text{CO}^\ominus\text{OH}] \rightarrow E + \text{HOOC CH}_2\text{CO}^\ominus\text{OH}$$

**(REACTION 4)**
tryptophan residues, their molar extinction coefficients were high (Table II). Microsequencing allowed the identification of the first 15 amino acids from the N terminus of the three malonamidases (Table V). However, in the case of Elb, four amino acids of the first 15 amino acids could not be identified, even after repeated attempts. The three malonamidases are clearly distinct from one another in sequences in the N-terminal region and their entire amino acid compositions, suggesting that they may be products from different genes.

Inhibition—E1a was competitively inhibited by acetate and propionate with $K_i$ values of 8.3 and 22.4 mM, respectively. However, compounds such as succinate, malate, citrate, etc., which are structurally similar to but larger than malonate had no effect on E1a activity. On the other hand, E2 was inhibited non-competitively by malonate with a $K_i$ value of 0.18 mM, indicating that the radioactivity was due to $[^14C]$malonate. The second approach to the identification of the acyl-enzyme intermediate was through an isolation of $[^14C]$malonyl-Ela by gel filtration (Fig. 2). The excluded peak of $[^14C]$malonate was co-chromatographed with the protein peak. Radioactivity retained in the protein was released by incubation with hydroxylamine, indicating that the radioactivity was due to $[^14C]$malonate. The fact that Ela catalyzes the direct formation of malonate from the reaction mixture containing $[^1-14C]$malonate, while 23% of that was converted to malonate was labeled with $^{18}O$. The malonate in the reaction mixture was isolated, and the $^{18}O$ content was analyzed by GC-MS (Fig. 3). 74% of malonate with two $^{18O}$ was entirely converted to $[^16O]$malonate, while 23% of that was converted to malonate with one $^{18O}$. This result clearly shows that malonyl-enzyme was formed. The bond character of the malonyl-Ela intermediate was determined using the $[^14C]$malonyl-Ela which had been isolated from the reaction mixture containing $[^1-14C]$malonate. The molar ratio of malonyl group/enzyme was found to be 1:1.

The fact that Ela catalyzes the direct formation of malonate from malonate and hydroxylamine suggests that the enzyme itself activates the carboxyl group of malonate by a functional group in its active site. The activation could be through an acyl-enzyme covalent intermediate. The first approach to the identification of the acyl-enzyme intermediate was through an isolation of $[^14C]$malonyl-Ela by gel filtration (Fig. 2). The excluded peak of $[^14C]$malonate was co-chromatographed with the protein peak. Radioactivity retained in the protein was released by incubation with hydroxylamine, indicating that the radioactivity was due to $[^14C]$malonate. The second approach to the identification of the intermediate was through an isotope exchange experiment (Reaction 4). Ela was incubated in the presence of $[^12C]$malonate (50% of the oxygen was labeled with $^{18}O$). The malonate in the reaction mixture was isolated, and the $^{18}O$ content was analyzed by GC-MS (Fig. 3). 74% of malonate with two $^{18O}$ was converted to $[^16O]$malonate, while 23% of that was converted to malonate with one $^{18O}$. This result clearly shows that malonyl-enzyme was formed. The bond character of the malonyl-Ela intermediate was determined using the $[^14C]$malonyl-Ela which had been isolated from the reaction mixture containing $[^1-14C]$malonate. The molar ratio of malonyl group/enzyme was found to be 1:1.

**Formation of Malonyl Enzyme**

The fact that Ela catalyzes the direct formation of malonoxyramate from malonate and hydroxylamine suggests that the enzyme itself activates the carboxyl group of malonate by a functional group in its active site. The activation could be through an acyl-enzyme covalent intermediate. The first approach to the identification of the acyl-enzyme intermediate was through an isolation of $[^14C]$malonyl-Ela by gel filtration (Fig. 2). The excluded peak of $[^14C]$malonate was co-chromatographed with the protein peak. Radioactivity retained in the protein was released by incubation with hydroxylamine, indicating that the radioactivity was due to $[^14C]$malonate. The second approach to the identification of the intermediate was through an isotope exchange experiment (Reaction 4). Ela was incubated in the presence of $[^12C]$malonate (50% of the oxygen was labeled with $^{18}O$). The malonate in the reaction mixture was isolated, and the $^{18}O$ content was analyzed by GC-MS (Fig. 3). 74% of malonate with two $^{18O}$ was converted to $[^16O]$malonate, while 23% of that was converted to malonate with one $^{18O}$. This result clearly shows that malonyl-enzyme was formed. The bond character of the malonyl-Ela intermediate was determined using the $[^14C]$malonyl-Ela which had been isolated from the reaction mixture containing $[^1-14C]$malonate. The molar ratio of malonyl group/enzyme was found to be 1:1.

**Table IV**

**Kinetic properties of malonamidases**

| Malonamidase | $K_m$ (mM) | $V_{max}$ (pmol/min/mg prot) | $K_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$mM$^{-1}$) |
|-------------|------------|-----------------------------|---------------------|----------------------------------|
| Ela         | 5.0        | 20                          | 0.2                 | 3.0                              |
| Elb         | 10.0       | 10                          | 0.1                 | 2.0                              |
| E2          | 20.0       | 5                           | 0.05                | 0.5                              |

*This reaction was assayed using phenol-hypochlorite method as described under "Experimental Procedures."*

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**Table V**

**N-terminals and amino acid compositions**

| Amino acid | Ela | Elb | E2 |
|------------|-----|-----|----|
| Cys*       | 2 (0.4) | 2 (0.4) | 2 (0.4) |
| Asx*       | 38 (6.5) | 28 (6.3) | 28 (6.3) |
| Glx*       | 58 (13.2) | 58 (13.2) | 58 (13.2) |
| Ser         | 25 (5.0)  | 25 (5.0)  | 25 (5.0)  |
| Gly         | 52 (11.7) | 52 (11.7) | 52 (11.7) |
| His         | 9 (2.2)   | 9 (2.2)   | 9 (2.2)   |
| Arg         | 16 (3.6)  | 16 (3.6)  | 16 (3.6)  |
| Thr         | 29 (6.5)  | 29 (6.5)  | 29 (6.5)  |
| Ala         | 25 (5.0)  | 25 (5.0)  | 25 (5.0)  |
| Pro         | 18 (4.1)  | 18 (4.1)  | 18 (4.1)  |
| Tyr         | 4 (1.0)   | 4 (1.0)   | 4 (1.0)   |
| Val         | 22 (5.0)  | 22 (5.0)  | 22 (5.0)  |
| Met         | 6 (1.3)   | 6 (1.3)   | 6 (1.3)   |
| Ile         | 16 (3.6)  | 16 (3.6)  | 16 (3.6)  |
| Leu         | 24 (5.4)  | 24 (5.4)  | 24 (5.4)  |
| Phe         | 17 (1.3)  | 17 (1.3)  | 17 (1.3)  |
| Trp*        | 31 (7.4)  | 31 (7.4)  | 31 (7.4)  |
| Lys         | 12 (2.8)  | 12 (2.8)  | 12 (2.8)  |

* X, non-identified residue.  
* Based on a monomer $M_r$, 58,900 for Ela, 51,600 for Elb, and 47,800 for E2.  
* Determined by performic acid oxidation. In the case of Elb, it was not determined.  
* The values represent both free and amide residues.  
* Determined by methanesulfonic acid hydrolysis. For Elb, it is all incredible value.

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**Fig. 2** Isolation of $[^14C]$malonyl-Ela complex by Sephadex G-25 gel chromatography. Fractions of 0.2 ml were collected. Aliquots of 100 ml were assayed for $^{14}C$ activity by scintillation counting and for protein using the Bradford microassay method (35).
Mass spectra of dimethyl malonyl ester prepared from isotope exchange experiments. Each malonate isolated from the reaction mixture was methylated with ethereal diazomethane before injection into GC. The two compounds had the same GC retention times. The scan was 50–140 atomic mass units with background subtracted. Panel A, control (no Ela); panel B, sample incubated with Ela.

to be 1.77, indicating that two malonyl groups are bound to one molecule of Ela. Since Ela is a homodimer, the result suggests that each subunit of the enzyme is malonylated. The Ela-bound malonate was rather stable upon application of heat (100 °C, 5 min) and strong alkali (final concentration, 0.1 N NaCl) than that of trichloroacetic acid (final concentration, 10%). At present, the functional group in the enzyme which activates the carboxyl group of malonate has not yet been clearly identified.

Immunological Characterization between Three Malonamidases

When immunoprecipitation experiments with the three malonamidases were performed with rabbit antiserum prepared against malonamidase E2, only the antigen itself (E2) precipitated. No cross-reactivity with the other enzymes, Ela and Elb, occurred. These results were also confirmed by the Ouchterlony immunodiffusion test and immunoblot analysis (Fig. 4). These results clearly indicate that E2 does not share any immunological identity with Ela and Elb in spite of the similarity in the type of reactions that they catalyze. Until now, however, the similarity between the immunological identities of Ela and Elb has not yet been examined.

DISCUSSION

In this report we describe the purification to homogeneity of novel malonamidase Ela, Elb, and E2 from B. japonicum and some of their properties. These enzymes can be classified as $\omega$-amidase based on the pattern of the reactions catalyzed; however, their substrate specificity for malonate and/or malonamide clearly distinguishes them from dicarboxylate $\omega$-amidase reported previously. These enzymes were found to catalyze all or some of the following three different reactions (Reaction 1, malonyl transfer to hydroxylamine; Reaction 2, hydroxaminolysis of malonamide; Reaction 3, hydrolysis of malonamate). An interesting point was that, although Ela and Elb acted on the same substrates, they displayed different relative rates of catalysis. The rate of hydrolysis activity of malonamate (Reaction 3) of Elb was 2.5-fold higher than the rate of its hydroxaminolysis activity of malonamate (Reaction 2), in contrast to the corresponding relative rates of Ela (Table II). Furthermore, these malonamidases were shown to be clearly distinct enzymes in molecular structures. This result implies that Ela and Elb must slightly differ from each other in the protein structural aspect of their active sites, whereas the functional groups involved in catalysis are similar.

E1a was found to form a malonyl-enzyme intermediate during catalysis through the isolation of $[^{14}C]$malonyl-E1a and the isotope exchange experiment. Although the nature of the functional group of the enzyme has not yet been elucidated, the inhibition study indicates that the functional group could be either an imidazole or a hydroxyl group. The formation of acyl-enzyme intermediate in dicarboxylate $\omega$-amidase has been also
proposed by kinetic experiment (24). The thiol group of cysteine residues was proposed to be involved in the catalysis by dicarboxylate \( \omega \)-amidase (25). However, various thiol-directed reagents did not affect the activity of malonamidase. Even though a malonyl-enzyme intermediate formed, the attempt to synthesize malonamate directly from NH\(_3\) instead of hydroxylamine in a test tube failed. In vivo, however, malonamate is very likely to be synthesized by the proper local concentration of "NH\(_3\)" or NH\(_3\) donor-like glutamine.

\[
\begin{align*}
E & \rightleftharpoons E-X-OCCH,COOH \\
E + HOOCCH,COOH & \rightarrow E-X-OCCH,COOH + E + HOOCCH,CONH_2 \\
malonate & \rightarrow H_2O \\
malonate & \\
\text{Reaction 5}
\end{align*}
\]

Although the formation of acyl-enzyme intermediate was only confirmed in \( E1a \) catalysis, \( E1b \) properly follows the same mechanism as \( E1a \).

It has been proposed before that one of the biological roles of malonamidases is as a malonate shuttle in symbiotic nitrogen metabolism (1). Under symbiotic conditions the plant keeps a high concentration of malonate in its nodule (14 mM) (9, 25–27). Considering the fact that nodules are packed with bacteroids, which cannot keep malonate in high concentration, the actual concentration of malonate in nodule plant cells may be much higher than the reported concentration. As reported previously, malonate in plant cells seems to be transported into bacteroids by diffusion (28). Recently, it has also been reported that the dicarboxylate carrier on a peribacteroid membrane recognizes malonate \( (K_m, 620 \pm 260 \mu M) \) (29). Since malonate is the most abundant organic acid in plant cell cytosol, the dicarboxylate carrier may preferentially transport it into a peribacteroid unit. As a consequence, malonate seems to be transported first into peribacteroid units by a dicarboxylate carrier, followed by its diffusion into bacteroid cells. Since malonate is a competitive inhibitor of succinate dehydrogenase \( (K = 17 \mu M) \), bacteroids would have to expel it. This is indirectly supported by the fact that the malonate in bacteroids amounted to only \( \sim 2\% \) of the total malonate in nodules (27). There are two possible routes for malonate metabolism in bacteroids: one is to malonyl-CoA by malonyl-CoA synthetase (14) and the other is to malonate. Even though the attempt to synthesize malonamate by \( E1a \) or \( E1b \) catalysis in a test tube failed, this reaction is very likely to occur in vivo. \( E1a \) forms a malonyl-enzyme intermediate which nucleophile, NH\(_3\), generated by nitrogenase, may attack easily. We have the evidence that malonamate exists in a cell-free extract of soybean nodule.\(^2\)

Another hypothesis cannot be ruled out. That is, some bacteroidal membrane contain ammonia monoxygenase which catalyzes the formation of hydroxylamine from ammonia (30, 31). It is this hydroxylamine instead of ammonia which may be the substrate of malonamidase, resulting in the formation of malonohydroxamate. \( E2 \) has very high hydroxaminolysis activity with malonamate. Its role is not clear, but it may possibly be involved in amidolysis or transmalonylation of some acceptor molecules in the periplasmic space or in the peribacteroid space. There is some evidence that \( E2 \) may be located in the periplasmic space and may be secreted into peribacteroid space.\(^2\) However, it is not clear whether \( E2 \)-type enzyme in plant cell reported previously (1) originated from \( B. japonicum \) bacteroids.

Although it has been proposed that transfer of ammonia from the nitrogen-fixing bacteroid to the host cytosol in soybean root nodules occurs by the simple diffusion of NH\(_3\) across the bacteroid and peribacteroid membranes (32–34), the research carried out until now has been done only with isolated bacteroids, and little is actually known about the in vivo transport of fixed nitrogen from bacteroids to plant cells. Hence, it is necessary to investigate malonamidases in all of their genetic and physiological aspects in order to define more clearly both of their roles in the self-protection against malonate and ammonia transport to the plant cell.

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6. Since malonate is a competitive inhibitor of succinate dehydrogenase \( (K = 17 \mu M) \), bacteroids would have to expel it. This is indirectly supported by the fact that the malonate in bacteroids amounted to only \( \sim 2\% \) of the total malonate in nodules (27). There are two possible routes for malonate metabolism in bacteroids: one is to malonyl-CoA by malonyl-CoA synthetase (14) and the other is to malonate. Even though the attempt to synthesize malonamate by \( E1a \) or \( E1b \) catalysis in a test tube failed, this reaction is very likely to occur in vivo. \( E1a \) forms a malonyl-enzyme intermediate which nucleophile, NH\(_3\), generated by nitrogenase, may attack easily. We have the evidence that malonamate exists in a cell-free extract of soybean nodule.

\(^2\) Y. S. Kim and S. W. Kang, unpublished data.