Partial Purification and Characterization of Glutamate Synthase from a Thermophilic *Bacillus*

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(Received 30 October 1981)

Glutamate synthase (GOGAT), glutamine synthetase (GS), NAD⁺-dependent glutamate dehydrogenase (GDH) and NAD⁺-dependent alanine dehydrogenase (AlaDH) activities were detected in cell extracts of *Bacillus stearothermophilus* PH24, a strain deficient in NADP⁺-dependent GDH. GDH and GOGAT activities were low under most growth conditions and GOGAT was not detectable in extracts of cells grown with amino acids as carbon and nitrogen source. AlaDH and GS activities were more variable, the former being high in cells grown on L-alanine as carbon and nitrogen source. GS was repressed during growth with high concentrations of NH₄Cl as nitrogen source but a corresponding increase in AlaDH activities suggests that this enzyme may replace NADP⁺-dependent GDH as the main enzyme for ammonia assimilation under these conditions.

GOGAT was purified 40-fold using affinity chromatography on NADPH-Sepharose. The molecular weight of the partially purified enzyme was estimated to be 160 000 and K_m values for NADPH, 2-oxoglutarate and L-glutamine were 22, 15 and 29 μ M, respectively. Glutamine could be replaced by NH₄Cl as nitrogen donor (K_m 44 mM) but the rate was only 10% to 15% that of the L-glutamine-dependent reaction. The pH optimum for glutamine-dependent activity was 8.0 and the temperature optimum 75 °C: the enzyme displayed a discontinuous Arrhenius plot over the range 30 °C to 75 °C. Azaserine, L-methionine sulphone and Cibacron Blue 3GA were all inhibitors and the enzyme was rapidly inactivated in the presence of NADPH when L-glutamine and 2-oxoglutarate were absent.

INTRODUCTION

Initially, many bacteria were thought to produce glutamate using the enzyme glutamate dehydrogenase (GDH; NADP⁺-dependent; EC 1.4.1.4) and, in bacilli lacking this enzyme, by production of alanine dehydrogenase (AlaDH; EC 1.4.1.1) and subsequent transamination (Hong *et al.*, 1959). Recent work, however, showed that, during growth with low concentrations of ammonia, glutamate is produced by the combined action of glutamine synthetase (GS; EC 6.3.1.2) and glutamate synthase (GOGAT; EC 1.4.1.13) (Tempest *et al.*, 1970; Nagatani *et al.*, 1971; Tempest *et al.*, 1973). This pathway operates in several bacilli (Elmerich & Aubert, 1971; Hemmilä & Mäntsälä, 1978; Deshpande & Kane, 1980) and GOGAT has been purified from the mesophilic organisms *Bacillus megaterium* (Hemmilä & Mäntsälä, 1978) and *Bacillus subtilis* (Deshpande & Kane, 1980).

No study of GOGAT from a thermophilic source has been made, although other ammonia assimilation enzymes have been studied (Wedler & Hoffman, 1974; Epstein & Grossowicz, 1975, 1976). For this reason a study was undertaken on *Bacillus stearothermophilus* PH24 to demonstrate the presence of GOGAT and its function in glutamate formation, and subsequently, to purify and examine the properties of this enzyme.

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METHODS

Materials. Sephadex G-25, Sephacryl S-300, Sepharose 6B and 2',5'-ADP-Sepharose were from Pharmacia, 2',5'-ADP and L-methionine sulphone were from Sigma and Cibacron Blue 3GA was from CIBA-Geigy.

Bacillus stearothermophilus strain PH24 was a gift from Dr J. A. Buswell of this department.

Bacterial growth. *Bacillus stearothermophilus* PH24 (Buswell & Twomey, 1975) was grown in a medium containing (g l^{-1}): KH_2PO_4 (0.1), K_2HPO_4 (0.4), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.02), yeast extract (0.05), and 1 ml trace elements solution (Barnett & Ingram, 1955) per litre; this medium was supplemented with carbon and nitrogen sources (see text). The organisms were grown in 21 baffled Erlenmeyer flasks containing 1 l medium. The flasks were incubated at 55 °C in an orbital incubator shaker (Gallenkamp) operated at 100 rev. min^{-1} . Cells were collected using a continuous-flow centrifuge (Alfa-Laval Co., Brentford, Middlesex, U.K.) and the slurry from this was cleared by further centrifugation at 15000 g for 15 min in an MSE High Speed 18. Collected cells were washed with buffer (25 mM- $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ pH 6.0 containing 1 mM-EDTA and 5 mM-2-mercaptoethanol) and stored frozen at -20 °C until required.

Preparation of cell extracts. Bacteria were thawed and suspended in the same buffer as used for washing (3 ml g^{-1}). The suspension was cooled to 2 °C, sonicated and cell debris was removed from the extract by centrifugation at 30000 g for 1 h at 4 °C.

Enzyme assays. All assays were carried out at 55 °C using a CE505 spectrophotometer (Cecil Instruments, Cambridge, U.K.) to measure the rate of dinucleotide oxidation at 340 nm. The reaction mixtures (volume 3 ml) were as follows. *GOGAT*: 50 mM- K^+ /HEPES pH 8.0, 0.1 mM-EDTA, 0.5 mM-2-oxoglutarate, 1 mM-L-glutamine and 60 μM -NADPH. *GDH*: 50 mM- K^+ /HEPES pH 7.5, 0.1 mM-EDTA, 0.5 mM-2-oxoglutarate, 100 mM- NH_4Cl and 60 μM -NADH. *AlaDH*: 50 mM- K^+ /HEPES pH 7.5, 0.1 mM-EDTA, 0.5 mM-pyruvate, 100 mM- NH_4Cl and 60 μM -NADH. The rate of dinucleotide oxidation in the absence of glutamine or NH_4Cl was subtracted from the observed activities to allow for NADPH or NADH oxidase activity in the extract. *GS* was assayed at 55 °C using the transferase system of Wedler & Hoffman (1974).

Enzyme activities. Enzyme activities are referred to as μmol dinucleotide oxidized $\text{min}^{-1} (\text{mg protein})^{-1}$ for dinucleotide-dependent enzymes and μmol γ -glutamyl hydroxamate formed $(5 \text{ min})^{-1} (\text{mg protein})^{-1}$ for the GS forward transferase reaction.

Protein determination. Protein was determined by the Lowry method using bovine serum albumin as the standard.

Affinity adsorbent synthesis. NADPH was immobilized on Sepharose 6B via the nicotinamide ribose by the following procedure. NADP⁺ (200 μmol) and sodium periodate (200 μmol) were each dissolved in 10 ml water and incubated at 5 °C for 1 h. Hexane diamine (8 mmol) was dissolved in 20 ml water and the pH of the solution was adjusted to 9.5 with HCl and then to 5.5 with 1 M-sodium acetate buffer, pH 5.0.

Periodate-oxidized NADP⁺ was added and the mixture was stirred at 20 °C for 2 h until Schiff's base formation occurred (indicated by the production of a deep yellowish-brown colour in the solution). The Schiff's base linkage between dinucleotide and diamine was reduced by adjusting the pH of the solution to 7.0 with 2 M-NaOH and adding 10 mg NaBH_4 over a period of 5 min. During this period the pH was maintained at 7.0 using 1 M-HCl.

The solution (now pale yellow) was adjusted to pH 10.0 with NaOH, diluted to 500 ml with distilled water and applied to a $5 \times 15 \text{ cm}$ column of DEAE-cellulose (DE-32) pre-washed with $(\text{NH}_4)_2\text{CO}_3/\text{NH}_4\text{HCO}_3$ buffer (2 M, pH 9.0) and then water. Following application of the reaction mixture, the column was washed with water until the effluent was free from hexane diamine [negative to the trinitrobenzene sulphonate test (Inman & Dintzis, 1969)]. The column was then eluted with a 0 to 1 M gradient of the ammonium carbonate buffer in a total volume of 500 ml and the major UV-absorbing peak was collected. This was reduced by rotary evaporation to 20 ml and precipitated by addition of 5 vol. ice-cold ethanol before being re-dissolved in $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ buffer (0.1 M, pH 9.5) and coupled to cyanogen bromide activated Sepharose 6B (Nishikawa & Bailon, 1975).

Electrophoresis. Disc-gel electrophoresis was done by the method of Hayes & Wellner (1969). Staining for protein was done by the method of Lumsden & Coggin (1977).

RESULTS

Enzyme activities under different growth conditions

The activities of the various enzymes in crude extracts of bacteria grown in the presence of different carbon and nitrogen sources were measured (Table 1). No NADP⁺-dependent GDH was detectable in this strain of *B. stearothermophilus* and the activity of NAD⁺-dependent GDH did not vary significantly under the test conditions. Growth in the presence of L-glutamate did not cause any increase in the activity of NAD⁺-dependent GDH, suggesting that this enzyme does not serve to deaminate L-glutamate and that this amino acid is metabolized by transamination.

Table 1. *Enzyme activities in crude extracts of B. stearothermophilus PH24 grown in the presence of different carbon and nitrogen sources*

The concentration of all carbon sources was 2 g l⁻¹ except in the case of the 2-oxoglutarate/fumarate mixture where the ratio was 2:1. Activities are expressed as IU (mg protein)⁻¹. Glutamate dehydrogenase was only present in the NAD⁺-dependent form.

Carbon source	NH ₄ Cl (g l ⁻¹)	GDH	AlaDH	GS	GOGAT
Fumarate	0.8	0.05	0.43	ND	0.01
Fumarate	0.2	0.05	0.12	0.5	0.02
2-Oxoglutarate	0.8	0.06	0.93	0.6	0.02
2-Oxoglutarate	0.2	0.17	0.15	0.7	0.02
2-Oxoglutarate/fumarate	0.8	0.07	0.51	0.5	0.03
Glutamate	0	0.08	0.30	1.3	ND
Alanine	0	0.07	2.80	ND	ND

ND, Not detected.

Activities of AlaDH were more variable. Growth with L-alanine as both carbon and nitrogen source had the effect of inducing the enzyme, suggesting that it has a catabolic function as previously postulated in *B. stearothermophilus* (Epstein & Grossowicz, 1976). However, cells grown in medium containing NH₄Cl at 0.8 g l⁻¹ (high) as nitrogen source also had elevated AlaDH activity when compared to those grown with NH₄Cl at 0.2 g l⁻¹ (low). It may be that, in the absence of NADP⁺-dependent GDH, AlaDH is involved in ammonia assimilation at high levels of ammonia and that glutamate is formed by transamination from alanine.

Although GS activity was only assayed by the transferase assay, it was evident that high levels of ammonia caused a decrease in the cell content of this enzyme, especially when fumarate was used as carbon source. Higher activities were obtained from cells grown on low ammonia and fumarate indicating that this enzyme is assimilatory under these conditions.

The activity of GOGAT was not significantly affected by ammonia levels in the medium or the type of carbon source supplied. GOGAT was undetectable during growth in the presence of amino acids. The mesophile *Bacillus megaterium* behaves similarly, producing GOGAT under all conditions except where amino acids are supplied in the growth medium (Elmerich & Aubert, 1971).

Purification and properties of glutamate synthase

Growth on a mixture of 2-oxoglutarate and fumarate in the presence of high NH₄Cl produced slightly more GOGAT than other conditions (Table 1). For this reason, the organism was routinely grown on this medium to provide material for enzyme purification.

The crude cell extract from 8 g cells was fractionated using (NH₄)₂SO₄ and the fraction precipitating between 40 and 50% saturation of this salt contained all of the GOGAT activity. This precipitate was collected by centrifugation, re-suspended in the pH 6.0 buffer and dialysed against the same buffer to remove the (NH₄)₂SO₄. The dialysed material was clarified by centrifugation and the supernatant was gel filtered through tandem columns (each 2.5 × 70 cm) of Sephacryl S-300 using the same buffer to elute. Active fractions, now containing only proteins in the 120 000 to 200 000 molecular weight range, were applied to a 1.5 × 5 cm column of 2',5'-ADP-Sepharose to remove unwanted NADP⁺-binding enzymes. The effluent from this first affinity column was applied directly to a 0.5 × 5 cm column of NADPH-Sepharose to which the GOGAT was adsorbed. The NADPH-Sepharose was washed with pH 6.0 buffer until the effluent was free from protein before the GOGAT was eluted using a 0 to 1 mM gradient of NADPH in 50 ml of the same buffer supplemented with 5 mM-L-glutamine and 5 mM-2-oxoglutarate. The two substrates were included to protect the

Table 2. *Partial purification of GOGAT from B. stearothermophilus PH24*

Fraction	Total protein (mg)	Specific activity [IU (mg protein) ⁻¹]	Purification factor	Yield (%)
Crude extract	276	0.028	1	100
(NH ₄) ₂ SO ₄ fraction	69.8	0.103	3.7	94
Sephacryl S-300 gel filtrate	16.8	0.346	12.5	76
Eluate from NADPH-Sepharose	3.2	1.125	40.3	42

GOGAT from damage by NADPH (see later); they were removed along with the NADPH by gel filtration through Sephadex G25.

The final preparation, as used for further studies, was estimated to be 10% pure by gel electrophoresis, there being three dark and several light bands in protein stains of disc gels. It was, however, purified 40-fold (Table 2) and was free from NADPH oxidase, NAD⁺-dependent GDH and other enzymes likely to interfere with the GOGAT assay.

Effect of pH on glutamate synthase activity

The optimum pH for GOGAT activity with L-glutamine as nitrogen donor was 8.0, with 50% of maximum activity being retained at pH 6.4 and pH 9.0.

Co-factor and substrate specificities

The enzyme required NADPH as co-factor and this could not be replaced by NADH; the apparent K_m for the dinucleotide was 22 μM (determined by the method of Eisenthal & Cornish-Bowden, 1974). The requirement for 2-oxoglutarate was also absolute (K_m 15 μM) although both L-glutamine (K_m 29 μM) and NH₄Cl (K_m 44 mM) could act as nitrogen donor. With 100 mM-NH₄Cl replacing the L-glutamine, the rate of dinucleotide oxidation was only 10 to 15% the rate seen under normal assay conditions.

No activity in the reverse direction (i.e. glutamine-forming) could be detected.

The dinucleotide, in the absence of the other substrates, was a potent inactivator capable of causing 50% inactivation in 5 min when present at a concentration of 1 mM. This inactivation was previously reported as occurring in the *Escherichia coli* enzyme (Mäntsälä & Zalkin, 1976) and was reduced by inclusion of 2-oxoglutarate or L-glutamine in the incubation mixture; inclusion of both substrates afforded complete protection.

Enzyme inhibitors

GOGAT from *B. stearothermophilus* PH24 was strongly inhibited by azaserine in both the L-glutamine- and NH₄Cl-dependent reactions. This observation confirms that the thermophilic enzyme resembles that from other sources (Mifflin & Lea, 1976) and that the activity is not due to an NADP⁺-dependent GDH.

The thermophilic enzyme was also strongly and competitively inhibited by L-methionine sulphone (K_i 22 μM) and Cibacron Blue 3GA (K_i 12 μM). However, no significant inhibition was caused by 2',5'-ADP, an NADP⁺-analogue which competitively inhibits *E. coli* GOGAT (K_i 106 μM ; Schmidt & Jervis, 1980). The reason for this difference in the effect of this dinucleotide analogue is unclear but it explains why the thermophilic enzyme failed to bind to an adsorbent based on this ligand, which was previously used in the successful purification of *E. coli* GOGAT (Schmidt & Jervis, 1980).

Effect of heat

The temperature optimum was 75 °C with 35% of the activity being lost during 10 min incubation at 80 °C. Over the temperature range 30 °C to 75 °C an Arrhenius plot shows a marked deviation from linearity, occurring at 58 °C and giving two different activation

Fig. 1. Arrhenius plot for GOGAT from *Bacillus stearothermophilus* in the range 30 °C to 75 °C.

energies (Fig. 1). Below 58 °C the activation energy is 82.8 kJ mol⁻¹ while above this temperature it falls to 27.8 kJ mol⁻¹. The form of the plot and the temperature at which the discontinuity occurs is similar to that seen in GS from *B. stearothermophilus* (Wedler & Hoffman, 1974).

Molecular weight

Using gel filtration (Andrews, 1965), the molecular weight was estimated at 160 000, a much lower value than that obtained for the enzymes from *E. coli* (800 000; Miller & Stadtman, 1972) or *Bacillus megaterium* (800 000; Hemmilä & Mäntsälä, 1978). However, it is similar to the estimated molecular weight of GOGAT from *Bacillus subtilis* (173 000; Deshpande & Kane, 1980).

DISCUSSION

From the results obtained, GOGAT appears to be important in ammonia assimilation in *B. stearothermophilus* PH24 just as it is in other bacteria under ammonia-limited conditions (Tempest *et al.*, 1973). Its presence at such low levels, however (Table 1), prompted a search for some alternative amidotransferase capable of utilizing glutamine for amino acid formation, but none was found.

The fact that GS is repressed at high ammonia levels suggests that some alternative means of assimilation exists under these conditions. No anabolic GDH is present as in *K. aerogenes* (Tempest *et al.*, 1970) but AlaDH activity is higher under these conditions and it is possible that this enzyme functions to assimilate ammonia despite previous reports that it is purely catabolic in this organism (Epstein & Grossowicz, 1976).

The GOGAT from strain PH24 appears to be basically similar to that from mesophilic sources already studied. The major difference is that the temperature optimum is much higher and similar to that of other *B. stearothermophilus* enzymes (Amelunxen & Murdock, 1978) and that the molecular weight is very low. Possibly the GOGAT enzymes from bacilli are of low molecular weight, since that from *B. subtilis* is only 173 000 (Deshpande & Kane, 1980).

and that from *B. megaterium* was found as a 200000 dalton dimer (Hemmilä & Mäntsälä, 1978).

Other properties, such as the inactivation by NADPH, suggest a similar catalytic centre to that in *E. coli* GOGAT, containing flavin, cysteine and non-haem iron: the inactivation caused by dinucleotide is dependent on the presence of these (Mäntsälä & Zalkin, 1976).

In conclusion it should be noted that the GOGAT from strain PH24 was difficult to purify, a similar difficulty being reported by Wedler & Hoffman (1974) with the GS from *B. stearothermophilus*. The affinity purification system developed for *E. coli* GOGAT and based on 2',5'-ADP-Sepharose (Schmidt & Jervis, 1980) was ineffective, and a new system was required. It is hoped that further refinements will result in higher purification factors being achieved.

L. Jervis wishes to thank the Royal Society for a Scientific Investigation Grant. C. N. G. Schmidt was in receipt of a Science Research Council Research Studentship.

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