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THE OCCURRENCE OF A CATABOLIC AND AN ANABOLIC ORNITHINE CARBAMOYLTRANSFERASE IN PSEUDOMONAS

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SUMMARY

The occurrence of two ornithine carbamoyltransferases (carbamoylphosphate:L-ornithine carbamoyltransferase, EC 2.1.3.3) in Pseudomonas is demonstrated by their separation with ammonium sulfate. The two enzymes are distinguished by their activities as a function of pH.

On the basis of the regulation of their synthesis, an anabolic function is assigned to one of these enzymes, a catabolic function to the other.

INTRODUCTION

The physiological role of ornithine carbamoyltransferase (carbamoylphosphate:L-ornithine carbamoyltransferase, EC 2.1.3.3) which catalyzes reaction I, has been reviewed recently[1,2].

Carbamoylphosphate + L-ornithine ⇌ L-citrulline + orthophosphate (I)

Fig. 1 summarizes the connections between this reaction and those to which it is related, in microbial metabolism at least.

The recent finding that the biosynthesis of carbamoylphosphate in *Escherichia coli*[3] and *Saccharomyces cerevisiae*[4] is catalyzed by an enzyme which is different from carbamate kinase (ATP:carbamate phosphotransferase, EC 2.7.2.2; reaction 12 in Fig. 1), allows one to consider that the biosynthesis of arginine is independent, in all its steps, from its catabolism. Indeed, the last step in which a common enzyme could have been involved was the ornithine–citrulline interconversion. We have reported briefly[5] that in this conversion the two functions (reactions 6 and 11 in Fig. 1) are performed by two distinct enzymes. The purpose of this and the following article is to give more information on these enzymes, their regulation and their specialization.

The forward reaction (carbamoylation) catalyzed by ornithine carbamoyl-

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transferase participates, in all known cases, in the synthesis of arginine[2]. The reverse reaction (phosphorolysis) was previously believed to be catalyzed by a distinct enzyme, "citrullinase" (or "citrulline ureidase"), one of the three components of the system known as "arginine dihydrolase", observed in a number of microorganisms including lactic acid bacteria, Pseudomonas and pleuropneumonia-like organisms[1,6-9]. REICHARD[10] has shown that a purified preparation of rat-liver ornithine carbamoyltransferase catalyzes the reaction in both directions. The backward direction is most conveniently studied by arsenolysis since the equilibrium is much in favor of the formation of citrulline (the equilibrium constant of reaction I is of the order of 10^5). The catabolic function of this enzyme has been suggested by the very high level of an inducible ornithine carbamoyltransferase and of carbamate kinase in *Streptococcus faecalis*[11,12].

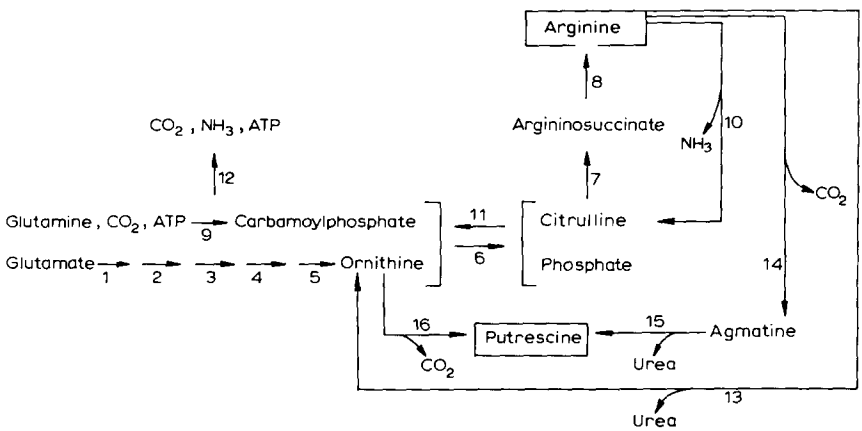

Fig. 1. Metabolic pathways related to ornithine carbamoyltransferase. The arrows indicate the direction in which the reaction is used by the cell. Reactions 1–9 are involved in the biosynthesis of arginine whereas reactions 10–12 represent the catabolic sequence pertinent to this work. This scheme results from data summarized in refs. 1–4. Reaction 13 is absent from Pseudomonas, but present in Bacillus[19]. Reactions 14–16 are present in *E. coli*[20].

This catabolic role of ornithine carbamoyltransferase is shown also in Pseudomonas, by the simultaneous presence of arginine deiminase (L-arginine iminohydrolase, EC 3.5.3.6, reaction 10 in Fig. 1)[13], by the ability of this organism to use arginine or citrulline as the unique source of carbon and nitrogen[14] and, finally, by the observation that its motility, lost in anaerobiosis, is restored when arginine or citrulline are introduced into the medium. This suggests the use of arginine or citrulline for the formation of ATP through an anaerobic process[15,16].

As the Pseudomonas are prototrophic for arginine, they must perform two reactions: (1) the carbamoylation of ornithine, functional in ordinary growth in minimal medium; and (2) the phosphorolysis of citrulline, performed when these bacteria use arginine or citrulline as source of nitrogen and ATP. In these circumstances, the carbamoylation is not necessary. The two functions of the reaction catalyzed by ornithine carbamoyltransferase operate under different, even opposed, conditions.

MATERIALS AND METHODS

Media

A mineral medium (No. 154) of the following composition (per l of medium) was used: KH_2PO_4, 1.4 g; $Na_2HPO_4 \cdot 12\ H_2O$, 14.3 g; NaCl, 0.6 g; K_2SO_4, 1.7 g; $MnSO_4 \cdot 4\ H_2O$, 0.55 mg; $MgSO_4 \cdot 7\ H_2O$, 50 mg; $FeCl_3 \cdot 6\ H_2O$, 5 mg. The pH of the medium was 7.3. To avoid precipitation, $MnSO_4$, $MgSO_4$ and $FeCl_3$ were added from concentrated solutions after sterilization of the medium. $FeCl_3$ was sterilized by filtration and added just before use. To this mineral medium, different carbon and nitrogen sources were added in the following combinations: (1) $(NH_4)_2SO_4$, 10^{-2} M and citrate, $2 \cdot 10^{-2}$ M; (2) L-arginine or L-citrulline, 0.4%. Growth was at 30° for *Pseudomonas fluorescens* IRC 204 and A.3.12, and at 37° for *Pseudomonas aeruginosa* ATCC 10145.

Microorganisms

Most of the experiments were performed with *P. fluorescens*, strain IRC 204. This bacterium was isolated after enrichment on medium No. 154 with citrulline as the carbon and nitrogen source. Some limited experiments were done with *P. fluorescens* (A.3.12, from R. STANIER) and *P. aeruginosa* (ATCC 10145).

Enzyme preparation

Exponentially growing cells in specified media were collected and resuspended in Tris 0.025 M (pH 7.5) at about 20 mg dry weight per ml. Small volumes (up to 3 ml of suspensions) were disrupted in an M.S.E. Mullard ultrasonic disintegrator; larger volumes (15 ml) were disrupted in a Raytheon 10-kcycles magnetostriction apparatus. If no further purification was intended the extracts were passed through a column of Sephadex G-50 (6 g, 15 cm high).

Enzyme activity

The activities were followed in the direction of carbamoylation; citrulline formation was determined by the colorimetric method of ARCHIBALD[17]. The synthesis of citrulline was performed as described by JONES, SPECTOR AND LIPMANN[18]. As pH was used as a variable, and sharp variations of activity with pH were observed, the incubation mixture was carefully adjusted to the desired pH. The assay mixture contained: 350 μmoles Tris (at the desired pH), 25 μmoles L-ornithine, 20 μmoles carbamoylphosphate. The pH was reajusted and the mixture kept at 0°. The enzyme (about 2 units) was added. The final volume was 2.5 ml. The reaction was started by raising the temperature to 30°, and was stopped after 15 and 30 min, by the addition of 2.5 ml of 1 M HCl. The excess of carbamoylphosphate was destroyed by heating for 10 min in a boiling-water bath. Similar tests without enzyme gave the chemical blank. A blank which was acidified before the addition of enzyme permitted a correction for pre-existing citrulline or other interfering material. As a rule both blanks were negligible. When necessary, the coagulated proteins were eliminated by centrifugation.

A unit of enzyme is defined as the amount of enzyme that catalyzes the formation of 1 μmole of citrulline per h under the conditions described above. Specific activities are expressed as units per mg of protein.

RESULTS

(1) The carbamoylation of ornithine as a function of pH

After growth of strain IRC 204 in a medium containing citrate as carbon source and NH_4^+ as nitrogen source (see *Media*), the enzymic activity showed two distinct pH optima (Fig. 2): one, very sharp, around pH 7.3, and a second, much broader, in the region of pH 8.5 to 9. After growth with arginine as carbon and ni-

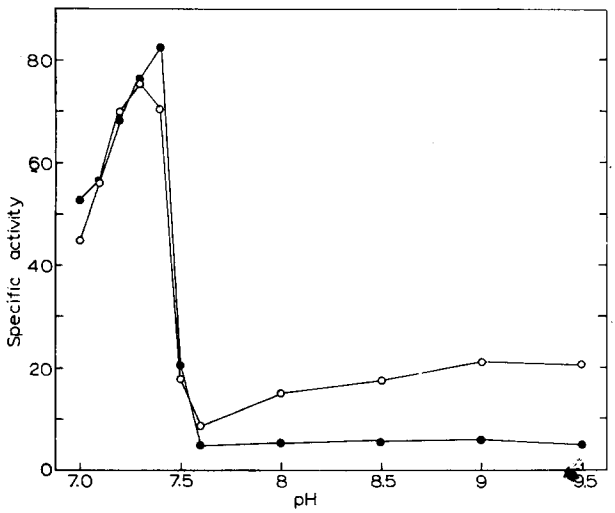

Fig. 2. Ornithine carbamoyltransferase activity of a crude cell-free extract of Pseudomonas IRC 204. ○—○, after growth on medium No. 154 supplemented with 0.01 M $(NH_4)_2SO_4$ and 0.02 M sodium citrate; ●—●, after growth on the same medium supplemented with 0.01 M $(NH_4)_2SO_4$, 0.02 M sodium citrate and 0.1% L-arginine.

trogen source or with citrate, NH_4^+ and arginine, the specific activity at pH 8.5 was reduced from 20 to 5; the activity around pH 7.3 was not significantly affected.

Similar experiments were made with *P. fluorescens* A.3.12, and with *P. aeruginosa* ATCC 10145 (see Figs. 3 and 4).

Two distinct regions of activity of variable size were always observed; but the activity in the alkaline region was always repressed by growth in the presence of arginine. In *P. aeruginosa*, the activity at neutral pH was markedly increased by growth in the presence of arginine.

These results suggest that the ornithine carbamoyltransferase activity in Pseudomonas results from the activity of two distinct enzymes. The enzyme with a pH optimum near neutrality has a catabolic function; that with an alkaline pH optimum has an anabolic function.

(2) The separation of the two ornithine carbamoyltransferases by $(NH_4)_2SO_4$ precipitation

The activities at neutral and alkaline pH are sharply separated with $(NH_4)_2SO_4$. Most of the neutral activity precipitates above 60% saturation, the alkaline activity

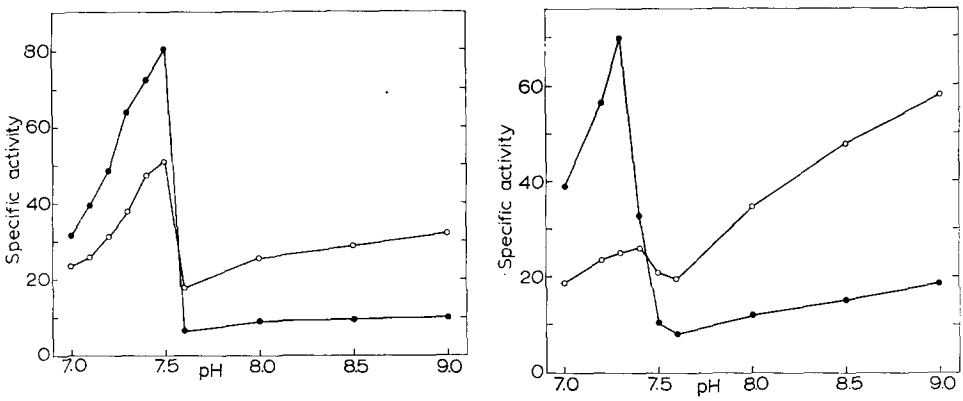

Fig. 3. Ornithine carbamoyltransferase activity of a crude cell-free extract of *P. fluorescens* A.3.12. ○—○, after growth on medium No. 154 supplemented with 0.01 M $(NH_4)_2SO_4$ and 0.02 M sodium citrate; ●—●, after growth on the same medium supplemented with 0.01 M $(NH_4)_2SO_4$, 0.02 M sodium citrate and 0.1% L-arginine.

Fig. 4. Ornithine carbamoyltransferase activity of a crude cell-free extract of *P. aeruginosa* ATCC 10145. ○—○, after growth on medium No. 154 supplemented with 0.01 M $(NH_4)_2SO_4$ and 0.02 M sodium citrate; ●—●, after growth on the same medium supplemented with 0.01 M $(NH_4)_2SO_4$, 0.02 M sodium citrate and 0.1% L-arginine.

below 45%. This is reported in the following experiment, summarized in Table I. The cells from 3 l culture (around 0.6 g dry weight) were sonicated in 15 ml Tris buffer at about 4° (as for all subsequent operations). The clear extract obtained after 6 min centrifugation at 20 000 × *g* was treated with 10% in volume of a 10% streptomycin sulfate solution. After 15 min stirring, the precipitate was discarded and the supernatant was brought to 76% saturation with solid $(NH_4)_2SO_4$. The precipitate was extracted with 10 ml of a 60% saturated $(NH_4)_2SO_4$ solution

TABLE I

SEPARATION OF THE TWO ORNITHINE CARBAMOYLTRANSFERASE ACTIVITIES OF PSEUDOMONAS IRC 204 BY $(NH_4)_2SO_4$ PRECIPITATION

Fraction	*Activity at neutral pH (optimum between 7.1 and 7.4)*		*Activity at pH 8.5*	
	Total activity (units)	*Specific activity (units/mg)*	*Total activity (units)*	*Specific activity (units/mg)*
Before separation	22 630	70.5	7360	24
56–76% saturation with $(NH_4)_2SO_4$	15 000	369	126	3.1
46–56% saturation with $(NH_4)_2SO_4$	322	8	366	9
36–46% saturation with $(NH_4)_2SO_4$	695	32	2600	116
0–36% saturation with $(NH_4)_2SO_4$	108	1.5	225	3.7

for 30 min. The remaining precipitate, after centrifugation, was then extracted successively with 45% and 35% saturated solutions. The true $(NH_4)_2SO_4$ saturation was corrected by refractometry (see Table I). All the solutions in $(NH_4)_2SO_4$ were then precipitated to 90% and the precipitates solubilized in Tris buffer and dialyzed. The enzymic activity was not affected by $(NH_4)_2SO_4$ as high as 1.5 M in the final incubation mixture. The fractions precipitated between 56 and 76% and between 36 and 46% $(NH_4)_2SO_4$ saturation were tested again over the whole range of pH. In each test, only one peak of activity was regained.

CONCLUSION

Cell-free extracts of Pseudomonas show two very distinct regions of ornithine carbamoyltransferase activity when studied as a function of pH. These activities can be separated by $(NH_4)_2SO_4$ precipitation. This leaves little doubt about the existence of two ornithine carbamoyltransferases in these organisms.

The regulation of the synthesis of these enzymes is different. In Pseudomonas IRC 204 the enzyme with an alkaline optimum is strongly repressed by exogenous arginine, while the activity at neutral pH remains unchanged. In *P. aeruginosa*, the alkaline activity is repressed by arginine but the neutral activity is increased in these conditions. These results suggest that the activity at pH 8.5 is due to an ornithine carbamoyltransferase with an anabolic function while the activity at neutral pH corresponds to an enzyme with a catabolic function. The latter enzyme, however, is not inducible in strain IRC 204.

This biplicity of enzymes which perform the same biochemical activity illustrates, once more, a way in which the independence of regulation is obtained in living cells. In the following paper a more detailed study shows the specialization of these enzymes and the integration of their function.

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