THE DUPLICATION OF ARGININE CATABOLISM AND THE MEANING OF THE TWO ORNITHINE CARBAMOYLTRANSFERASES IN BACILLUS LICHENIFORMIS

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SUMMARY

Arginine deiminase and carbamate kinase activities are shown to be present in cell-free extracts of Bacillus licheniformis. This gives the rationale for the occurrence of an arginine-inducible ornithine carbamoyltransferase in this organism and suggests that, in vivo and under the proper conditions, this enzyme is able to catalyze the phosphorolysis of citrulline. This also shows that this Bacillus species has two arginine catabolic pathways. This duplication appears to be under the control of O_2.

INTRODUCTION

Bacillus licheniformis is known (1) to possess two ornithine carbamoyltransferases (EC 2.1.3.3). One of these enzymes is assumed to participate in the synthesis of arginine from glutamate, by catalyzing the formation of citrulline and phosphate from ornithine and carbamoylphosphate. It is regulated like a typical anabolic enzyme in that its synthesis is repressed by arginine. The second enzyme is more of a problem. It is inducible by arginine and repressed by glucose, and would be expected to have a catabolic function, as in the clear-cut case of Pseudomonas (2, 3) and in Streptococcus (4, 17) and Mycoplasma (5). In these organisms the arginine catabolic pathway involves the conversion of arginine to citrulline and ammonia, the formation of ornithine and carbamoyl-phosphate from citrulline and phosphate, and the formation of ATP,

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CO_2 and NH_3 from carbamoylphosphate and ADP. These steps are catalyzed by an arginine deiminase (EC. 3.5.3.6), the catabolic ornithine carbamoyltransferase and a carbamate kinase (EC 2.7.2.2) respectively.

It has been shown that in Bacillus licheniformis, among other Bacillus species (6, 7) whole cells excrete ammonia or citrulline and ornithine. However, investigators (1) failed to detect arginine deiminase in extracts containing the inducible transferase, and to show the capacity of the latter enzyme to function in the direction of citrulline phosphorolysis. Furthermore, B. licheniformis, among other Bacilli, is able to degrade arginine via an arginase (EC 3.5.3.1) and this pathway, because it leads to the formation of glutamate, allows the organism to grow on arginine as sole nitrogen source (8, 9). For these reasons, some doubt remained as to the function of the inducible ornithine carbamoyltransferase. It should be mentioned that recent work in this laboratory has shown the capacity of the B. licheniformis inducible transferase to function in the direction of catabolism (Stalon and Legrain, in prep.), and that a carbamate kinase has been found in Bacillus subtilis (10).

The present study demonstrates the presence of arginine deiminase and carbamate kinase in cell-free extracts of B. licheniformis, thus establishing the catabolic function of the inducible ornithine carbamoyltransferase and the existence of two differently regulated pathways of arginine catabolism.

MATERIALS AND METHODS

Strain used : Bacillus licheniformis ATCC nr. 14580.

Growth conditions : The cells were grown at 37°C in half-filled culture flasks when good oxygenation of the cultures was desired. Poor oxygenation of the cultures was obtained by filling the flasks almost to the top. All cultures were agitated.
All cultures contained the basic salts medium nr. 154 (2). To this medium various sources of carbon and nitrogen were added. These are specified in each case in the section "Results".

Harvest and extraction :
Cultures were harvested around 5.10^8 cells/ml except for the poorly oxygenated cultures containing glucose, which were harvested around 3.10^8 cells/ml. The cells were centrifuged and the pellets resuspended in 50 mM phosphate buffer at pH 7.5. The cell suspensions

were sonicated for 10 minutes with a Mullard Sonic Oscillator, and the cell debris were eliminated by centrifugation.

Enzyme assays : Arginase :

Arginase activity determinations were performed by measuring the urea formed in a reaction mixture of 2.0 ml containing Na-glycine buffer adjusted to pH 9.0, arginine and $MnCl_2$ at the following concentrations : glycine : 100 mM, arginine 50 mM, $MnCl_2$: 5 mM. The arginine solution was used to start the reaction which was stopped after 10 minutes at 37°C by addition of 2.0 ml 1 M HCl. Urea was measured by the method of Archibald (11).

Ornithine carbamoyltransferase :

Ornithine carbamoyltransferase determinations were performed by measuring the citrulline formed in a reaction mixture of 2.0 ml containing ethylenediaminetetraacetate-NaOH buffer adjusted to pH 8.5, ornithine and carbamoylphosphate at the following concentrations : ethylenediaminetetraacetate:60 mM, ornithine : 5 mM, carbamoylphosphate : 20 mM. The reaction was started by the addition of the carbamoylphosphate solution. It was stopped after 10 minutes at 37°C by addition of 2.0 ml 1 M HCl. Citrulline was assayed by the method of Archibald (11).

Arginine deiminase :

Arginine deiminase determinations were performed by measuring the citrulline formed in a reaction mixture containing citrate buffer adjusted to pH 5.4, arginine and $MnCl_2$ at the following concentrations : arginine :50 mM, citrate : 50 mM, $MnCl_2$: 20 mM. $MnCl_2$ enhanced the activity of the enzyme and increases the reliability of the assay. Urease was added (0.1 mg/ml) to prevent the interference of urea with the colorimetric assay. The reaction was started with the addition of the arginine solution. The reaction was stopped after 15 minutes at 37°C by 2.0 ml of 6 % trichloracetic acid. Precipitated protein was eliminated by centrifugation. Citrulline was measured by the method of Archibald (11).

Carbamate kinase :

Carbamate kinase activities were performed by measurement of the quantity of $^{14}CO_2$ formed in 2.0 ml of a reaction mixture containing Tris-HCl buffer at pH 7.0, ADP, $MgCl_2$ and $\left[^{12}C\right]$ + $\left[^{14}C\right]$ carbamoylphosphate at the following concentrations : Tris : 50 mM, ADP : 10 mM, $MgCl_2$: 40 mM, total carbamoylphosphate : 5 mM. The method has been described elsewhere (13), and was modified for the use of carbamoylphosphate as radioactive substrate by addition of 0.02 mmoles of ornithine and 400 units of E. coli ornithine carbamoyltransferase two minutes before the reaction was stopped, in order to destroy the carbamoylphosphate which had not reacted. The radioactivity measurements were carried out in a Beckman 100 C scintillator.

Protein content was measured by the method of Folin (12).

RESULTS

The levels of arginase, ornithine carbamoyltransferase, arginine deiminase and carbamate kinase activities obtained on various media under conditions of oxygen abundance and oxygen limitation are summarized in table 1.

Obviously , a distinction should be made between what occurs in well- and poorly oxygenated cultures. Whereas glucose and NH_4^+ both act as strong repressors of the synthesis of the inducible ornithine carbamoyltransferase when oxygen is abundant, high levels of this enzyme are obtained in poorly oxygenated cultures on the same media. It is interesting to note that arginase levels are relatively low in poorly oxygenated cultures, and that in cells grown on glucose plus arginine, the deiminase pathway seems to function to the partial exclusion of the arginase pathway. Arginine deiminase and carbamate kinase were detected in all poorly oxygenated cultures containing arginine, that is whenever the level of inducible transferase was high. This, we believe, establishes the catabolic rôle of the inducible transferase, and the existence of a second pathway for the arginine catabolism. The deiminase appears to be a relatively unstable enzyme and cannot be stored. It is of some interest that the ions Mn^{2+} and Mg^{2+} enhance the activity of the enzyme and are necessary to prevent the decrease in the specific activity as the dilution of the enzyme is increased. Fig. 1 gives an idea of the effect of these ions on enzyme activity. Whether or not this observation has any structural significance must await further study.

DISCUSSION

With the detection of arginine deiminase and carbamate kinase activities in cell-free extracts of Bacillus licheniformis, we have established the catabolic function of the arginine-inducible ornithine carbamoyltransferase present in this organism. The long-term purpose of such a study is to improve our understanding of the evolutionary processes which determine the selection of a given pathway, and the relationships between the various organisms which have acquired a same enzymatic system.

In this respect, the case of Bacillus licheniformis is interesting. Here we have an organism with (at least) two possible

Table 1 : Specific activity of arginase, ornithine carbamoyltransferase, arginine deiminase and carbamate kinase under various conditions [1] (expressed in µmoles/hour/mg protein formed)

Culture conditions	OTCase [2] activity	Arginase activity	Arginine deiminase activity	Carbamate kinase activity
Glucose 20 mM + arginine 40 mM, O_2 abundant	0.7	180	< 0.1	1.5
Idem, O_2 scarce	2560	32	4.5	29
Glutamate 20 mM + arginine 20 mM, O_2 abundant	175	477	< 0.1	2
Idem, O_2 scarce	4000	240	8	64
Arginine 40 mM, O_2 abundant	36	498	< 0.1	n.m. [3]
Idem, O_2 scarce	323	340	0.2	n.m.
Glutamate 20 mM + arginine 20 mM + NH_4^+ 50 mM, O_2 abundant	20	680	< 0.1	n.m.
Idem, O_2 scarce	940	184	4	n.m.
Glutamate 40 mM, O_2 abundant	16 (a)	2.50	< 0.1	n.m.
Idem, O_2 scarce	17	13	< 0.1	0.5
Glucose 20 mM + $(NH_4)_2SO_4$ 50 mM, O_2 abundant	14 (a)	3	< 0.1	1

(1) Under conditions of oxygen limitation our measurements presented strong fluctuations in cultures grown on the same media. This is probably due to the method used for oxygen limitation, which does not allow a rigorous control of oxygenation. Qualitatively, however, the results were reproducible.
(2) OTCase : ornithine carbamoyltransferase.
(3) n.m. : not measured.
(a) stands for anabolic transferase. In the other cases, mainly the catabolic transferase is present but a mixture of the two cannot be excluded.

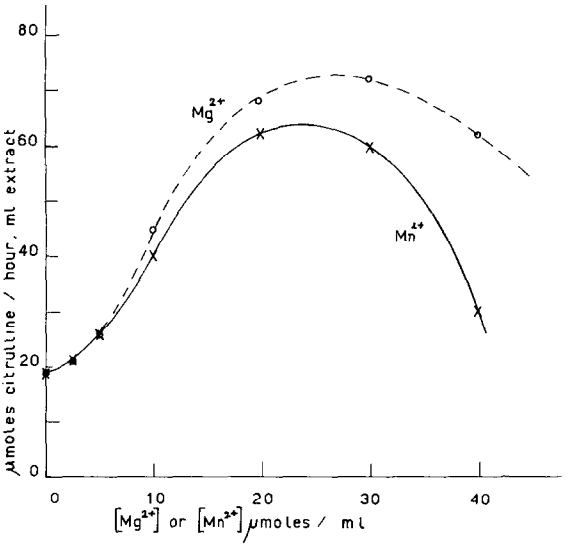

Fig. 1 : Activation effect of Mg^{2+} and Mn^{2+} concentrations on arginine deiminase activity. pH 5.8, [arginine] = 10 mM, [citrate] = 50 mM.

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pathways for arginine catabolism. The two pathways are regulated quite differently, the presence of oxygen apparently favouring the arginase pathway and causing the repression of the enzymes of the deiminase route, while the opposite occurs when oxygen is scarce.

Factors such as glucose and the ammonium ion which act as strong repressors of the catabolic transferase in the presence of oxygen do not prevent the appearance of high levels of this enzyme in poorly oxygenated cells. The deiminase pathway is a source of energy for essentially fermentative organisms like Clostridium botulinum (14) and Streptococcus faecalis (15). It is interesting to observe that in Pseudomonas the highest levels of catabolic ornithine carbamoyltransferase were obtained from cells deprived of oxygen, despite the fact that this organism is a strict aerobe (15). Bacillus licheniformis can ferment glucose, but cannot grow on all of the media used in our experiments when the oxygenation of the cultures is reduced too far.

The presence of carbamate kinase in extracts of B. licheniformis would indeed indicate that the deiminase pathway may serve as

a source of energy under conditions where respiration becomes difficult. Why two pathways are necessary for B. licheniformis remains unknown. No arginase has been detected in Pseudomonas.

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