BBA 65557

THE SPECIALIZATION OF THE TWO ORNITHINE CARBAMOYLTRANSFERASES OF PSEUDOMONAS

F. RAMOS, V. STALON, A. PIÉRARD AND J. M. WIAME
Institut de Recherches du C.E.R.I.A., Bruxelles, and Laboratoire de Microbiologie de l'Université de Bruxelles, Brussels (Belgium)*

(Received August 2nd, 1966)

SUMMARY

The two ornithine carbamoyltransferases (carbamoylphosphate:L-ornithine carbamoyltransferase, EC 2.1.3.3) of Pseudomonas are integrated into different metabolic sequences. This integration appears both in their regulation and in their activities. In Pseudomonas IRC 204, the catabolic ornithine carbamoyltransferase is subject to catabolite repression in the same way as arginine deiminase (L-arginine iminohydrolase, EC 3.5.3.6) and carbamate kinase (ATP:carbamate phosphotransferase, EC 2.7.2.2). The catabolic ornithine carbamoyltransferase *in vivo*, has little or no biosynthetic function. The anabolic ornithine carbamoyltransferase, unlike other similar enzymes, is unable to perform the catabolic phosphorolysis.

INTRODUCTION

The existence of isodynamic enzymes appears at present to be a normal mechanism by which the cell switches common reactions to their different biological ends[1]. This mechanism may operate when the two functions are anabolic[2–4], amphibolic[5], or catabolic[6,7]. In the example described here and in the preceding paper[8], as for the earliest clear observation of such enzyme multiplicity[9], one function is anabolic, the other catabolic. This multiplicity allows the specialization of these enzymes, which can be exhibited by the regulation of their synthesis, by the regulation of their activities, by the possibility of different locations in the cell and also by the proper characteristics of their kinetics toward their substrates.

Although not strictly speaking isodynamic, since they may involve different cofactors or prosthetic groups, good examples of this specialization are the two glutamic dehydrogenases, one working with NAD, the other with NADP[10,11], or the succinic dehydrogenase and fumarate reductase[12]. The two ornithine carbamoyltransferases are specialized in the same way but most probably without involving different cofactors or prosthetic groups. This is the subject of this article.

A preliminary report of this work has appeared[13].

* C/o C.E.R.I.A., Bruxelles 7, Belgique.

MATERIALS AND METHODS

Organisms and growth media

Pseudomonas IRC 204 has been described in the preceding article[8]. From this strain, two arginine auxotrophic mutants were obtained, IRC 204RS1 by ultraviolet irradiation, and IRC 204RS5 by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment. Both mutants were selected by the penicillin procedure[14]. Mutant IRC 204RS1 lacks the anabolic ornithine carbamoyltransferase whereas mutant IRC 204RS5 is blocked in the arginine biosynthetic pathway at a step anterior to that enzyme.

Media and growth conditions for Pseudomonas were described in the preceding article[8]. In addition to batch cultures, the regulation of enzyme synthesis was studied in chemostat. In this continuous culture, medium No. 154 was supplemented with $1 \cdot 10^{-2}$ M $(NH_4)_2SO_4$ and a limiting amount of citrate (the carbon source) in order to release the catabolite repression. The concentration of citrate was 1 g per l of $Na_3C_6H_5O_7 \cdot 5\ H_2O$, giving approximately 0.2 mg of cell (dry weight) per ml. The culture volume was 180 ml. Aeration was obtained with a magnetic stirrer, and the flow rate was limited by means of a Sigmamotor Co. digital pump.

Some experiments were performed with lactic acid bacteria, for comparative purposes. The classical strain Streptococcus sp. ATCC 8042 strain P-60 (sometimes erroneously designated as *Leuconostoc mesenteroides* P-60), was grown on the medium of STEELE *et al.*[15].

Strain P4XB2 of *Escherichia coli* K-12 is non-repressible for the arginine biosynthetic pathway[16]. It is grown on medium No. 132 (ref. 17) containing 0.5% glucose.

Preparation of carbamate kinase

Carbamate kinase was prepared from *Streptococcus faecalis* NCIB 6459 according to a modification of the procedure of JONES[18] which successively involved streptomycin-nucleate precipitation, $(NH_4)_2SO_4$ precipitation between 60 and 80% saturation and acetic acid precipitation at pH 3.8. After dialysis and lyophilization, the preparation contained 630 units (μmoles carbamoylphosphate decomposed per h) of kinase and 2.3 units of ornithine carbamoyltransferase (from 500 units in the crude extract) per mg of protein. The slight ornithine carbamoyltransferase contamination did not increase the blank when this preparation was used to test the phosphorolysis of citrulline.

Determination of enzymic activities

(a) The assay procedure for ornithine carbamoyltransferase in the direction of the carbamoylation of ornithine has been described in the preceding article[8].

(b) Determination of ornithine carbamoyltransferase in the backward direction (phosphorolysis of citrulline). As the carbamoylation goes almost to completion, the reverse reaction has been studied by arsenolysis or by coupling the phosphorolysis with the production of ATP in the presence of added carbamate kinase and ADP.

High activities can be measured by following either the disappearance of citrulline, or the appearance of ornithine, CO_2 or NH_3. When the activities are low, the sensitivity of the method can be increased by determining the $^{14}CO_2$ formed from [*carbamoyl*-^{14}C]citrulline.

Phosphorolysis was performed in Warburg manometric vessels with two side-arms. The flask contained the following reagents in a total volume of 1 ml: sodium phosphate (pH 6.7), 25 μmoles; sodium citrate (pH 6.7), 100 μmoles; $MgCl_2$, 5 μmoles; ADP, 5 μmoles; L-[*carbamoyl*-^{14}C]citrulline, 25 μmoles ($4 \cdot 10^4$ counts/min); carbamate kinase, 15–30 units; the sample of ornithine carbamoyltransferase to be estimated. This reaction mixture was completed with 100 μmoles Tris in the experiments on the influence of pH on phosphorolysis, for the range of pH 6.7 to 8. One side-arm contained 10 μmoles of Na_2CO_3, the other 900 μmoles of H_2SO_4 and the center well 800 μmoles of carbonate-free NaOH. The vessels were not connected to manometers but closed with glass stoppers immediately after the addition of the enzyme. They were incubated for 15 or 30 min at 30°, and the reaction was stopped by pouring successively the H_2SO_4 and the Na_2CO_3 into the flask. The absorption of CO_2 by NaOH was allowed to proceed for 1 h and the content of the center well was transferred to a tube into which the carbonate was precipitated with 40 μmoles of $BaCl_2$. The $BaCO_3$ was collected on a filter paper, and washed with water and acetone. The radioactivity was measured with a Nuclear–Chicago low-background gas-flow counter.

Arsenolysis was followed by the determination of the production of ammonium with the Nessler reagent[21]. The reaction mixture, at pH 6.7, contained 100 μmoles of L-citrulline and 500 μmoles of arsenite in a total volume of 1.4 ml. The addition of 0.6 ml of enzyme started the reaction which was performed at 30° for 10, 20 and 30 min. The reaction was stopped by the addition of 0.2 ml of 4 M $HClO_4$ and the proteins were removed by centrifugation. An aliquot of 0.2 ml was used for the determination of NH_3. The sensitivity of the method could be increased, if necessary, by proceeding as for the phosphorolysis, using the determination of $^{14}CO_2$.

(c) Carbamate kinase activities were measured by the manometric determination of the rate of CO_2 production in the presence of carbamoylphosphate and ADP, according to KALMAN AND DUFFIELD[22], except that the mixture contained 200 μmoles of acetate buffer. After treatment on Sephadex G-50, the cell-free extract had an ADP-independent CO_2 production which was less than 10% of the CO_2 production in the presence of ADP.

(d) Arginine deiminase activities were determined by measuring, by means of the colorimetric determination of ARCHIBALD[23], the production of citrulline from arginine. The further destruction of citrulline by phosphorolysis and carbamoylphosphate breakdown was avoided by the removal, by Sephadex treatment, of any inorganic phosphate and ADP from the extract. The reaction mixture contained in a final volume of 1 ml: 100 μmoles sodium citrate (pH 5.8); 10 μmoles of L-arginine–HCl and the cell-free extract. The mixture kept at 0° before incubation was then incubated for 15 and 30 min at 30°. The reaction was stopped and the proteins were coagulated by the addition of 1 ml of 1 M HCl.

Units of activity

All units of activity are defined as the amount of enzyme that catalyzes the synthesis of 1 μmole of product per h. Specific activities are expressed in terms of units per mg of protein.

RESULTS

(a) The catabolite repression of the catabolic ornithine carbamoyltransferase of Pseudomonas IRC 204

In the preceding article[8] it was concluded that the ornithine carbamoyltransferase with a neutral optimal pH has a catabolic function. The demonstration was indirect and based on the fact that this enzyme was, at least in *P. aeruginosa*, inducible by arginine, while the other ornithine carbamoyltransferase was repressible by that amino acid. A further indication of this catabolic role is shown in Table I, where it appears that, when growth is limited by the carbon source—citrate in these experiments—a typical derepression of the synthesis of this ornithine carbamoyltransferase occurs. Derepression is a function of the catabolite pool since increasing the flow rate of the chemostat (thus increasing the pool of the limiting catabolite) leads to repression. Further, the two enzymes, arginine deiminase and carbamate kinase, which, in addition to ornithine carbamoyltransferase, belong to the arginine catabolic sequence, are subjected to the same regulation. The variations of the three enzymes are co-ordinated. The anabolic ornithine carbamoyltransferase, in contrast, varies in the opposite direction. The catabolic behavior of carbamate kinase poses the same type of question as ornithine carbamoyltransferase. Is the biosynthesis of carbamoylphosphate performed by this same kinase or by a glutamino-carbamoyl-

TABLE I

REGULATION OF THE TWO ORNITHINE CARBAMOYLTRANSFERASES OF PSEUDOMONAS IRC 204

The procedure for the growth in chemostat is described in MATERIALS AND METHODS. The two ornithine carbamoyltransferase activities were differentiated by their optimal pH (ref. 8). Both activities were determined in the direction of the synthesis of citrulline.

Type of culture	*Units of enzymic activity*			
	Catabolic enzymes			*Biosynthetic ornithine carbamoyltransferase*
	Arginine deiminase	*Catabolic ornithine carbamoyltransferase*	*Carbamate kinase*	
Batch				
Minimal	—	32	—	22
Minimal + arginine	—	30	—	6.8
Chemostat				
Growth rate*:				
$v_{max}/12$	31	460	36	8.3
$v_{max}/10.5$	23	330	35	6.5
$v_{max}/8.4$		428		9.3
$v_{max}/2$		87		5
$v_{max}/1.2$		21		22
$v_{max}/1.2$	2	33	4	24
$v_{max}/1.2$	3	17	2.5	24

* The growth rates in chemostat are expressed in terms of the fractions of the growth rate (v_{max}) obtained by growing the organism in batch culture on the same medium (see MATERIALS AND METHODS).

phosphate synthetase as in *E. coli*[24]. We have observed a slight (0.5 unit per mg) but positive activity of this glutamine-dependent carbamoylphosphate synthesis in Pseudomonas IRC 204. The use of one-step mutants simultaneously auxotrophic for arginine and uracil should give a definitive answer to this question[25].

(b) Further purification of the ornithine carbamoyltransferases

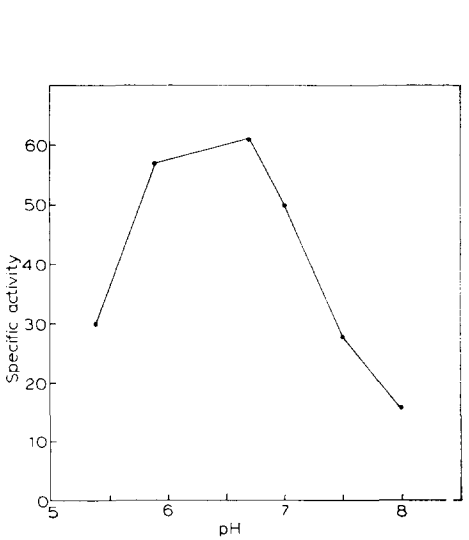
The study of the kinetics of the ornithine carbamoyltransferases required large amounts of the purified enzymes. The first steps in the purification which have been described previously[8] were applied to extracts of cells whose growth had been adjusted to obtain a maximal level of the ornithine carbamoyltransferases.

The cellular content of the catabolic enzyme was increased by means of the partial derepression which occurs at the end of the exponential growth on a limiting amount of citrate. $(NH_4)_2SO_4$ precipitation between 60 and 90% saturation, followed by a heat denaturation of 30 min at 55°, brought the specific activity to about 2000 units per mg of protein.

For the anabolic ornithine carbamoyltransferase the enzyme level was increased by growing the auxotrophic strain IRC 204RS5 in a chemostat with ornithine as the limiting nutrient. Starting from a crude extract with 80 units of enzyme per mg of protein, two consecutive fractionations with $(NH_4)_2SO_4$ between 30 and 50% saturation gave a preparation with a specific activity of 230 units per mg of protein.

(c) The reversibility of the ornithine carbamoyltransferase activities

In the cell the two ornithine carbamoyltransferases catalyze opposite reactions.



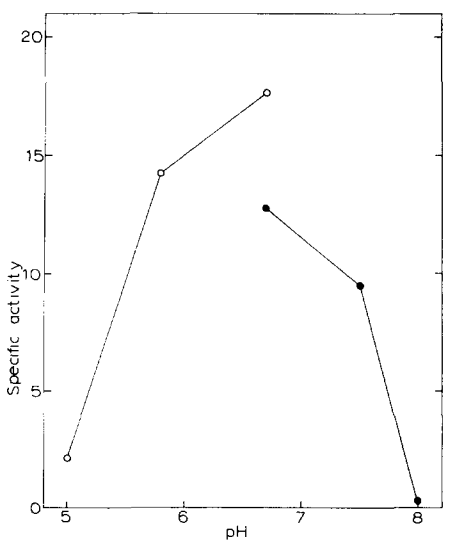


Fig. 1. Influence of pH on the citrulline arsenolysis activity of the catabolic ornithine carbamoyltransferase of Pseudomonas IRC 204. Arsenolysis is followed by the determination of NH_3 produced (see MATERIALS AND METHODS).

Fig. 2. Influence of pH on the citrulline phosphorolysis activity of the catabolic ornithine carbamoyltransferase of Pseudomonas IRC 204. The determination of the activity was performed as described in MATERIALS AND METHODS. ○—○, reaction mixture buffered with citrate; ●—●, reaction mixture buffered with citrate–Tris.

TABLE II

THE REVERSIBILITY OF THE REACTION CATALYZED BY THE ORNITHINE CARBAMOYLTRANSFERASES OF VARIOUS ORIGINS

Activity determinations were performed as described in MATERIALS AND METHODS.

Origin and type of preparation	*Method used for the backward reaction*	*Activity at the optimal pH for 1 mg protein*		*Ratio of activities (Forward) / (Backward)*
		Forward reaction	*Backward reaction (pH 6.7)*	
Purified enzyme from rat liver[19]	Arsenolysis of [^{14}C]citrulline	990 (pH 7.3)	30	33
E. coli P4XB2	Arsenolysis, NH_3 determination	5700 (pH 8.0)	106	55
Streptococcus P-60	Arsenolysis, NH_3 determination	280 (pH 8.0)	5.7	50
Pseudomonas IRC 204				
1. The catabolic enzyme (purified as described in section b)	a. arsenolysis, NH_3 determination	300 (pH 7.3)	38	7.9
	b. arsenolysis of [^{14}C]citrulline	320 (pH 7.3)	43	7.5
	c. phosphorolysis of [^{14}C]citrulline	300 (pH 7.3)	15	20
	d. phosphorolysis CO_2 manometry	320 (pH 7.3)	18	18
2. The anabolic enzyme, purified from Pseudomonas IRC 204RS5 (see section b)	Phosphorolysis of [^{14}C]citrulline	230 (pH 8.5)	0.12	1900

For this reason it was interesting to compare the kinetics in both directions of the two Pseudomonas enzymes and of other transferases of various origins.

The pH optimum of the activity of the Pseudomonas catabolic transferase in the backward direction lies around pH 6.7, whatever method, phosphorolysis or arsenolysis, is used for measuring this activity (Figs. 1 and 2). This pH optimum is broad in contrast with the sharp optimum, at pH 7.3, observed with the same enzyme in the opposite direction[8].

The forward and backward activities of various enzymic preparations are compared in Table II. The activity determinations were performed for each enzyme at its pH optimum, and the ratio of the activities in both directions was calculated for each preparation. The most striking result was obtained with the Pseudomonas anabolic enzyme which displayed practically no activity in the backward direction, in contrast to all the other known ornithine carbamoyltransferases. The inability of this enzyme to catalyze the breakdown of citrulline was checked over the whole range of pH. As shown in Fig. 3, the saturation curve of the enzyme by ornithine is strictly Michaelian, and is in all respects similar to that of the catabolic ornithine carbamoyltransferase. The K_m's for ornithine of both enzymes are of the same order ($1.5 \cdot 10^{-3}$ to $4 \cdot 10^{-3}$ M).

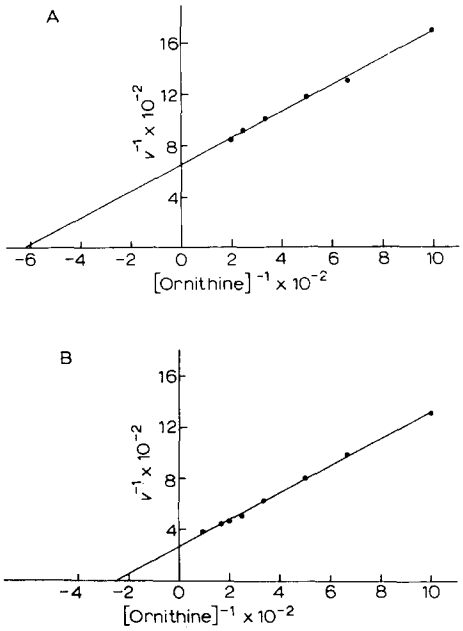


Fig. 3. Activity of the ornithine carbamoyltransferase of Pseudomonas IRC 204 as a function of ornithine concentration. The rate v is expressed as μmoles of citrulline formed per h. For both enzymes, 20 units were used for each determination. A, Lineweaver–Burk plots for the catabolic enzyme; B, Lineweaver–Burk plots for the anabolic enzyme.

(d) The physiological function of the catabolic ornithine carbamoyltransferase. The behavior of the mutant IRC 204RS1

The mutant IRC 204RS1 has no anabolic ornithine carbamoyltransferase but a normal level of the catabolic enzyme. The generation time of this mutant on minimal medium is 516 min. It is 54 min when arginine is added to the medium, equivalent to that of the parent strain on minimal medium. Arginine can be replaced by citrulline but not by ornithine. This behavior is unexpected since the catabolic enzyme is able to catalyze the synthesis of citrulline *in vitro*, and this enzyme is always present in relatively high amount in the cell.

From this we must conclude that this enzyme, *in vivo*, is in such a state that it does not function (see DISCUSSION).

DISCUSSION

The two ornithine carbamoyltransferases of Pseudomonas appear to be highly specialized. Their syntheses are subjected to the classical mode of regulation: the anabolic enzyme is repressed by arginine and the catabolic enzyme is either induced or catabolically repressed by this amino acid.

The specialization of these enzymes is still more striking if one considers their activities, since, at least *in vivo*, both enzymes catalyze the reaction in one direction only. The anabolic enzyme is unable to catalyze the phosphorolysis of citrulline, *in vitro*, in contrast with all the ornithine carbamoyltransferases known at present.

The irreversibility of both reactions, at least *in vivo*, deserves some comment. The inability of the catabolic enzyme to perform an efficient synthesis of citrulline *in vivo* could be viewed as the result of compartmentation. Such compartmentation has been suggested by the work of DAVIS[26,27] which shows that in *Neurospora crassa* there are two independent pools of carbamoylphosphate, respectively specific for arginine and pyrimidine biosyntheses. It is well established that in fungi two different enzymes catalyze the synthesis of carbamoylphosphate[3].

In the present example, the very narrow range of pH (around pH 7.3) over which the synthesis of citrulline can occur with the catabolic enzyme, in contrast to the broad range of pH (optimal 6.7) over which the catabolic direction can occur, may exclude a biosynthetic function. The two enzymes, arginine deiminase and carbamate kinase, which also operate in arginine catabolism, likewise have acidic pH optima and are regulated co-ordinatively with the catabolic ornithine carbamoyltransferase. The three enzymes could thus belong to an "acidic compartment".

Another explanation could be that metabolic effectors modify the kinetics of the enzyme with the result of a physiological irreversibility, as observed for isocitric dehydrogenase[28]. This may also apply to the irreversibility of anabolic transferase. A more complete kinetic study including the determination of the saturation curve by carbamoylphosphate, which has not been accurately achieved so far, and the search for allosteric effectors[29] of both enzymes is in progress.

Since the first report of the existence of the two ornithine carbamoyltransferases[13], another example of duplication of this enzyme has been observed in *Bacillus licheniformis*[30]. As the genus Bacillus possesses the inducible arginine catabolic pathway through arginine permease, arginase (L-arginine ureohydrolase, EC 3.5.3.1), ornithine transaminase (L-ornithine:2-oxoglutarate aminotransferase, EC 2.6.1.13) and pyrroline dehydrogenase (L-Δ^1-pyrroline-5-carboxylate:NADP oxidoreductase)[31], the necessity for a catabolic ornithine carbamoyltransferase is not obvious. The physiological significance of this inducible transferase remains to be explained.

In some instances, although there are two functions to be fulfilled, only one ornithine carbamoyltransferase has been found. This is so in *Halobacterium salinarium*[32].

Physiological evidence obtained in this laboratory shows that in a lactic acid bacterium such as *Lactobacillus fermentii* (strain 36, ATCC 9338), both the biosynthesis and the catabolism (*via* citrulline) of arginine occur. The existence of a repressible argininosuccinatelyase (L-argininosuccinate arginine-lyase, EC 4.3.2.1) in this organism suggests that the biosynthetic pathway is under repression by arginine. The catabolic sequence is inducible by external arginine, the carbamate kinase increasing by a factor of 60 and the transferase by a factor of 100. However, no evidence for the existence of two ornithine carbamoyltransferases has been obtained so far (F. RAMOS AND J. M. WIAME, unpublished observations).

ACKNOWLEDGEMENT

This work was supported by the "Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture (I.R.S.I.A.)".

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