Nitrogen Assimilation by *Bacillus licheniformis* Organisms Growing in Chemostat Cultures

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SUMMARY

Medium composition influenced the synthesis and breakdown of glutamic acid and alanine by *Bacillus licheniformis* examined in a chemostat. With ammonia in growth-limiting quantities as the sole nitrogen source, glutamate was synthesized from 2-oxoglutarate via glutamine synthetase and glutamine amide 2-oxoglutarate amino transferase (NADP; oxidoreductase), and alanine was formed from glutamate and pyruvate by transamination. With excess nitrogen, as either alanine or glutamate, a carbon-limited culture synthesized respectively NAD-linked alanine dehydrogenase or NADP-linked glutamate dehydrogenase, and the latter two enzymes performed only a catabolic role. If, however, nitrogen was supplied as alanine or glutamate to a nitrogen-limited culture, then synthesis of alanine dehydrogenase and glutamate dehydrogenase was repressed. Correlations were drawn between the nature of the growth environment, the composition of the amino acid pools and the synthesis of the above mentioned enzymes in *B. licheniformis*.

INTRODUCTION

In bacteria, the biosynthesis of 2-amino acids from ammonia is generally supposed to occur through the reductive amination of the corresponding 2-keto acids, these reactions being catalysed by the amino acid dehydrogenases, principally glutamate dehydrogenase (GD) (EC 1.4.1.4) but also alanine dehydrogenase (AD) (EC 1.4.1.1) (see, for example, Goldman, 1959; Meister, 1965; Germano & Anderson, 1968). Hong, Shen & Braunstein (1959) found that glutamate dehydrogenase-deficient mutants of some Gram-positive bacteria could readily grow and assimilate ammonia providing they contained alanine dehydrogenase; therefore they concluded that organisms of the genus *Bacillus* could synthesize alanine by direct amination of pyruvate, and thence glutamate, by transamination. However, the importance of GD in ammonia assimilation has been stressed by, amongst others, Piérard & Wiame (1960). Freese & Oosterwyk (1963) concluded that, in addition to its main role in ammonia assimilation, a physiological function of AD was to catalyse the catabolism of L-alanine, since AD⁺ but not AD⁻ strains of *Bacillus subtilis* could utilize L-alanine as a source of carbon and nitrogen.

Recently an alternative pathway for ammonia assimilation has been discovered in which AD and GD do not participate (Tempest, Meers & Brown, 1970). *Aerobacter aerogenes* organisms produced glutamic acid by the synthesis of glutamine from glutamic acid and ammonia, followed by the reductive transfer of the glutamine amide nitrogen to 2-oxo-

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glutaric acid. These two reactions were catalysed by the enzymes glutamine synthetase (EC 6.3.1.2) and glutamine amide 2-oxoglutarate amino transferase (GOGAT).

Previous investigations into the control of synthesis of AD and GD used strains of bacteria which probably also synthesized GOGAT, and the presence of this enzyme would affect the validity of the conclusions drawn by previous authors. The original work on GOGAT (Tempest *et al.* 1970) was done with *Aerobacter aerogenes*, a species which lacks AD (Wiame, Piérard & Ramos, 1962; Tempest *et al.* 1970). Therefore it seemed appropriate to reinvestigate the physiological relationship between AD, GD and GOGAT in a species that may (and, indeed, does) contain all three enzymes. The present communication reports the results of such an investigation using a strain of *Bacillus licheniformis*.

METHODS

Organism. *Bacillus licheniformis* (NCIB8016) was maintained on nutrient agar slopes by monthly subculture at 30°.

Cultural conditions. The organisms were grown in 3 l chemostats based on the Biotec FL 103 laboratory fermenter (Biotec AB, Postbox 16152, Stockholm 16, Sweden). Media were designed to give about 2 mg dry bacteria/ml (Evans, Herbert & Tempest, 1970). The culture was maintained automatically at pH 7.0 by the addition of 4 M-NaOH. The temperature was maintained at 34° throughout, and the air flow rate adjusted to give an oxygen solution rate greater than 250 mm-O₂/l/h – more than that required to maintain fully aerobic growth (Pirt, 1957). The rate of flow of medium into the growth vessel was regulated at the required value using a Watson–Marlow MHRE Mk. 3 pump (Watson–Marlow Ltd, Falmouth, Cornwall).

Extraction of enzymes. Culture effluent was collected directly from the fermenter, and the organisms were harvested and washed (once in chilled 1 % (w/v) NaCl) by centrifuging at 4°. Extracts were then prepared by disrupting suspensions (about 50 mg dry wt of organisms/ml, in 50 mM-tris-HCl buffer, pH 7.6 plus 10 mM-mercaptoethanol) in a sonicator (Biosonik III, Bronwell Scientific, Rochester, New York 14603, U.S.A.) at 60 % of maximum power for 10 min at 4°. The bacterial paste was centrifuged (30 min at 10,000g, 4°) to sediment the unbroken organisms and debris, and the resulting supernatant fluid was dialysed overnight at 4° against the tris buffer containing mercaptoethanol as specified above. A clear fluid was obtained which contained about 20 mg protein/ml and the required enzymes.

Assays of enzyme activity. GD and GOGAT were assayed, and pH activity curves determined by the spectrophotometric methods described previously (Meers, Tempest & Brown, 1971). AD activities were assayed using the method of Yoshida & Freese (1965). *K_m* values were determined by conventional methods (Dixon & Webb, 1964).

Analytical procedures. The free amino acid pool content and composition was assessed by the method described previously (Tempest, Meers & Brown, 1971), except that a JEOLCO amino acid analyser (model 5/AH; 84 Vesterbrogade, 1620 Copenhagen V, Denmark) was used in conjunction with an electronic integrator (JEOLCO, model DK). Protein was estimated by the Biuret method of Stickland (1951).

RESULTS

Bacillus licheniformis (NCIB8016) was selected as a representative Gram-positive bacillus able to grow well on a defined simple salts medium and produce AD, GD and GOGAT.

Table 1. *Michaelis constants for some of the substrates for alanine dehydrogenase (AD), glutamate dehydrogenase (GD) and glutamine amide, 2-oxoglutarate amino transferase (GOGAT) measured at the appropriate optimum pH values for maximum activity*

Enzyme	Optimum pH value	Michaelis constants (mM) for				
		Ammonia	Glutamine	Pyruvate	2-oxo- glutarate	Alanine
AD (aminating)	7.6	300	—	0.33	—	—
GD (aminating)	8.1	12	—	—	1.7	—
GOGAT	8.0	—	3.0	—	0.5	—
AD (deaminating)	9.5	—	—	—	—	1.2

Properties of alanine dehydrogenase, glutamate dehydrogenase and glutamine amide 2-oxoglutarate amino transferase measured with the primary substrates

The effect of pH on the activity of AD, GD and GOGAT was examined with extracts of glucose-limited *Bacillus licheniformis* organisms ($D = 0.2 \text{ h}^{-1}$). Each enzyme had a marked optimum (in the aminating reaction) near to pH 7.6 (Table 1), and this value was selected for use in the routine enzyme assays. The optimum pH for the activity of the AD from *Bacillus subtilis* is lower for the amination reaction than for the deamination reaction (Yoshida & Freese, 1965), and the same was true for the enzyme from *Bacillus licheniformis* (Table 1).

The rates of the three enzymatic reactions were examined as functions of the concentration of the appropriate amino donor and 2-keto acid substrates, and typical Michaelis-Menten relationships were observed (Table 1).

The aminating activity of AD was 50 % inhibited by 10^{-5} M -alanine and the deaminating activity was 50 % inhibited by 10^{-6} M -pyruvate, but no inhibition was detectable with less than 10^{-4} M -ammonia. Similar results were obtained by Goldman (1959), who proposed reaction mechanisms for AD which explain the differential inhibition by ammonia and pyruvate. GD and GOGAT were inhibited in their aminating activity by glutamate to an extent similar to that described previously for the enzymes from other species of bacteria (Meers *et al.* 1971).

Potassium or magnesium ions inhibited the activity of all three enzymes. The extent of inhibition (by given concentrations of the metal ions) was quantitatively similar to previously reported data for AD from *Bacillus subtilis* (Yoshida & Freese, 1965) and for GOGAT and GD from various bacterial species (Meers *et al.* 1971). The inactivation by metal ions was reversed by cysteine or mercaptoethanol, and the latter compound (at 10 mM concentration) was incorporated into all of the buffers used during the extraction, dialysis, and determination of the enzymes, as described in Methods.

AD was specific for NAD^+ , whereas GD and GOGAT were specific for NADP^+ ; no reaction was observed with the alternative coenzymes.

The 'steady state' levels of AD, GD and GOGAT in chemostat cultures of Bacillus licheniformis

When growing in the magnesium-limited medium (containing glucose and ammonia) *Bacillus licheniformis* synthesized AD, GD and GOGAT (Table 2). However, of the three enzymes, only GD had high specific activity when compared with the activities of each enzyme under most other growth conditions. This observation is consistent with the suggestion (Meers *et al.* 1971) that, under conditions of ammonia excess, bacteria use the enzyme

Table 2. *The activities of alanine dehydrogenase (AD), glutamate dehydrogenase (GD) and glutamine amide, 2-oxoglutarate amino transferase (GOGAT) in Bacillus licheniformis*

The bacteria were grown in chemostats under carbon-, nitrogen- or magnesium-limited conditions with various substrates as carbon and nitrogen sources. Enzyme activities are expressed as nmoles of NADH (for AD) or NADPH (for GD or GOGAT) oxidized/mg protein/min, corrected for small endogenous NADH or NADPH oxidase activities.

Growth-limiting factor	Energy source	Nitrogen source	Dilution rate (h ⁻¹)	Specific activities of		
				AD	GD	GOGAT
Magnesium	Glucose	Ammonia	0.04	21	14	6
			0.31	14	42	11
Carbon	Glucose	Ammonia	0.05	75	9	11
			0.19	134	33	17
Carbon	Alanine	Alanine	0.05	4500	7	28
			0.10	4700	19	26
			0.20	4700	34	22
Nitrogen	Glucose	Ammonia	0.05	38	< 5	11
			0.10	53	< 5	20
			0.22	37	12	41
Nitrogen	Glucose/ alanine	Alanine	0.05	118	< 5	21
			0.19	152	< 5	24

GD to aminate 2-oxoglutarate directly to glutamate. However, GOGAT activity was low but none the less appreciable in these magnesium-limited organisms (Table 2), and it is possible that in this species the cyclic mechanism for nitrogen assimilation (Tempest *et al.* 1970) was responsible for some glutamate synthesis even under conditions of nitrogen excess. The comparatively high Michaelis constant of AD for ammonia (Table 1) makes it unlikely that ammonia was incorporated into bacteria by means of the reaction catalysed by this enzyme.

The glucose-limited organisms also contained high GD activity when ammonia was the sole source of nitrogen, and the significant difference between these and the magnesium-limited bacteria was in the AD levels. When the organisms were grown on the same nitrogen source and at the same growth rate, in every case the levels of AD in carbon-limited bacteria were higher than in magnesium- or nitrogen-limited bacteria (Table 2).

Ammonia-limited organisms produced high levels of GOGAT. This is consistent with the suggestion of Meers *et al.* (1971) that GOGAT is necessary for ammonia assimilation under conditions where the extracellular ammonia concentration is extremely low.

When glucose was replaced by alanine as the energy source for a carbon-limited culture, much ammonia was excreted by the bacteria and the AD level increased markedly (Table 2). Thus it seemed that alanine was catabolized directly to pyruvate and ammonia. However, the availability of alanine as a substrate was not in itself the only factor prescribing high AD levels; nitrogen-limited organisms, metabolizing alanine as the growth-limiting nitrogen source, had a comparatively low specific activity of AD (Table 2). The pool alanine concentrations in the carbon (alanine)- and nitrogen (alanine)-limited cultures were similar (i.e. both between 3 and 4 mM at a dilution rate of 0.2 h⁻¹).

Fig. 1. Changes in the pool concentrations of ammonia (■), glutamate (○), glutamine (●), aspartate (△) and alanine (▲) following the addition of $(\text{NH}_4)_2\text{SO}_4$ (approximately 30 mM final concentration) to a steady-state nitrogen (ammonia)-limited culture of *Bacillus licheniformis* growing in a chemostat at a dilution rate of 0.2 h^{-1} (34° , pH 7.0, carbon source glucose). An arrow marks the time when the sterile $(\text{NH}_4)_2\text{SO}_4$ solution was added to the culture.

Changes in 'pool' amino acid contents and enzyme activities of Bacillus licheniformis following the addition of specific substrates to growing cultures

To investigate further the routes involved in amination and deamination in *Bacillus licheniformis*, solutions of several key substrates were pulsed into the chemostat cultures and subsequent changes in amino acid pool content and distribution were observed. These results were then correlated with concomitant changes in the specific activities of AD, GD and GOGAT.

Under ammonia-limited growth conditions the organisms contained measurable quantities of AD, GD and GOGAT when grown at a dilution rate of 0.2 h^{-1} (Table 2). Excess ammonia pulsed into such a culture effected a rapid, 200-fold increase in the concentration of glutamine. This result is consistent with the previously reported observation that nitrogen-limited bacteria contain high concentrations of glutamine synthetase (Meers & Tempest, 1970), and that the initial step in amination under these growth conditions is the formation of glutamine. However, in *Bacillus licheniformis* there then followed a gradual accumulation of alanine (Fig. 1) but not glutamate. This alanine could have been formed by transamination from glutamate, or by reductive amination from ammonia, or conceivably by the reductive transfer of the amide group of glutamine to pyruvate by a reaction analogous to that catalysed by GOGAT. The latter possibility was examined directly and found not to occur.

When alanine was pulsed into a glucose-limited culture in which ammonia was the nitrogen source, then deamination of an amino acid was necessary before the alanine could be metabolized as an energy source. The removal of ammonia from alanine could occur either by direct deamination of alanine or by the deamination of glutamate following a

Fig. 2 (a) Changes in the specific activity of AD (▲), GD (■) and GOGAT (●) following the addition of alanine (approximately 30 mM final concentration) to a steady-state carbon (glucose)-limited culture of *Bacillus licheniformis* growing in a chemostat at a dilution rate of 0.2 h^{-1} (34° , pH 7.0, nitrogen source NH_3). The units of GD and GOGAT activity are expressed as nmoles/min/mg protein, and the units of AD activity as moles $^{-5}$ /min/mg protein. An arrow marks the time of addition of alanine.

(b) Changes in the pool concentration of ammonia (■), glutamate (○), glutamine (●), aspartate (△) and alanine (▲) following the addition of alanine to a *Bacillus licheniformis* culture. The details of this experiment were as described in Fig. 2(a).

transamination reaction between 2-oxoglutarate and alanine, as suggested by Shen, Hong & Braunstein (1959). In fact, the alanine pulse stimulated a rapid increase in the concentration of AD, but caused no significant change in the concentration of GD (Fig. 2a). This indicated that alanine was deaminated directly to pyruvate and ammonia. This conclusion was reinforced by the finding that the extracellular and intracellular concentrations of alanine decreased to a steady-state level in 3 h at the same time as the pool, and extracellular, ammonia concentrations increased. During this 3 h period there was no increase in the pool concentration of glutamate; indeed, during the first hour the pool glutamate level decreased (Fig. 2b).

When glutamate (30 mM final concentration) was pulsed into a glucose-limited culture instead of alanine, there was some increase in the level of GD, but little change in the specific activities of AD or GOGAT (Fig. 3a). The period when the greatest specific activities of GD were evident coincided with the time of maximum ammonia production (Fig. 3b). This result was thus analogous to that obtained when alanine was added to a carbon-limited culture, but the changes were quantitatively not as marked.

The changes which occurred when the bacteria were taken from conditions of nitrogen excess to nitrogen limitation are shown in Fig. 4. Glucose (20 mM final concentration) was pulsed into a glucose-limited culture containing alanine as its sole nitrogen source. At the same time as the pulse was added, the medium was changed so as to bring about ammonia-limited growth conditions after a short period of unrestricted growth. This experiment was thus essentially the reverse of the alanine pulse experiment illustrated by Fig. 1, and the results of the two experiments were complementary. On becoming nitrogen-limited the level

Fig. 3. Changes in (a) specific enzyme activity and (b) the amino acid pool of *Bacillus licheniformis* organisms following the addition of sodium glutamate (approximately 20 mM final concentration) to a carbon (glucose)-limited chemostat culture (dilution rate 0.2 h^{-1} , 34° , pH 7.0, nitrogen source NH_3). The symbols and units of specific enzyme activity are as described for Fig. 2.

Fig. 4. Changes in the specific activity of AD ($\blacktriangle$), GD ($\blacksquare$) and GOGAT ($\bullet$) following the change of a *Bacillus licheniformis* culture from carbon to nitrogen-limited growth conditions. At the time marked by an arrow excess sterile glucose solution was added to the culture, and at the same time the medium entering the chemostat was changed from that designed to produce carbon-limited growth with alanine as the carbon source to that producing ammonia-limited growth in the chemostat with glucose as the carbon source. The dilution rate was maintained at 0.2 h^{-1} throughout the experiment.

of AD in the organisms fell sharply, whereas the level of GOGAT reached a transient peak level that was substantially higher than was observed at any other time during the course of this work. The level of GD again did not change significantly.

DISCUSSION

The properties of AD, GD, and GOGAT, extracted from *Bacillus licheniformis* were similar to those from other species of *Bacillus* (see, for example, Yoshida & Freese, 1965; Meers *et al.* 1971). From these data alone it is not possible to be sure which reactions they do in fact catalyse *in vivo*. As a result of growing *B. licheniformis* in a chemostat under steady-state conditions, and then noting the changes which occurred following a controlled disturbance of the dynamic equilibrium of the cultures, roles can be suggested for these three functionally related enzymes.

It has previously been suggested that GOGAT functions, in ammonia-limited *Aerobacter aerogenes*, as an integral part of a cyclic mechanism which leads to the assimilation of low concentrations of ammonia (Tempest *et al.* 1970). Recent work by Elmerlich & Aubert (1971) leads to the suggestion that GD⁻ strains of *Bacillus megaterium* synthesized glutamate by the same mechanism. The present results are consistent with these conclusions. Thus, low concentrations of ammonia derepressed GOGAT synthesis (Table 2; Fig. 4), but an increase in the concentration of glutamate in the amino acid pool of *Bacillus licheniformis* did not significantly affect GOGAT synthesis (Fig. 3), showing that the synthesis of this enzyme was not subject to end-product repression. However, the pool glutamate level was invariably high (see Fig. 2-4), and this could explain why quantitatively large, but proportionately small, changes in glutamate concentration did not have as marked an effect on enzyme synthesis as did quantitatively similar, but proportionately larger increases in the initially low pool alanine concentration (compare Fig. 2 and 3). Thus, one important factor influencing both the levels of GOGAT activity in *B. licheniformis* organisms and the specific activity of glutamine synthetase measured in *A. aerogenes* organisms (Meers & Tempest, 1970) was the availability of ammonia. Since both enzymes are co-operatively concerned in glutamate synthesis, under ammonia-limited growth conditions, this result is predictable. However, the activity of glutamine synthetase is also subject to complicated control by enzyme-catalysed chemical modification (Holzer *et al.* 1970), and Deuel *et al.* (1970) have shown that glutamine synthetase synthesis in *Bacillus subtilis* is derepressed by ammonia-limitation. Therefore the activity of this enzyme seems primarily to control the flux of metabolites at this important linkage point between carbon and nitrogen metabolism in bacteria.

The control and function of GD synthesis in *Bacillus licheniformis* cannot be as readily explained. Tempest *et al.* (1970) and Meers *et al.* (1971) concluded that GD functioned in ammonia incorporation under growth conditions of ammonia excess when the high K_m of this enzyme for ammonia did not restrict its activity. The present results (Tables 1, 2) are consistent with those reported previously with other species of bacteria (Meers *et al.* 1971), but in addition it was observed that when glutamate was added to a carbon-limited chemostat culture then the specific activity of GD increased and direct deamination of glutamate took place (Fig. 3). Thus, GD functions (at least in *B. licheniformis*) in either an aminating or in a deaminating direction, and thereby operates as a 'valve', preventing an excessive increase in the pool glutamate level. This latter function is in addition to that previously suggested (Tempest *et al.* 1970).

No evidence was obtained to support the suggestion (Fairhurst, King & Sewell, 1956;

Hong *et al.* 1959; Shen *et al.* 1959; Goldman, 1959; Freese & Oosterwyk, 1963; Meister, 1965; Germano & Anderson, 1968) that AD plays a significant role in incorporating free ammonia into cellular protein. Preparations of AD extracted from various bacteria invariably have a high K_m value for ammonia (Wiame *et al.* 1962; Yoshida & Freese, 1965), and the value found for *Bacillus licheniformis* was 300 mM (Table 1). However, the organisms could grow and assimilate ammonia when the intracellular ammonia concentration was less than 1 mM (Fig. 1 and Tempest *et al.* 1971), at which concentration no AD activity could be measured *in vitro* (J. L. Meers & L. K. Pederson, unpublished observation). Under ammonia-limited growth conditions the enzymes involved in ammonia assimilation would probably be derepressed; under these conditions the specific activity of AD was small (Table 2). AD was, however, synthesized to a greater extent when the organisms were carbon-limited, and the highest specific activity for this enzyme was measured in organisms provided with alanine as the sole energy source for a carbon-limited culture. However, the availability of alanine as a substrate was not the sole factor prescribing high AD levels, because nitrogen-limited organisms metabolizing alanine as the growth-limiting nitrogen source in the presence of excess glucose had a comparatively low specific activity of AD (Table 2). These results infer that AD synthesis was repressed by the presence of catabolites and induced (or derepressed) by alanine. The catabolic activity of AD was furthermore subject to severe end-product inhibition by pyruvate, but not by ammonia. The results reported here strongly support the conclusion that the essential physiological function of AD is to catabolize L-alanine to produce pyruvate which can then readily be used as a carbon and energy source.

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