THE DEMONSTRATION OF TWO DISCRETE ENZYMES CATALYZING THE SYNTHESIS OF GLUTAMINE AND γ-GLUTAMYLMETHYLAMIDE IN PSEUDOMONAS MS

M. E. Levitch

Department of Microbiology, Meharry Medical College, Nashville, Tn. 37208, U.S.A.

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SUMMARY

Cells of Pseudomonas MS grown on methylamine synthesize both glutamine and γ-glutamylmethylamide. The former activity is detected by the γ-glutamyl transferase assay. Chromatography of extracts of methylamine-grown cells on DEAE cellulose yields 4 peaks of γ-glutamyl transferase activity, but only a single peak of γ-glutamylmethylamide synthetase activity, which coincides with one of the peaks of γ-glutamyl transferase activity. Glucose-grown cells have very minimal (analogue) activity for γ-glutamylmethylamide synthesis, but exhibit the same 4 peaks of γ-glutamyl transferase activity from DEAE cellulose chromatography.

The isolation of an unusual amino acid, γ-glutamylmethylamide[1] from cells of Pseudomonas MS, was reported by Kung and Wagner (1). The organism is a methylotroph which can grow on methylamine, dimethylamine, trimethylamine, trimethylsulfonium chloride, or on more common carbon sources such as glucose. When grown on glucose the organism does not produce γ-glutamylmethylamide. Extracts of cells grown on methylamine (but not glucose), contain an enzymatic activity which catalyzes reaction 1:

(1) Glutamate + ATP + CH_3NH_2 $\xrightarrow{M^{++}}$ Glutamylmethylamide + ADP + P

Where M^{++} is a divalent cation, usually either Mg^{++} or Mn^{++}. The extracts

[1] γ-Glutamylmethylamide will be abbreviated as GMAD.

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of cells grown on methylamine or on glucose also catalyze the glutamine synthetase reaction 2:

$$(2)\quad \text{Glutamate} + \text{ATP} + NH_3 \xrightarrow{Mg^{++}} \text{Glutamine} + \text{ADP} + P$$

The two enzymatic activities were never separated, and the only evidence that two distinct enzymes are responsible for the two reactions is the data of Kung and Wagner (1) showing varying ratios of the two activities under different growth conditions. Separation of the two activities is reported in this communication.

MATERIALS AND METHODS

All chemicals were obtained from commerical sources and used without further purification. Pseudomonas MS was grown as described by Wagner et al (2), using as a carbon source either methylamine hydrochloride or glucose. Cells were washed once in buffer and suspended in the same buffer at a ratio of 0.25g per ml. The buffer used was 0.01M potassium phosphate buffer (pH 7.0) containing ATP (0.25mM), methylamine hydrochloride (1mM) $MnCl_2$ (1mM), L-Glutamate (1mM), and dithioerythritol (1mM). Cells were broken by sonication with an MSE Sonic Oscillator. Cell debris and unbroken cells were removed by centrifugation at 32,000 xg for 1 hr.

Glutamine synthetase was measured by the γ-glutamyl transfer assay of Woolfolk, Shapiro and Stadtman (3). The γ-glutamylmethylamide synthetase activity was determined by a modification of the method of Kung and Wagner (1). The reaction mixture contained, in a final volume of 1.0 ml., L-glutamate (0.01M), ATP (0.005M), $MnCl_2$ (0.005M), mercaptoethanol (0.028M), imidazole buffer (0.1M, pH 7.0), ^{14}C-methylamine hydrochloride (0.005M, 0.1 μCi/μmole), enzyme extract and water. Samples were incubated at 37°C for 1 hr. in a water bath, and the reaction was stopped by the addition of an equal volume of cold 95% alcohol. If a precipitate appeared, the sample was centrifuged. Aliquots of 0.4 ml. were plated on ridged planchets with 0.1 ml. of 1N NaOH and heated to dryness on a hot plate. Planchets were counted in a Nuclear-Chicago gas flow counter. Values for zero time controls were substracted from the incubated samples to yield net values.

DEAE cellulose (Whatman DE52) was used for column chromatography after it was equilibrated with the acidic and basic components of the indicated buffer according to the manufacturer's instruction.

RESULTS AND DISCUSSION

Previous attempts to separate the two activities by salt fractionation were unsuccessful, although some glutamine synthetase (γ-glutamyl transferase) fractions had greatly reduced γ-glutamylmethylamide synthetase activity.

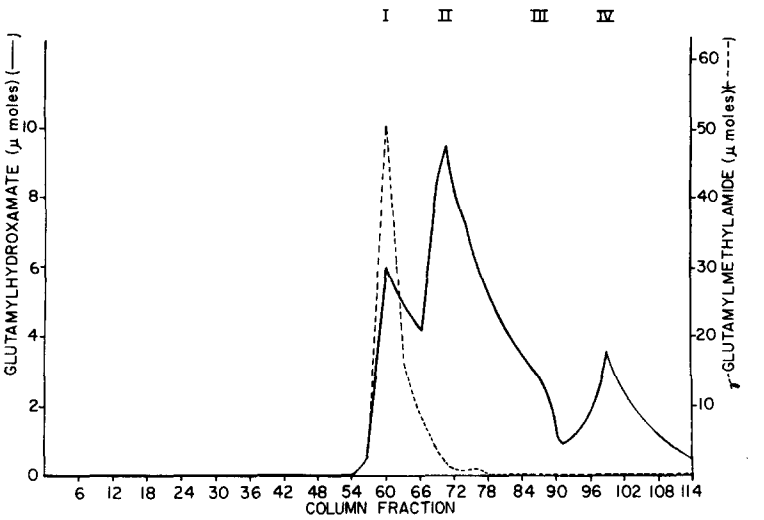

Figure 1. Chromatography of methylamine-grown cell extract. A 10 ml sample of a sonic extract of methylamine-grown Pseudomonas MS (51 mg of protein) was chromatographed at 4°C on a DEAE cellulose column (2.5 x 27 cm) equilibrated with substrate buffer, eluted with a linear gradient of 0-0.6 M KCl in substrate buffer at a rate of 1.3 ml per min. Fractions were 10 ml. Enzymes were assayed as described in Methods. Activity is plotted as μmoles of glutamylhydroxamate formed per 15 mins. per fraction, or μmoles GMAD formed per hr. per fraction.

Since the latter activity is more susceptible to inactivation, it was not clear whether this represented a separation of activities or merely an inactivation of one of the two activities of a bifunctional enzyme. No preparation of γ-glutamylmethylamide synthetase has ever been obtained free of glutamine synthetase activity, as measured by either the γ-glutamyl transfer assay of Woolfolk et al (3) or the hydroxamate assay of Elliot (4). Figure 1 shows a graph of the enzyme activities found in column fractions following DEAE cellulose chromatography of a crude sonic extract of P. MS cells grown on methylamine. Four peaks of γ-glutamyl transferase activity can be seen on the graph, at tubes 60, 71, 87, and 102. Only one peak of γ-glutamylmethylamide synthetase activity was found, coinciding with the first γ-glutamyl transferase peak. If the synthesis of GMAD were attributable to glutamine synthetase, one would expect to find proportional activity in each

of the four γ-glutamyl transferase peaks, i.e., the ratio of the two activities would be constant in all fractions. There is very little γ-glutamylmethylamide synthetase activity in the other three peaks of γ-glutamyl transferase shown in fig. 1. The ratio of the two activities in peak 1 is at least fifty times that of each of the other three peaks of fig. 1. Therefore, I conclude that the syntheses of glutamine and γ-glutamylmethylamine are catalyzed by two distinct enzymes.

Is the γ-glutamyl transferase activity in peak 1 an intrinsic property of the γ-glutamylmethylamide synthetase enzyme? If one examines reactions (1) and (2), the similarities are apparent, two of the three substrates being identical. The γ-glutamyl transferase reaction (reaction 3, below)

(3) Glutamine + $HN_2OH \xrightarrow{AsO_4^{\equiv},\ ADP,\ Mn^{++}}$ γ-Glutamylhydroxamate + NH_3

catalyzed by the glutamine synthetase in Escherichia coli is thought to proceed via either of two enzyme-bound intermediates, according to Rhee and co-workers (5). They propose two possible mechanisms, one in which γ-glutamyl arsenate (or phosphate, depending on the anion present) is the enzyme-bound intermediate; or an activated complex of γ-glutamylarsenyl-ADP as the enzyme-bound intermediate. An assumption that the two enzymes under study in P. MS may each have the same enzyme-bound intermediate is not unreasonable. It seems reasonable that a number of glutamyl derivatives could serve as substrates for the γ-glutamyl transferase reaction, catalyzed by either the glutamine synthetase or the γ-glutamylmethylamide synthetase.

Cells grown on glucose + ammonium salts have very low levels of γ-glutamylmethylamide synthetase activity (the specific activity is less than 0.5% of the methylamine-grown cells). If this low level of γ-glutamylmethylmide synthetase represents the repressed level of the enzyme in glucose-grown cells, should one expect to see all or a major portion of this activity in the same peak 1 fractions of a DEAE cellulose chromatogram of an extract from cells grown on glucose? Chromatography of an extract from

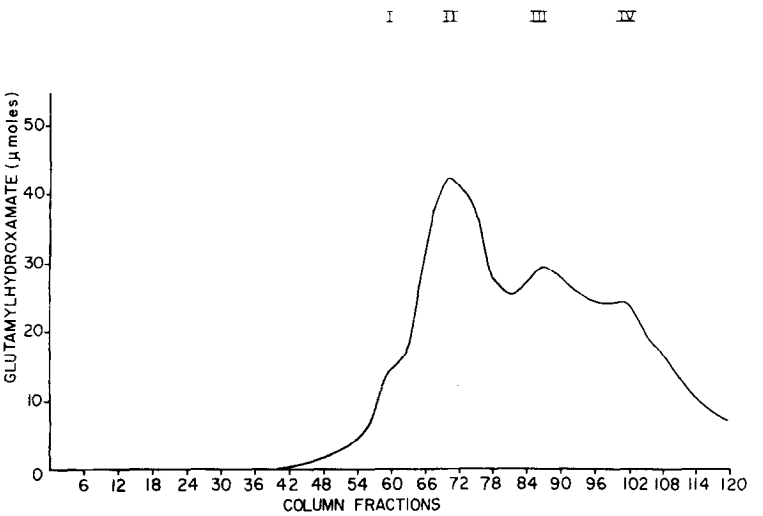

Figure 2. Chromatography of extract of glucose-grown cells. A 10 ml sample of a sonic extract of glucose-grown Pseudomonas MS (54 mg of protein) was chromatographed at 4°C on a DEAE cellulose column (2.5 x 27 cm) equilibrated with substrate buffer, eluted with a linear gradient of 0-0.6 M KCl in substrate buffer at a rate of 1.3 ml per min. Collected 10 ml fractions. The enzyme assays were described in Methods. Activity is plotted as μmoles of glutamylhydroxamate formed per 15 mins. per fraction.

glucose-grown cells on DEAE cellulose is shown in figure 2. The chromatogram shows four peaks of γ-glutamyl transferase activity, occurring in the same fractions as those of the chromatogram from methylamine-grown cells. These fractions were assayed for γ-glutamylmethylamide synthetase activity. The data (not shown) indicated low but statistically significant activity in peaks I, II, and III with activities ranging from 0.20 to 0.26 μmole per hr. per fraction. The failure to find γ-glutamylmethylamide synthetase activity in peak IV may be attributable to the fact that these activities represent the lower limits of detection of the assay. Because of the constancy of this low level of activity, it is plausible that this represents the substitution of methylamine for the natural substrate, ammonia, in the glutamine synthetase reaction. In other organisms glutamine synthetase (L-glutamate: ammonia ligase (ADP), EC 6.3.1.2) has been reported to synthesize GMAD in the presence of methylamine (3,6,7). The enzyme from

E. coli, for example, has approximately 10% of the activity with methylamine, compared to ammonia, in the biosynthetic assay (3).

The significance of the multiple peaks of γ-glutamyl transferase activity is not known. They may represent isoenzymes of glutamine synthetase, or other enzymes capable of catalyzing the γ-glutamyl transfer reaction under the conditions used in these studies. Does the appearance of γ-glutamylmethylamide activity in the peak I area of the chromatogram of methylamine-grown cells indicate some alteration of the protein found in peak I of glucose-grown cells, which exhibits primarily γ-glutamyl transferase activity, or is the method incapable of separating two enzymes found in peak I? One must also consider the possibility that the four peaks of γ-glutamyl transferase activity represents various stages of degradation of the glutamine synthetase (8).

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