SYNTHESIS AND INTERACTION WITH *ESCHERICHIA COLI* L-ORNITHINE CARBAMOYLTRANSFERASE OF TWO POTENTIAL TRANSITION-STATE ANALOGUES

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1. Introduction

It has been observed [1] that the N-terminal amino acid sequences of *Escherichia coli* L-ornithine carbamoyltransferase (OTCase, EC 2.1.3.3.) and of L-aspartate carbamoyltransferase (ATCase, EC 2.1.3.2.) exhibit some homology.

The respective reactions catalyzed by these two enzymes are basically the same and stable phosphonic analogues of the transition state in the reaction catalyzed by OTCase should inhibit the enzyme in a similar fashion as *N*-(phosphonoacetyl)-L-aspartate (PALA) inhibits ATCase [2]. In this paper we present the synthesis and the study of the interaction of δ-*N*-(phosphonoacetyl)-L-ornithine (PALO) and of the corresponding decarboxylated analogue, *N*-(phosphonoacetyl)-putrescine (PAPU), with *Escherichia coli* OTCase.

2. Materials and methods

2.1. *Synthesis of PALO*

A synthesis of PALO, using the cupric complex of L-ornithine has been reported [3], but not described. We used the following procedure: 30 mmol L-ornithine·HCl (Sigma) were allowed to form a cupric complex as in [4]. Phosphonoacetyl chloride (40 mmol) was prepared as in [5]. The acid chloride was added dropwise, as a 6 ml solution in dry dioxane, to the cooled (4°C) solution of the cupric complex. This operation was maintained at pH 8–9. After completion of the reaction, copper was displaced by bubbling with H_2S [4]. After discarding the copper sulfide, the solution was concentrated and desalted on a column (2.5 × 40 cm) of Dowex (Bio-Rad AG 50 W × 8 200–400 mesh H^+ form) eluted with 125 ml water. The eluate was evaporated under reduced pressure. The residue obtained was twice recrystallized from dioxane.

Yield: 0.6 g (8%) C_7 H_{15} N_2 P O_6

Calculated: C, 33.08%; H, 5.95%; N, 11.02%; P, 12.18%

Found: C, 33.31%; H, 6.10%; N, 10.72%; P, 12.04%

Spectral data: ν cm^{-1} (KBr) 1650 (amide C=O)

2.1.1. Thin layer chromatography

One spot on polyethylene imine-impregnated cellulose plates, as in [2].

2.1.2. Acid hydrolysis

(6 N-HCl, 12 h, 105°C) 1 μmol product gave 0.99 μmol L-ornithine (determined by enzymatic conversion to L-citrulline) and 1.02 μmol phosphonoacetic acid.

2.2. *Synthesis of PAPU*

Putrescine free-base (Fluka) 50 mmol in 20 ml dry dioxane were allowed to react with 10 mmol phosphonoacetyl chloride. Under these conditions, one may expect that a significant portion of putrescine is only mono-substituted. The resulting precipitate was filtered and extensively washed with dry dioxane. It

was then dissolved in 75 ml water and desalted in the same way as for PALO. The eluate of this last step was evaporated under reduced pressure to dryness, taken up in 10 ml water, adjusted to pH 8, and further fractionated on a column (2.2 × 40 cm) of Dowex (Fluka AG 1 × 8 200–400 mesh Cl^- form) equilibrated with 10 mM Tris–HCl buffer, pH 8. Elution was with a 1 litre linear gradient of 0–1 M NaCl in the equilibration buffer. The phosphorus and amino positive fractions which emerged in the gradient were again desalted on Dowex AG 50 (see above). The eluate from this last step was evaporated to dryness and recrystallized from dioxane.

Yield: 0.3 g (14%) C_6 H_{15} N_2 P O_4

Calculated: C, 34.29%; H, 7.19%; N, 13.33%; P, 14.74%

Found: C, 34.18%; H, 6.91%; N, 13.01%; P, 15.09%

Melting point: 151–153°C

Spectral data: ν cm^{-1} (KBr) 1640 (amide C=O)

2.2.1. Thin layer chromatography

One spot in the system used for PALO.

2.2.2. Amino group analysis

0.97 μmol $-NH_2$ was found in 1 μmol product by the quantitative trinitrobenzene sulfonate procedure [6].

2.2.3. Phosphorus analysis

0.99 μmol phosphorus was found in 1 μmol of the product by the method in [7].

2.3. *OTCase*

Escherichia coli strain W OTCase was purified as in [8]. Initial velocity determinations of the forward enzyme-catalyzed reaction were made by [^{14}C]citrulline estimation at pH 8 in 150 mM Tris–HCl buffer at 37°C [8].

3. Results and discussion

The inhibition of OTCase by PALO was competitive versus carbamoylphosphate (fig.1A) and non-competitive versus L-ornithine (fig.2A). These obser-

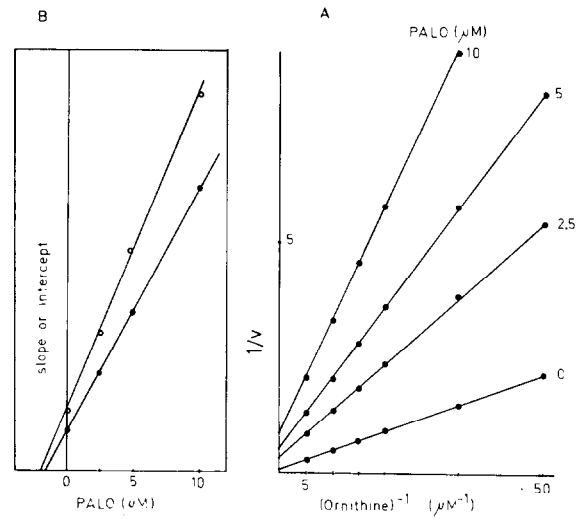

Fig.1. Inhibition of OTCase by PALO. (A) Reciprocal velocity with respect to reciprocal carbamoylphosphate concentration. Ornithine concentration is taken equal to 0.20 mM. The concentrations of PALO are shown. ν is nmol citrulline/10 min incubation. (B) Replot of the slopes with respect to PALO concentration.

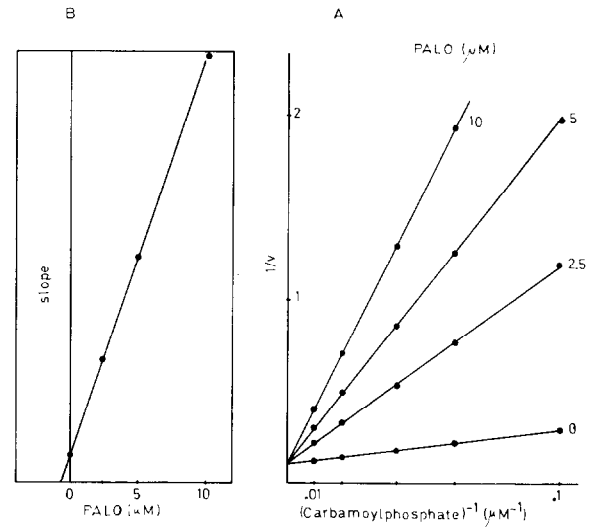

Fig.2. Inhibition of OTCase by PALO. (A) Reciprocal velocity with respect to reciprocal ornithine concentration. Carbamoylphosphate concentration is taken equal to 25 μM. The concentrations of PALO are shown. ν is nmol citrulline/10 min incubation. (B) Replot of the slopes (•) and the $1/\nu$ intercepts (○) with respect to PALO concentration.

vations are in agreement with an ordered binding of the substrates, carbamoylphosphate adding first [8]. Replot of the slopes for the inhibition versus carbamoylphosphate (fig.1B) gave an apparent dissociation constant of 0.77 μM for the inhibitor.

The replot of the slopes and the intercepts for the inhibition versus L-ornithine (fig.2B) gave respective values of 2.0 μM and 1.7 μM. The real dissociation constant (K_i) was calculated assuming the ordered mechanism and using the Cleland formulation [9]. K_i values of 0.77 μM and 0.78 μM were respectively deduced from the inhibition versus carbamoylphosphate and L-ornithine. Moreover, PALO protects OTCase against thermal denaturation at 66°C in 150 mM Tris–HCl buffer, pH 8 (fig.3). The half-life of the enzyme (7 min in the absence of PALO) increases to about 14 min in the presence of 1 μM PALO. The enzyme becomes virtually heat-insensitive in the presence of 30 μM PALO (half-life > 15 h).

OTCase inhibition by PAPU follows the same patterns as for the inhibition by PALO (not shown), but with a mean real K_i value of 0.79 mM. The PAPU molecule, which could contain some of the expected features of the transition state analogue for OTCase, seems in fact to be only a 'fundamental state' analogue if we consider the true dissociation constant of 15 μM for carbamoylphosphate, the substrate most tightly bound to OTCase [8]. In contrast, PALO (K_i 0.77 μM) binds 20-times more tightly to OTCase than does carbamoylphosphate. With ATCase, the transition state analogue PALA binds a 1000-times more tightly to the enzyme than does carbamoylphosphate [2].

Nevertheless, these values are far from those which could be expected on the basis of the transition state theory [10]. In our opinion, a completely new design for more realistic transition-state analogues must be sought.

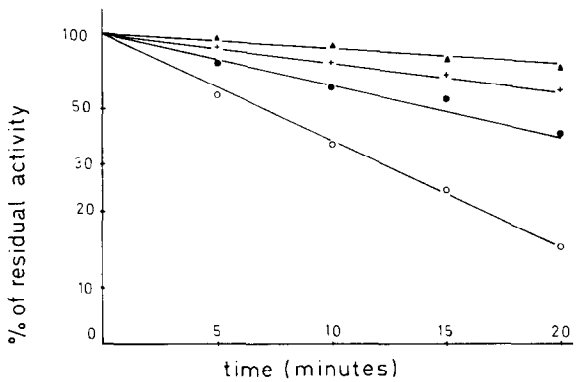

Fig.3. Effect of PALO on heat inactivation of OTCase at 66°C. The enzyme was added to a heated 150 mM Tris–HCl buffer, pH 8, containing PALO. At various times, aliquots of the solution were removed and the OTCase activity estimated. The data are plotted as the log of the residual activity versus time of heating. (○) No PALO; (●) 1 μM PALO; (+) 7 μM PALO; (▲) 10 μM PALO.

Acknowledgements

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