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THE REGULATION AND KINETICS OF THE TWO ORNITHINE TRANSCARBAMYLASE ENZYMES OF *BACILLUS LICHENIFORMIS*

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SUMMARY

1. The regulation of the two ornithine transcarbamylase enzymes (carbamoylphosphate: L-ornithine carbamoyltransferase, EC 2.1.3.3) of *Bacillus licheniformis* was studied. Ornithine transcarbamylase I is repressed by L-arginine, L-citrulline and L-ornithine during growth on glucose. Ornithine transcarbamylase II is induced by L-arginine (but not by L-citrulline or L-ornithine) in post-logarithmic phase cells or in cells growing on glutamate. Glucose addition represses this induction in the glutamate medium.

2. The ornithine transcarbamylase I was purified about 10 fold and ornithine transcarbamylase II about 8 fold.

3. The two ornithine transcarbamylases have the same pH optimum (8–9) and no significant differences could be detected in Michaelis–Menten constants for either substrate.

4. The back reaction (citrulline → ornithine) was not catalyzed by either ornithine transcarbamylase.

5. Arginine deiminase was not detected in extracts of these cells.

6. The physiological role of the inducible ornithine transcarbamylase (ornithine transcarbamylase II) is not known.

INTRODUCTION

The two functions of ornithine transcarbamylase (carbamoylphosphate: L-ornithine carbamoyltransferase, EC 2.1.3.3) have been clearly stated recently[1]. The enzyme catalyzes the synthesis of citrulline in the arginine biosynthetic pathway in *Bacillus licheniformis*[2] and in all other systems[1]. The catabolism of arginine in microbes takes different forms, one of which utilizes ornithine transcarbamylase operating in the reverse direction. *B. licheniformis*[2] and *B. subtilis*[3] degrade arginine *via* an arginase (L-arginine amidinohydrolase, EC 3.5.3.1), thus circumventing the ornithine transcarbamylase.

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However, it was reported that an ornithine transcarbamylase activity could be induced by L-arginine during the late-exponential phase of growth of *B. licheniformis*[4]. Subsequently, it was shown that this organism can produce two DEAE-cellulose separable ornithine transcarbamylases, ornithine transcarbamylase I being heat stable and ornithine transcarbamylase II being inducible and heat labile[5]. The presence of two ornithine transcarbamylases has been reported in Pseudomonas[1,6] where it is clear that one has an anabolic function and the other a catabolic one. Since we have recently shown[7,8] that an ornithine transcarbamylase is not necessary for the catabolism of arginine in *B. licheniformis*, it was of interest to study the control and enzymic properties of the two ornithine transcarbamylases in order to more fully understand the function of these enzymes. Unfortunately, no definitive role for the induced ornithine transcarbamylase II can be proposed, and we must conclude that the control systems of the cell allow the production of an enzyme that may not be required.

MATERIALS AND METHODS

Culture conditions and cell free extracts

B. licheniformis strain A-5 was used throughout this investigation. The growth conditions and cell free extract preparations used in the induction and repression studies in which cells were grown on glucose or glutamate are described by LAISHLEY AND BERNLOHR[8].

Cells used for the purification of the inducible ornithine transcarbamylase II were grown in 1-l lots in a 20 mM glucose, 50 mM ammonium lactate and mineral salts medium[9] supplemented with 10 mM L-arginine. Growth was at 37° in a warm room on a New Brunswick gyrotary shaker. After the cells had finished growing on glucose, the culture was harvested by centrifugation at 13 200 × *g* at 0° for 10 min. The supernatant solution was discarded and the cells suspended in 8 ml distilled water. Cell-free extracts were prepared by breaking the cells in a MSE sonic oscillator at 2° for 2 min followed by centrifugation at 22 000 × *g* for 10 min. The supernatant solution was saved for the enzyme purification steps. The growth conditions and cell free extracts for the detection of the arginine deiminase were the same as described for ornithine transcarbamylase II with the exception that the cell free extract was dialyzed against 4 l of 3 mM succinate buffer (pH 6) *plus* 1 mM mercaptoethanol for 5.5 h.

The growth conditions and cell-free preparations for the repressible ornithine transcarbamylase I enzyme purification were the same as described for ornithine transcarbamylase II with the exception that the cells were harvested during the logarithmic phase of growth in a minimal salts medium[9] containing 60 mM glucose and 100 mM ammonium lactate. This high level of glucose was used to insure the absence of ornithine transcarbamylase II.

Enzyme assays and protein determination

Ornithine transcarbamylase activity was routinely assayed as described by ROGERS AND NOVELLI[10]. The reaction mixture was assayed for citrulline by the method of ARCHIBALD[11] as modified by KNIVETT[12]. However, for the enzyme purification and kinetic studies the more sensitive assay method of HUNNINGHAKE AND GRISOLIA[13] for citrulline was used. To assay the enzyme in the reverse direction, cit-

rulline to ornithine, the modified arsenolysis procedure of SCHIMKE *et al.*[14] was used and the appearance of ornithine was measured by the the method of RATNER[15].

Arginine deiminase was determined by the modified procedure of OGINSKY[16]. The assay mixture contained 40 μmoles L-arginine, 100 μmoles Tricine buffer (pH 6.0) or 100 μmoles succinate buffer (pH 6.0), enzyme preparation and distilled water to 1 ml. The reaction mixture was stopped after 10 min at 37° with 0.2 ml 60% $HClO_4$, 0.8 ml distilled water was added and citrulline determined[12]. All protein concentrations were measured by the method of LOWRY *et al.*[17].

Radioactive dilution experiment for detection of arginine deiminase

The procedure for growing, harvesting, and preparing cell-free extracts is described above. Three reaction mixtures contained 40 μmoles L-arginine, 100 μmoles succinate buffer (pH 6.0) 5.0 μC uniformly ^{14}C-labelled L-arginine (spec. activity: 174 mC/mmole) cell-free extract preparation (13.5 mg protein) and distilled water to 1.0 ml in Servall heavy-walled glass centrifuge tubes. A fourth tube contained all of the above mentioned compounds with the exception of the enzyme. Incubation was at 37°. At 15-, 30-, and 60-min intervals samples were removed and the reaction terminated by immersion in boiling water for 5 min. The tubes were centrifuged at 12 000 × *g* for 5 min and the supernatant solution was collected. This experiment was repeated as described above with the addition of 20 μmoles L-citrulline to the reaction mixture. This control would allow the trapping of radioactive citrulline if it were being formed from arginine.

The clear supernatant solutions and the appropriate standards (arginine, ornithine, and citrulline) were spotted (20 lambda) on Whatman No. 1 chromatography paper. Ascending paper chromatography was performed using a solvent system consisting of 100 ml phenol and 20 ml water, and in an atmosphere provided by a beaker containing 20 ml 0.3% $(NH_4)OH$. After 11 h, the paper chromatograms were removed and allowed to dry overnight. The chromatograms were cut into one inch strips and the strips containing the standards were sprayed with ninhydrin to locate the amino acids. The remaining strips containing the reaction mixture were run through a Baird Atomic strip scanner Model 432A to locate and identify the end-products by the radioactive profiles on the chart paper, for comparison with the standards.

Purification of two ornithine transcarbamylases

Ornithine transcarbamylase II. The procedure for growing, inducing, harvesting, and preparing cell-free extracts is described above. All purification work was performed at 5° or below, unless noted.

The cell-free extract (7.0 ml) was placed on a Sephadex G-50 column (18 cm × 3 cm) which had been previously buffered with 5 mM Tris–HCl (pH 7.8) *plus* 1 mM mercaptoethanol. The protein was eluted from the column with the same solution and the enzyme collected in 5-ml samples in tubes numbered 3–10, and pooled. To this pooled sample was added solid $(NH_4)_2SO_4$ to 60% saturation. The suspension was centrifuged at 22 000 × *g* for 5 min. The supernatant solution was saved and solid $(NH_4)_2SO_4$ added to 95% saturation and centrifuged as above. The sediment was dissolved in a small volume of 5 mM Tris–HCl (pH 7.8) *plus* 1 mM mercaptoethanol. The major portion of the enzyme activity was located in the 60–95% $(NH_4)_2SO_4$ fraction. This was dialyzed against 4 l 3 mM Tris–HCl (pH 7.8) *plus* 1 mM mercapto-

TABLE I

PARTIAL PURIFICATION OF ORNITHINE TRANSCARBAMYLASE II

Purification step	*Volume (ml)*	*mg protein/ml*	*Spec. act. (units/mg protein)**	*Total units**
Crude extract	7	30.5	13.9	2960
Sephadex G-50	35	5.7	11.0	2200
60–95% $(NH_4)_2SO_4$	2.7	5.0	37.0	500
DEAE-cellulose	53	0.084	94.0	428

* 1 unit of enzyme catalyzes the formation of 1 μmole citrulline per min.

ethanol for 3 h, and the dialysate was placed on a DEAE-cellulose column which had been buffered as described for the Sephadex column. The protein was eluted from this column by a linear 0.0–0.5 M KCl (*plus* 1 mM mercaptoethanol) gradient and collected in 5-ml samples. The enzyme (the activity was found in Tubes 35–45) was collected and pooled for the kinetic studies. A summary is given in Table I.

Ornithine transcarbamylase I. The cell-free extract (17 ml prepared from two 1-l cultures of cells) was placed in a 50-ml erlenmeyer flask, heat-treated by constant rotation of the enzyme preparation in the flask for 10 min in a 60° water-bath. This heat-treated preparation was centrifuged at 105 000 × *g* for 30 min. The clear supernatant solution was fractionated with solid $(NH_4)_2SO_4$ to 45% saturation. The suspension was centrifuged at 22 000 × *g* for 5 min. The supernatant solution was saved and solid $(NH_4)_2SO_4$ added to 95% saturation. After centrifugation as above the

TABLE II

PARTIAL PURIFICATION OF ORNITHINE TRANSCARBAMYLASE I

Purification step	*Volume (ml)*	*mg protein/ml*	*Spec. act. (units/mg protein)**	*Total units**
Crude extract	17.0	22.5	0.178	68
Heat treated, 60°	11.0	7.7	0.428	36.1
45–95% $(NH_4)_2SO_4$	2.9	8.0	0.750	17.4
DEAE-cellulose	48	0.045	1.85	4.0

* 1 unit of enzyme catalyzes the formation of 1 μmole citrulline per min.

sediment was dissolved in a small volume of 5 mM Tris–HCl (pH 7.8) *plus* 1 mM mercaptoethanol. Further purification by DEAE-cellulose chromatography was the same as described for the inducible ornithine transcarbamylase, but the enzyme was eluted into Tubes 22–36. A summary is given in Table II.

Materials

All chemicals were of reagent grade purity. L-Arginine, L-citrulline and L-ornithine were chromatographically homogeneous preparations from Mann Research Labs, Inc. Uniformly ^{14}C-labelled L-arginine was purchased from Nuclear Chicago.

RESULTS

Repression of ornithine transcarbamylase I

The addition of L-arginine, L-citrulline or L-ornithine to a *B. licheniformis* culture growing on a glucose–salts medium causes a significant repression of the ornithine transcarbamylase activity. Previous studies[5] have shown that this activity is due to ornithine transcarbamylase I. Fig. 1 illustrates this effect. Growth of the

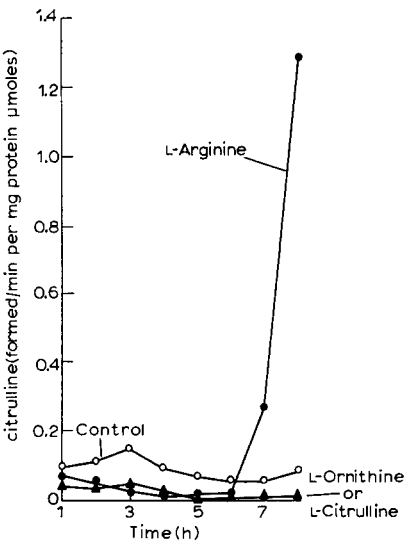


Fig. 1. Regulation of ornithine-transcarbamylase synthesis in cells growing on salts *plus* 60 mM glucose. Amino acids were added as indicated at zero time to a final concentration of 10 mM. Growth was complete at 6 h. Samples were removed, extracts prepared and the activity determined as described in MATERIALS AND METHODS. Protein concentrations for the assays ranged from 6.8 to 680 µg/ml.

cells in this experiment continued until the 6th h, so it is concluded that all three amino acids can cause repression of ornithine transcarbamylase I during the entire growth period.

Induction of ornithine transcarbamylase II

Fig. 1 also demonstrates the induction, after growth is completed, of a high ornithine transcarbamylase activity. Chromatography of extracts from 7- to 8-h cultures indicates that ornithine transcarbamylase I remains low while ornithine transcarbamylase II is induced[5]. L-Arginine addition causes this induction while L-citrulline and L-ornithine do not. Since the ornithine transcarbamylase II is not induced until growth is complete (glucose is exhausted from the medium[18,19]), the phenomenon of glucose-mediated catabolite repression was suggested. Thus, cells were grown in a glutamate–salts medium and inoculated into flasks containing the glutamate medium to which additions were made. Fig. 2 shows the results of this experiment. L-Arginine causes the induction of ornithine transcarbamylase-II activity while L-ornithine represses ornithine transcarbamylase-I activity. Control cultures

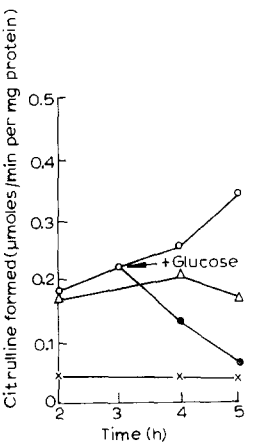
had about twice the activity of cells grown on the glucose medium. Glucose addition to a glutamate and L-arginine culture produced a pronounced repression of the induction of the ornithine transcarbamylase. Thus, ornithine transcarbamylase II is induced by arginine and is under catabolite repression control. Neither L-citrulline nor L-ornithine are inducers and the cell apparently does not convert them to L-arginine at a rate sufficient to cause induction.

We have recently described, in detail, the regulation of the arginine catabolic pathway in *B. licheniformis*[8]. This pathway converts arginine to glutamic acid in the following manner:

$$\text{L-Arginine} \xrightarrow{\text{arginase}} \text{ornithine} \xrightarrow{\text{transaminase}} \text{pyrroline-5-carboxylate} \xrightarrow{\text{dehydrogenase}} \text{glutamic acid.}$$

All of the enzymes of this pathway are under catabolite repression control and all of the enzymes are induced by L-ornithine and L-arginine[8]. In fact, L-ornithine is a better inducer than L-arginine on some media. Thus, it is clear that the control pattern of the arginine catabolism system is different from that of ornithine transcarbamylase II.

As a further check on the validity of the assumption that ornithine transcarba-



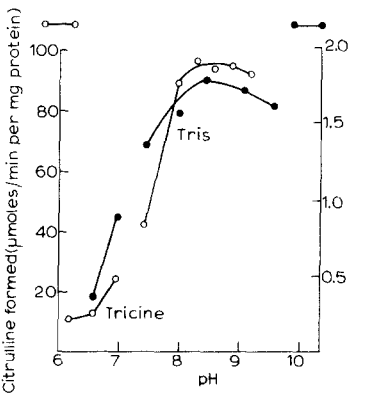


Fig. 2. Induction and catabolite repression of ornithine transcarbamylase synthesis. Cells were grown on the salts medium *plus* 50 mM glutamic acid. At zero time L-arginine was added (15 mM) to two cultures (○--○) and L-ornithine added (15 mM) to a third (×—×). A fourth culture (△—△) contained no additions. At 3 h, 30 mM glucose was added to one (●—●) of the arginine cultures. Growth was in mid-log phase at 5 h. Other details as in Fig. 1 except that 26 to 90 μg protein/ml were used.

Fig. 3. The effect of pH on the ornithine transcarbamylase activities. Ornithine transcarbamylase I (●—●); ornithine transcarbamylase II (○—○). The assay is described in MATERIALS AND METHODS except for the use of 200 mM buffer at the indicated pH. The enzyme preparations were DEAE-cellulose fractions.

mylase II is not a part of this arginine catabolic pathway, a number of attempts were made to demonstrate an arginine deiminase (L-arginine iminohydrolase, EC 3.5.3.6) activity in extracts of cells that contain ornithine transcarbamylase II. No deiminase activity was detected by either method described in MATERIALS AND METHODS, and we conclude that *B. licheniformis* cells cannot degrade arginine to citrulline. A sub-

strate for the ornithine transcarbamylase II operating in the reverse direction would not be present in the cells.

Kinetics of the two ornithine transcarbamylases

Ornithine transcarbamylase I apparently functions in *B. licheniformis* as a biosynthetic enzyme. The experiments on regulation that are described above do not allow us to draw a conclusion as to the function of ornithine transcarbamylase II. Therefore, both activities were partially purified and the pH optimum and apparent K_m constants for both substrates were determined.

TABLE III

SUMMARY OF DATA ON THE TWO ORNITHINE-TRANSCARBAMYLASE ENZYMES

Characteristic	*Ornithine transcarbamylase I*	*Ornithine transcarbamylase II*
Regulation	Repressed by L-arginine, L-ornithine and L-citrulline.	Induced by L-arginine only. Under catabolite repression control.
pH optimum	8.5	8.5
K_m (ornithine)	0.71 mM	0.46 mM
K_m (carbamylphosphate)	0.10 mM	0.29 mM
Reversibility	No	No

Both ornithine-transcarbamylase activities demonstrate a relatively broad pH optimum of about 8.5 as shown in Fig. 3. Both enzymes displayed normal Michaelis–Menten kinetics and K_m values were calculated from the slopes of reciprocal plots (Table III).

We also attempted to demonstrate catalysis of the reverse reaction, *i.e.*, citrulline → ornithine by arsenolysis, using both enzyme preparations. In no case was this reverse reaction observed, even though incubation conditions (pH, ionic strength) were significantly varied.

Our results on the characteristics of ornithine transcarbamylase I and ornithine transcarbamylase II are summarized in Table III.

DISCUSSION

On the basis of different heat stabilities, the manner of purification, and mechanism of regulation of biosynthesis, the two ornithine-transcarbamylase activities clearly reside in different protein molecules. They are indistinguishable, however, in regard to kinetic criteria.

From the information available, it is reasonable to assume that ornithine transcarbamylase I serves a biosynthetic function in the cell. The repression by L-arginine, the pH optimum and the K_m values are all similar to reported literature values of biosynthetic ornithine transcarbamylase. The enzymes from *Streptococcus lactis*[20] and *Escherichia coli*[21] have pH optima of about 8.5 and K_m values for ornithine that are in the same range as those reported here. Of the two ornithine-transcarbamylase enzymes found in Pseudomonas species[1,6], the one with a pH optimum of 8.5 was judged to be biosynthetic.

The role of ornithine transcarbamylase II is much more obscure. This enzyme has kinetic constants that are the same as ornithine transcarbamylase I. It is also unable to catalyze the back reaction that would be necessary if it were to have a catabolic function. However, ornithine transcarbamylase II is induced by arginine and the synthesis is under catabolite repression control, thus suggesting a catabolic function.

We are left with a dilemma that cannot be resolved with the available information. However, one hypothesis can be offered. *B. licheniformis* may contain duplicate ornithine-transcarbamylase genes under different mechanisms of control.

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