Role of Glutamine Synthetase in the Uptake and Metabolism of Methylammonium by Azotobacter vinelandii

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Methylammonium is a substrate for the ammonium transport system of Azotobacter vinelandii. During cellular uptake methylammonium is rapidly converted to a less polar metabolite (E. M. Barnes, Jr., and P. Zimniak, J. Bacteriol. 146:512–516, 1981). This metabolite has been isolated from A. vinelandii and identified as γ-glutamylmethylamide by mass spectroscopy, 1H nuclear magnetic resonance spectroscopy, and chromatography with the authentic compound. Escherichia coli also accumulated γ-glutamylmethylamide during methylammonium uptake. The biosynthesis of γ-glutamylmethylamide in vitro required methylammonium, ATP, L-glutamate, and a soluble cell extract from A. vinelandii. The enzyme responsible for γ-glutamylmethylamide synthesis was glutamine synthetase. In a crude extract, L-methionine-DL-sulfoximine was equipotent in inhibiting the activities for γ-glutamyltransferase and for the synthesis of glutamine and γ-glutamylmethylamide. Likewise, an antiserum against the glutamine synthetase of E. coli precipitated the transferase and both synthetic activities at similar titers. During repression by growth of cells on ammonium medium, the synthesis of glutamine and γ-glutamylmethylamide in vitro was also inhibited coordinately. A partially purified preparation of glutamine synthetase from A. vinelandii utilized methylammonium as substrate (Km = 78 mM, Vmax = 0.30 μmol/min per mg), although less efficiently than ammonium (Km = 0.089 mM, Vmax = 1.1 μmol/min per mg). The kinetic properties of glutamine synthetase with methylammonium as substrate as well as the insensitivity of this activity to inhibition by Ti⁺ were strikingly different from methylammonium translocation. Thus, methylammonium (ammonium) translocation and intracellular trapping as glutamylamides are experimentally distinguishable processes.

Methylamine is used experimentally both as a substrate for ammonium transport (2, 5, 6, 13, 17, 19) and in measurement of intracellular pH (1, 7) in microorganisms. It is commonly assumed that methylamine is not metabolized, although specialized C1-utilizing microorganisms readily metabolize it. Two pathways for methylamine oxidation by methylo trophic bacteria are known (3). One of these is a mechanism involving direct conversion to formaldehyde via methylamine dehydrogenase as in Pseudomonas AM1 (4). In the second, intermediates, γ-glutamylmethylamide (GMAD), N-methyl glutamate, and N-methylalanine accumulate as in Pseudomonas MS (15, 20).

In the course of our studies of the ammonium transporter in Azotobacter vinelandii, a N2-fixing aerobe, we found that methylammonium is rapidly converted to an impermeant metabolite (2). This is surprising since A. vinelandii is not a methylo troph, nor is it able to utilize methylammonium as a nitrogen source. In this paper we report the identification of this metabolite as GMAD and the identification of glutamine synthetase as the enzyme responsible for its synthesis. The properties of methylammonium as a substrate for glutamine synthetase are also described and compared with those for membrane transport.

MATERIALS AND METHODS

A. vinelandii OP (ATCC 13705) was grown as previously described (2), except that media were supplemented with a trace metal solution (16). The uptake assay of [14C]methylammonium was adapted from the method of Barnes and Zimniak (2) (see legend to Fig. 1). For isolation and purification of the methylammonium metabolite, the uptake assay was scaled up and modified. Cells (500 to 1,000 mg of protein) were suspended in 30 ml of 10 mM sodium phosphate (pH 7.4)–0.1 mM MgCl2. Sodium succinate was added to give 2 mM, and humidified oxygen was bubbled through the cell suspension at room temperature. Over a period of 30 min, a concentrated solution (total, 6 ml) containing 4.8 mmol of succinate and 40 μmol of

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methylvammonium chloride (50 mol% C\textsubscript{2}H\textsubscript{5}NH\textsubscript{2} and 0.11 mol% \textsuperscript{14}C\textsubscript{2}H\textsubscript{5}NH\textsubscript{2}, equivalent to 70 mCi/mol) was added in small portions every 2 min. After the last addition was made, the suspension was oxygenated for another 15 min and centrifuged. The pellet was suspended in 20 ml of water and heated for 5 min in a boiling water bath. After dilution with water, the mixture was centrifuged (15 min at 70,000 × g), and the pellet was reextracted with water. The combined supernatants, which contained 60 to 80\% of the initial radioactivity, all located in the metabolite spot as determined by thin-layer chromatography (2), were lyophilized, and the residue was extracted twice with hot methanol. The methanol extract was dried, and the residue was dissolved in 1 ml of water and centrifuged. The material so obtained was subjected to a series of chromatographic purification steps: Sephadex G-15 (0.8 by 85 cm) in water, followed by a preparative high-pressure liquid chromatography silica gel column eluted with CH\textsubscript{3}OH-H\textsubscript{2}O-CH\textsubscript{2}COOH (80:19:1) and, in the next run, with CH\textsubscript{3}OH H\textsubscript{2}O (9:1), followed by a C\textsubscript{18} reversed-phase column eluted with water. A single radioactive peak emerged which on concentration yielded 0.4 mg of material and contained 6.4\% of the initial radioactivity. Assuming for the half-deuterated compound a mean molecular weight of 161.5 (see below) and one labeled methyl group per molecule, the material was pure as judged by its radioactivity.

Thin-layer chromatography of the material, with and without prior dansylation, was previously reported (2). Authentic GMAD was synthesized chemically by cyclization of glutamic acid to pyroglycamic acid and by its subsequent reaction with methylamine by the method of Lichtenstein (10).

\textsuperscript{1}H nuclear magnetic resonance (NMR) spectra were recorded at 100 MHz with a Varian XL-100-15 NMR spectrometer interfaced to a Nicolet TT-100 data system. Samples for analysis (30°C) were dissolved in deuterated water. Chemical shifts were expressed in parts per million from external tetramethylsilane.

Mass spectra were recorded with a Finnigan-MAT double-focusing high-resolution mass spectrometer outfitted with a combination field desorption—field ionization—electron ionization ion source. Spectra were obtained in the field desorption mode with a benzonitrile-activated tungsten wire emitter; the amino acids field desorbed at 16 mA of current. The spectra were peak matched with histidine as the mass standard.

For enzyme assays, a crude extract of \textit{A. vinelandii} was prepared by sonication (40-W output, 5 min) of cells suspended in 25 mM imidazolium chloride buffer (pH 7.1) containing 1 mM MnCl\textsubscript{2}. The homogenates were centrifuged at 40,000 × g for 30 min, and the supernatant was decanted. Protein was determined by the method of Lowry et al. (11). For kinetic studies, glutamine synthetase was purified through the acid phase in a single step (16). \gamma-Glutamyltransferase activity was measured by the hydroxamate method (14). The biosynthetic activity of glutamine synthetase in crude extracts was assayed by phosphate release (14). In some cases with the \textsuperscript{14}Cmethylvammonium substrate, the incubation mixture was adsorbed on Dowex-50 columns, and GMAD was eluted as described by Kung and Wagner (9). For kinetic studies of the purified glutamine synthetase, the biosynthetic activity was determined by a continuous spectrophotometric assay with pyruvate kinase and lactic dehydrogenase (14).

The procedures of Stevenson and Silver (17) were followed for the growth of \textit{Escherichia coli} B and measurement of methylamine uptake. A crude antiserum against the glutamine synthetase of \textit{E. coli} was provided by Earl Stadtman (National Heart, Lung, and Blood Institute). A \gamma-globulin fraction was prepared by ammonium sulfate precipitation (40% saturation at 0°C) followed by dialysis. The final volume of the \gamma-globulin fraction was the same as that of the original serum. A control preimmune sheep serum was treated similarly. For immune precipitation, the \gamma-globulin fraction was serially diluted with a buffer containing 100 mM KCl, 1 mM MnCl\textsubscript{2}, and 10 mM imidazolium chloride (pH 7.1). A crude cell extract (150 \mu l, 55 \mu g of protein) was incubated for 2 h at 0°C with 30 \mu l of diluted \gamma-globulin. These incubations were then centrifuged (15,000 × g for 5 min), and the supernatants were assayed for glutamine synthetase activities.

### RESULTS

A typical time course for the uptake of \textsuperscript{14}Cmethylvammonium by intact cells of \textit{A. vinelandii} is shown in Fig. 1A. During this experi-

![FIG. 1. Uptake and metabolism of \textsuperscript{14}Cmethylvammonium by \textit{A. vinelandii}. Assay mixtures contained 10 mM N-(tris(hydroxymethyl)methyl)glycine (adjusted to pH 7.8 with NaOH), 1 mM MgCl\textsubscript{2}, 8 mM sodium succinate, 0.1 mM \textsuperscript{14}C\textsubscript{2}H\textsubscript{5}NH\textsubscript{2}Cl (6.1 \muCi/mmol), and intact cells (50 \mu g of total protein) in a final volume of 0.1 ml. (A) Total intracellular radioactivity (O) was determined after termination of the assay by filtration (see the text) at the times shown. (B) Distribution of intracellular radioactivity as GMAD (●) and CH\textsubscript{3}NH\textsubscript{2}* (△) was determined by thin-layer chromatography of extracts from filters (see the text). The data are expressed as percentages of the total counts detected on the chromatogram.](http://jb.asm.org/)
ment, cellular extracts were analyzed as shown in Fig. 1B. After 1 min of uptake, methylammonium accounted for 34% of the total radioactivity; the remainder was associated with a less polar metabolite. With time, the metabolite constituted an increasing fraction of the intracellular radioactivity and represented 95% of the total after 10 min. Only a single metabolite was detected. Thin-layer chromatography of both the native and dansyl derivative as well as high-pressure liquid chromatography on reversed-phase and silicic acid columns revealed only a single 14C-labeled species (data not shown). This simple pattern for methylammonium metabolism is consistent with the observation that this compound cannot serve as a nitrogen source for A. vinelandii (Jayakumar and Barnes, unpublished results).

In experiments similar to those shown in Fig. 1, the metabolite was labeled by incorporation of methylammonium containing 50 mol% C3H7NH2 and 0.11 mol% 14CH3NH2. From the cell extracts, the metabolite was purified to homogeneity by high-pressure liquid chromatography and then analyzed by field desorption mass spectroscopy. This yielded a characteristic doublet (M+H = 161; M2+H+ = 164) and an accurate mass of 160.077 ± 0.018. Among other possibilities, this suggested the formula C6H12N2O3 (M = 160.085), consistent with GMAD. GMAD was synthesized chemically (10). The 1H-NMR spectrum of this authentic compound closely resembled that of the isolated metabolite. Both exhibited a prominent narrow resonance at 2.89 ppm attributable to the N-methyl group. The single α proton, the two β protons, and the two γ protons gave multiplets centered at 3.77, 2.61, and 2.29 ppm, respectively. Likewise, the mobilities of the metabolite and of its dansyl derivative on thin-layer chromatography corresponded to those for GMAD. Amino acid analysis of perchloric acid extracts of A. vinelandii incubated with methylammonium also revealed substantial amounts of a substance which coeluted with GMAD. (Jayakumar and Barnes, Fed. Proc. 42:1941, 1983). This compound was not found in control cells incubated with N2 or NH4+. Under conditions similar to those of the experiments shown in Fig. 1, E. coli B accumulated GMAD at a rate of 0.3 nmol/min per mg of protein. This represents about 20% of the activity observed with A. vinelandii.

The in vitro synthesis of GMAD by an extract from A. vinelandii could also be demonstrated (Table 1). The synthesis of GMAD from methylammonium required both ATP and glutamate, suggesting the involvement of glutamine synthetase or an analogous activity. Glutamine synthetase was assayed as a Mn2+-dependent γ-glutamyltransferase activity (from glutamine to NH2OH) as well as a Mg2+-dependent biosynthetic activity. As shown in Table 2, A. vinelandii extracts contained significant levels of both activities, as was previously reported (8, 16). Methylammonium was an alternate substrate for the biosynthetic activity in crude extracts and yielded activities about half those found with NH4+ as substrate. During growth of A. vinelandii on NH4+, glutamine synthetase is regulated by adenylylation (8, 16). This inhibits the biosynthetic activity (NH4+ as substrate) and stimulates the transferase activity (Table 2). With methylammonium as biosynthetic substrate, the activity was also dramatically reduced in NH4+-cultured cells (Table 2). Although the degree of inhibition appears more pronounced for the methylammonium-dependent than for the ammonium-dependent activity, the rather low rates observed place the differences nearly within experimental error.

The effects of methionine sulfoximine, a potent inhibitor of glutamine synthetase (12), on these activities was also examined (Fig. 2). At 1

| Table 1. Synthesis of GMAD from a cell-free extract of A. vinelandii |
|-------------------------------|-------------------------------|
| **Additions** | **P, released** |
| Completea | 0.17 |
| CH3NH3+ | 0.02 |
| Glutamate | 0.02 |
| ATP | 0.00 |
| Cell extract | 0.00 |

a Expressed as micromoles of P, formed from ATP per 30-min assay. In some cases, 14CH3NH3+ was employed as substrate; the radioactive reaction product chromatographed with authentic GMAD (see text).

b Complete assay contained 50 mM imidazolium chloride (pH 7.6), 100 mM L-glutamate, 50 mM MgCl2, 6.25 mM ATP, 125 mM CH3NH2Cl, and 25 μg of protein from a cell-free extract (see the text) in a final volume of 0.2 ml.

| Table 2. Regulation of glutamine and GMAD synthetase by ammonium |
|-------------------------------|-------------------------------|
| **Nitrogen source** | **Transferase activity** | **Biosynthetic activity** |
| NH2 | CH3NH2 |
| N2 | 0.41 ± 0.02 | 0.28 ± 0.02 | 0.14 ± 0.01 |
| NH4+ | 2.92 ± 0.03 | 0.057 ± 0.009 | 0.014 ± 0.004 |

a A. vinelandii was grown on media (see text) without fixed nitrogen or with 10 mM NH4Cl.
b Expressed as micromoles per minute per milligram of protein.
c Crude extracts were assayed in the presence of 50 mM MgCl2 and either 50 mM NH4Cl or 200 mM CH3NH2Cl as described in Table 1, footnote b. Formation of P, from ATP was measured.
FIG. 2. Effect of methionine sulfoximine on glutamine and GMAD synthetase activities in crude extracts of A. vinelandii. A soluble extract (90 μg of protein) was incubated for 30 min at 25°C with L-methionine-DL-sulfoximine at the final concentrations shown in the presence of 5 mM ATP-50 mM MgCl₂-25 mM imidazolium chloride (pH 7.1) in a final volume of 0.2 ml. Samples from these incubations were assayed for transferase activity (A, △) and for biosynthetic activity (B) with 50 mM NH₄Cl (○) or 125 mM CH₃NH₃Cl (●) as described in Table 2, footnote c. All activities are expressed as percentages of activities in controls which lacked inhibitors.

μM, this compound produced a 60% inhibition of the rates of GMAD synthesis and of transferase activity; 65% inhibition was observed for glutamine synthesis. The effects of methionine sulfoximine on all three activities were essentially the same at each concentration tested.

Immune precipitation experiments with an antiserum against the E. coli glutamine synthetase were also conducted. The γ-globulin fraction of this antiserum was previously shown to cross-react with the A. vinelandii enzyme (18). This observation was confirmed (Fig. 3A); 32% of the total transferase activity (represented by 55 μg of crude extract) was precipitated by 1 μl of immune γ-globulin. The biosynthetic activity with both ammonium and methylammonium substrates was precipitated at essentially the same titer. In no case did γ-globulin from preimmune serum reduce activity.

The kinetic parameters of methylammonium and ammonium as substrates were determined with a partially purified preparation of A. vinelandii glutamine synthetase. Both substrates gave linear, monophasic Eadie-Hofstee plots (data not shown). Methylammonium gave a \( V_{\text{max}} \) of 0.30 μmol/min per mg, which was 27% of that observed for ammonium. The \( K_m \) for methylammonium (78 mM) was strikingly higher than that for ammonium (0.089 mM). This suggests that the apparent affinity of glutamine synthetase for methylammonium is approximately 0.1% of that for ammonium.

DISCUSSION

A metabolite of methylammonium which was previously shown to accumulate in A. vinelandii (2) has now been identified as GMAD. This was demonstrated by isolation of the metabolite followed by mass spectroscopy, NMR spectroscopy, and cochromatography with chemically synthesized GMAD. Although Gordon and Moore (6) reported that only chemically unaltered methylammonium accumulates in the same

FIG. 3. Immune precipitation of glutamine and GMAD synthetase activities. Crude extracts from A. vinelandii were incubated (see the text) with γ-globulin from antiserum to the E. coli glutamine synthetase (△, ○, ●) or a preimmune pooled serum (△, □, ■). The volume of γ-globulin solution shown is based on the original serum volume and calculated by the serial dilution factor. After centrifugation, samples were assayed for transferase activity (A, △ and ○) or biosynthetic activity (B) with 50 mM NH₄Cl (●, ■) or with 125 mM CH₃NH₃Cl (○, □) as described in Table 2, footnote c. All activities are expressed as percentages of activities in controls which lacked γ-globulin.
strain of *A. vinelandii*, the chromatographic method which they employed affords small resolution of methylammonium and GMAD. We were also able to identify GMAD as the major metabolite of methylammonium in *E. coli*. This compound has been found in *Rhizobium* sp. under similar conditions (5).

We have demonstrated that the biosynthesis of GMAD by cell extracts required both glutamate and ATP. The enzyme responsible for this reaction was glutamine synthetase, the major activity of ammonia assimilation in *A. vinelandii*. Glutamine synthetase was measured in crude extracts as biosynthetic activities with ammonium and methylammonium substrates. Both biosynthetic activities responded similarly to inhibition during ammonium repression experiments, inactivation by methionine sulfoximine, and immune precipitation by a specific antiserum. Likewise, a partially purified preparation of glutamine synthetase from *A. vinelandii* utilized methylammonium as a substrate. Woolfolk et al. (20) have shown previously that methylammonium is a weak substrate for pure preparations of glutamine synthetase from *E. coli*. These findings are quite different from those of Kung and Wagner (9), who reported that a distinct GMAD synthetase is induced in *Pseudomonas MS* during growth on methylamines.

The observation that methylammonium is rapidly converted to GMAD places significant restrictions on its use as a transport substrate or probe for measurement of intracellular pH. Both our laboratory (2) and others (6, 19) have found methylammonium to be a useful substrate for the ammonium transport system of *A. vinelandii*. However, the majority of radioactivity which accumulates intracellularly is contained in GMAD, which is much less permeable than methylammonium (Fig. 1 and reference 2). Thus, we consider the uptake of methylammonium to be a two-step process involving a mediated translocation of solute across the cell membrane followed by its intracellular conversion to GMAD. Since the latter step represents a metabolic trapping reaction which prevents exodus of radiolabel, GMAD synthesis is likely to become rate limiting for the accumulation of label at uptake times beyond 1 min (Fig. 1). We have also demonstrated recently that GMAD itself is an inhibitor of methylammonium uptake (Jayakumar and Barnes, Fed. Proc. 42:1941, 1983), which introduces an additional complication for these measurements.

Two groups of investigators have suggested a role for glutamine synthetase in the translocation of methylammonium. Kleinschmidt and Kleiner (8) based this idea on the finding that a small fraction of glutamine synthetase activity is associated with membranes from *A. vinelandii*. A similar view by Gober and Kashket (5) is supported by the observation that methionine sulfoximine inhibits the uptake of methylammonium by *Rhizobium* sp. On the other hand, we have found a number of properties which distinguish methylammonium (ammonium) transport from glutamine synthetase. (i) The apparent affinity of uptake sites for methylammonium (Km = 25 μM [2]) is more than 3 orders of magnitude higher than the corresponding affinity for glutamine synthetase (Km = 78 mM). (ii) The uptake of methylammonium is strongly inhibited by Tl+ (Ki = 13 μM [2]), whereas glutamine synthetase activity in vitro and ATP synthesis in vivo are not significantly affected by Tl+ concentrations up to 200 μM (data not shown). (iii) Glutamine and glutamic analogs inhibited methylammonium uptake at concentrations which had no effect on glutamine synthetase activity (Jayakumar and Barnes, Fed. Proc. 42:1941, 1983). Inhibition of uptake by methionine sulfoximine, also a structural analog of glutamine, is therefore likely to occur at membrane sites distinct from glutamine synthetase. (iv) Although both methylammonium uptake and glutamine synthetase biosynthetic activities are reduced by growth of cells in ammonium medium, only the uptake activity is reduced in cells exposed to methylammonium (Jayakumar and Barnes, Fed. Proc. 42:1941, 1983). This demonstrates independent regulation of the two processes. Although these properties point to a transport component which clearly differs from glutamine synthetase, they do not rule out some involvement of the synthetase in methylammonium translocation. It is apparent that the GMAD metabolic trap must be eliminated experimentally for us to explore this possibility as well as provide for unequivocal measurements of methylammonium translocation. Such experiments are in progress.

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LITERATURE CITED


