Purification and Characterization of γ-Glutamylmethylamide Synthetase from *Methylophaga* sp. AA-30

Toshio Kimura, Isao Sugahara, Katsuyuki Hanai, and Yuuko Tonomura

Faculty of Bioreources, Mie University, Tsu, Mie 514, Japan

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γ-Glutamylmethylamide synthetase [l-glutamate : methylamine ligase (ADP-forming), EC 6.3.4.12] was purified about 70-fold from a cell-free extract of *Methylophaga* sp. AA-30 by ammonium sulfate fractionation, Octyl-Sepharose column chromatography, and Sephacryl S-300 gel filtration. Only a single protein band was detected after SDS-polyacrylamide gel electrophoresis of the purified preparation; the band was at a position corresponding to a molecular weight of 56,000. The molecular weight of the enzyme was calculated to be 440,000 by Superose 6HR gel filtration, so we suggest that the enzyme is an octomer of identical subunits. The enzyme had maximum activity at pH 7.5 and 40°C. It could use ethylamine and propylamine instead of methylamine as the substrate, but it could not use d-glutamate or l-glutamate instead of l-glutamate.

The occurrence of γ-glutamylmethylamide (GMA) in nature was first reported by Konishi and Takahashi. They showed that in tea (*Thea sinensis*) plants, GMA is synthesized from methylamine and l-glutamate, and that the N-methyl carbon of GMA is a precursor in caffeine synthesis. Kung and Wagner reported that in *Pseudomonas* strain MS, GMA is an intermediate in the metabolism of methylamine, synthesized from glutamate and methylamine by the action of GMA synthetase [l-glutamate : methylamine ligase (ADP-forming), EC 6.3.4.12] in the presence of ATP and Mn²⁺. Several species of bacteria grown with methylamine as the sole source of carbon and energy synthesize GMA by GMA synthetase. The reaction catalyzed by GMA synthetase is similar to that catalyzed by glutamate synthetase, which can catalyze the synthesis of GMA from glutamine and methylamine, so there is some doubt whether GMA synthetase is different from glutamate synthetase. Leitch suggested that two different enzymes catalyze the synthesis of glutamine and GMA in *Pseudomonas* strain MS but failed in purifying the enzymes.

We have reported on enzymes catalyzing the synthesis and decomposition of GMA in a marine methylotroph, *Methylophaga* sp. AA-30, which was grown with methylamine. GMA synthetase is an inducible enzyme; it is detected only when the microorganism is grown with methylamine as the sole source of carbon and energy. Details about properties of GMA synthetase are not known because the enzyme is labile. Here, we report that ammonium sulfate protected the enzyme from inactivation, and give results of the purification and characterization of GMA synthetase from *Methylophaga* sp. AA-30.

Materials and Methods

Microorganism and culture conditions. *Methylophaga* sp. AA-30 isolated from coastal water was grown in a mineral salt medium containing (per liter of distilled water): KCl, 0.2 g; MgSO₄·7H₂O, 0.3 g; CaCl₂·2H₂O, 0.01 g; NaCl, 25 g; methylamine·HCl, 15 g; H₃PO₄, 0.1 g; FeSO₄·7H₂O, 0.01 g; ash of yeast extract, 0.005 g; cyanocobalamin, 1 µg; CuSO₄·5H₂O, 20 µg; ZnSO₄·7H₂O, 40 µg; H₂BO₃, 40 µg; NiSO₄·6H₂O, 40 µg; Na₂MoO₄·2H₂O, 40 µg; MnCl₂·4H₂O, 40 µg; and CoSO₄·7H₂O, 40 µg. The medium was prepared as a solution. A medium containing the above nutrients except for FeSO₄·7H₂O, ash of yeast extract, and methylamine·HCl was heat-sterilized, and after being cooled, a filter-sterilized 50% (w/v) methylamine·HCl solution and a filter-sterilized HCl solution (0.1 N) containing FeSO₄·7H₂O and ash of yeast extract were added to the medium to give the composition stated above. The pH of the medium was adjusted to 7.0 with 3 N NaOH. Culture was for about 2 weeks in a jar fermentor (B. E. Marubishi, MD-500) containing 6 liters of the medium under the following conditions: agitation, 300 rpm; aeration, 3 liters/min; dilution rate, 0.15 hr⁻¹; temperature, 35°C; pH, 7.0 (controlled with 3 N NaOH). The cells were harvested by centrifugation and stored at −85°C until use. About 100 g of wet cells was obtained from 20 liters of the culture medium.

Assay of enzyme activity. GMA synthetase activity was assayed by measurement of GMA produced in a reaction mixture containing 200 µl of 0.5 M Tris·HCl buffer (pH 7.5), 20 µl of 1.0 M sodium l-glutamate, 50 µl of 0.5 M methylamine·HCl, 10 µl of 2.5 M 2-mercaptoethanol, 40 µl of 0.1 M MnCl₂, 40 µl of 0.1 M ATP, 440 µl of distilled water, and 200 µl of an enzyme solution during incubation at 30°C for 10 min. The reaction was stopped by the addition of 2 ml of 5% sulfosalicylic acid and 1 ml of 0.1 M lithium citrate buffer (pH 2.2), and the GMA formed was measured with an amino acid analyzer (Atto Co., Ltd., MLC-203). The amount of the enzyme that gave 1 µmol of GMA per minute under these conditions was defined as 1 unit. Glutamine synthetase activity was assayed by measurement of the rate of hydroxamate formation from l-glutamate and hydroxylamine as described by Bender et al. The amount of glutamine synthetase that gave 1 µmol of glutamylhydroxamate per minute was defined as 1 unit. Protein was measured by the method of Bradford with bovine serum albumin as the standard.

Purification of GMA synthetase. The enzyme was purified from *Methylophaga* sp. AA-30 cells as described below. All operations were done at 0 to 5°C unless otherwise specified.

1) Extraction. Frozen cells (80 g, wet weight) were suspended in 160 ml of 50 mM Tris·HCl buffer (pH 7.5) containing 1 mM dithioerythritol and disrupted with an ultrasonicator (Heat Systems-Ultrasonics Inc., W-75). After centrifugation at 20,000 × g for 20 min, 10 ml of 50% (w/v) streptomycin sulfate solution was added to the supernatant. Precipitated materials were removed by centrifugation at 20,000 × g for 30 min.

2) Ammonium sulfate fractionation. The crude extract was fractionated with ammonium sulfate. Proteins precipitating between 40 and 50% saturation were collected and dissolved in 40 ml of 20 mM imidazole·HCl buffer (pH 6.3) containing 5 mM 2-mercaptoethanol and 1.2 M ammonium sulfate.

3) Octyl-Sepharose CL-4B column chromatography. The enzyme solution was put on an Octyl-Sepharose CL-4B column (26 × 100 mm) equilibrated with 20 mM imidazole·HCl buffer (pH 6.3) containing 5 mM 2-mercaptoethanol and 1.2 M ammonium sulfate. The column was washed with 300 ml of this buffer and elution was done at a flow rate of 60 ml/hr.
with a reverse linear gradient of ammonium sulfate from 1.2 M to 0 M in
400 ml of the same buffer. Protein concentration in the effluent was
monitored by measurement of OD_{280} with a Mini-UV monitor (Atto,
SI-1541). Ten-milliliter fractions were collected and active fractions
(fraction Nos. 24—34) were mixed with ammonium sulfate at the final
concentration of 1.2 M. The solution was rechromatographed on an Octyl-
Sepharose CL-4B column (ø16 × 200 mm) under the same conditions
as before. Active fractions (about 90 ml) were dialyzed overnight against
20 mM imidazole—HCl buffer (pH 6.3) containing 5 mM 2-mercaptoethanol
and 1.0 M ammonium sulfate and concentrated to 8 ml by ultrafiltration
with an Amicon ultrafilter (UK-30).
4) Sephacryl S-300 chromatography. The concentrated enzyme
solution (8 ml) was then fractionated with a Sephacryl S-300 column
(ø26 × 900 mm) equilibrated with 20 mM imidazole—HCl buffer (pH 6.3)
containing 5 mM 2-mercaptoethanol and 1.0 M ammonium sulfate. Elution
was at a flow rate of 20 ml/hr with the same buffer, and 10-ml fractions
were collected. Active fractions (fraction Nos. 27—31) were concentrated
10-fold by ultrafiltration with an Amicon ultrafilter (UK-30) and
glycerin was added to the concentrated enzyme solution at the concen-
tration of 20% (v/v). The enzyme solution was stored at —85°C, and
was stable for one month under these conditions. The enzyme solution
was filtered through a Bio-Gel P-6DG column equilibrated with the buffer
to be used in that experiment just before use.

Electrophoresis. SDS-polyacrylamide gel electrophoresis was done in
12.5% polyacrylamide gel slab gels with the Tris—glycine buffer system
described by Laemmli.14 The protein was stained with Coomassie brilli-
ant blue R-250. The marker proteins used were: cytochrome c monomer,
mol. wt. 12,400; cytochrome c dimer, mol. wt. 24,800; cytochrome c
trimmer, mol. wt. 37,200; cytochrome c tetramer, mol. wt. 49,600; and
cytochrome c hexamer, mol. wt. 74,400.

Molecular weight determination. The molecular weight of the native
enzyme was estimated by Superose 6HR gel filtration with a FPLC
chromatography (Pharmacia LKB). The purified GMA synthetase
preparation (about 50 µg protein/50 µl) was put on a Superose 6HR
column equilibrated with 50 mM potassium phosphate buffer (pH 7.0)
containing 0.15 M NaCl. Proteins were eluted with the same buffer at
the flow rate of 0.2 ml/min at room temperature. Standard proteins (each
100 µg/50 µl) were put on a Superose 6HR column as described above.
TD standard proteins used were (mol. wt.): thyroglobulin (670,000),
yeast glutamate dehydrogenase (290,000), γ-globulin (158,000), pig heart
lactate dehydrogenase (140,000), and ovalbumin (44,000). The molecular
weight of the enzyme was estimated from a calibration curve prepared
with these standard proteins.

Chemicals. GMA, γ-glutamylmethyamine, and γ-glutamylpropylamide
were synthesized chemically by the procedure of Leichtenstein.15 Octyl-
Sepharose CL-4B and Sephacryl S-300 were products of Pharmacia LKB
Biotechnology (Uppsala, Sweden). The protein assay kit was purchased
from Bio-Rad Laboratories (Richmond, Calif.). All other chemicals
were of analytical grade and were purchased from Nacalai Tesque, Inc.
(Kyoto).

Results
Effect of ammonium sulfate on the stability of GMA synthetase
When crude extracts of strain AA-30 were stored at 4°C in 20 mM
imidazole—HCl buffer (pH 6.3) for 48 hr, the residual activity of GMA
synthetase was lowered by about half. This enzyme was labile and it was necessary
to stabilize the enzyme before purification. The enzyme was
stabilized by the addition of ammonium sulfate at high concentrations of 1 M or more. More than 90% of the
original activity remained after the enzyme was kept at 0°C in 20 mM piperazine—HCl buffer (pH 5.5) for a week,
when the buffer contained 1 M ammonium sulfate. Stabiliza-
tion was observed with sodium sulfate but not with ammonium chloride (data not shown), indicating that
sulfate ion participated in the stabilizing effect.

Purification of GMA synthetase
The purification of GMA synthetase is summarized in Table I. GMA synthetase was purified 73.6-fold from the
crude extract with a yield of 19.4%. When the final en-
yme preparation was treated by SDS-polyacrylamide
gel electrophoresis, a single protein band was observed
(Fig. 1). Figure 2 shows the elution profiles of glutamine
synthetase and GMA synthetase in the first Octyl-Sepharose
CL-4B column chromatography. GMA synthetase was eluted in fractions No. 25 to 32, and γ-glutamyltransferase
was eluted in fractions No. 19 to 23. γ-Glutamyltransferase
activity was not detected in the GMA synthetase fractions.

Molecular weight
The molecular weight of the purified enzyme was calculated to be 440,000. After SDS-polyacrylamide gel

![Fig. 1. Electrophoretic Pattern of Purified GMA Synthetase.](image-url)
Electrophoresis of the purified enzyme preparation, a protein band was found at a position corresponding to a molecular weight of 56,000 (Fig. 1), so we concluded that the enzyme had eight identical subunits.

Effects of pH and temperature on enzyme activity and stability

GMA synthetase had maximum activity at the pH range of 7.5 to 8.0 and was most stable between pH 4.5 and 6.0 (Fig. 3). Figure 4 shows the dependence of the activity and heat stability of this enzyme on temperature. Activity was maximum at 40°C (10 min reaction). The enzyme was stable at up to 30°C, and lost its activity at 50°C.

Effects of metal ions

A number of metal ions were tested for their ability to replace Mn²⁺ in the enzymatic synthesis of GMA (Table II). GMA synthetase had activity in the presence of Fe²⁺, but not in the presence of Mg²⁺, Ca²⁺, Co²⁺, or Ni²⁺.
**Substrate specificity**

The purified enzyme could use ethylamine and propylamine instead of methylamine as the substrate (Table III). It catalyzed the formation of l-glutamate from L-glutamine and ammonium ion (Table III), although it did not form L-glutamylhydroxamate from L-glutamine and hydroxylamine (Fig. 2). The Michaelis constant, $K_m$, for ammonium chloride was $1.37 \times 10^{-2} \text{M}$, which was much higher than that for methylamine. The enzyme had little or no ability to use D-glutamate, L-aspartate, or L-glutamine instead of L-glutamate as the substrate (Table III).

**Discussion**

We found that high concentrations of sulfate ion protect GMA synthetase from inactivation, and thus succeeded in purifying the enzyme from *Methylophaga* sp. AA-30 by Octyl-Sepharose CL-4B chromatography and Sephacryl S-300 gel filtration in the presence of ammonium sulfate at high concentrations. Salting-out-type salts including ammonium sulfate seem to facilitate the formation of hydrophobic interaction in proteins. Studies of salt-dependent proteins in extremely halophilic bacteria have suggested that salting-out-type salts may stabilize the enzymes of halophiles by strengthening hydrophobic interactions, giving a more tightly folded conformation.\(^{17}\)

Hochstein and Dalton\(^{18}\) reported that NADH oxidase from a halophilic bacterium was protected by salting-out-type salts including ions such as SO$_4^{2-}$ and Cl$^-$. GMA synthetase may have such salt-dependent properties.

Studies\(^{19}\) done with a crude GMA synthetase preparation from *Pseudomonas* strain MS suggested that the enzyme required Mn$^{2+}$ for its activity, and that Ca$^{2+}$, Co$^{2+}$, and Fe$^{2+}$ could replace Mn$^{2+}$. We found, however, that the enzyme in *Methylophaga* sp. AA-30, did not function with Ca$^{2+}$ or Co$^{2+}$ as it did with Mn$^{2+}$. GMA synthetase, whatever its origin, requires Mn$^{2+}$ and ATP for its activity, and can use Fe$^{2+}$ in place of Mn$^{2+}$.

The enzyme used ethylamine or propylamine as its substrate instead of methylamine, which suggests that the enzyme has broad substrate specificity with respect to one of its substrates. The enzyme seemed to have strict substrate specificity for the other substrate, L-glutamate.

It is not clear whether GMA synthetase is different from glutamine synthetase. Our purified GMA synthetase used ammonia fairly rapidly in place of methylamine, but the $K_m$ for ammonium chloride was much higher than that for methylamine. The purified enzyme did not form L-glutamylhydroxamate from L-glutamine and hydroxylamine, and was separated from an enzyme (probably real glutamine synthetase) that catalyzed the formation of hydroxylamates with low concentrations of hydroxylamine ($K_m$ was 0.18 mM; other data not shown) by Octyl-Sepharose CL-4B column chromatography (Fig. 2). We concluded that the purified enzyme differed from glutamine synthetase and that it catalyzed the synthesis of GMA in *Methylophaga* sp. AA-30 cells under physiological conditions.

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**References**