N^{δ}-(PHOSPHONACETYL)-L-ORNITHINE, A POTENT TRANSITION STATE ANALOGUE INHIBITOR OF ORNITHINE CARBAMOYLTRANSFERASE

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Received April 26,1977

SUMMARY

N^{δ}-(Phosphonacetyl)-L-ornithine, a transition state analogue for the reaction catalyzed by ornithine carbamoyltransferase (EC 2.1.3.3), was synthesized. It strongly inhibited bovine liver ornithine carbamoyltransferase. The inhibition was competitive with respect to carbamoyl-phosphate; the apparent K_m values for carbamoyl-phosphate were 15 μM in 0.05 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate (pH 7.2) and 33 μM in 0.1 M Tris-HCl (pH 8.5), and the inhibition constants at pH 7.2 and 8.5 were 7.1 and 4.7 nM, respectively. The inhibition was non-competitive with L-ornithine, the other substrate of the enzyme. This analogue may provide an effective reagent for the elucidation of carbamoyl-phosphate metabolism and its regulation in the liver of ureotelic animals.

During the last several years, transition state analogues for several enzymes have been synthesized and tested, and their tight binding to enzymes has been demonstrated (1-4). Collins and Stark (5) synthesized N-(phosphonacetyl)-L-aspartate, an analogue for the aspartate carbamoyltransferase (EC 2.1.3.2) reaction. It has proved to be a potent inhibitor of the enzymes from Escherichia coli (5) and hematopoietic mouse spleen (6, 7). Ornithine carbamoyltransferase, the second enzyme of the urea biosynthetic pathway, catalyzes basically the same reaction as aspartate carbamoyltransferase (8). Therefore, N^{δ}-(phosphonacetyl)-L-ornithine (PALO), which has most of the structural features of the two substrates or the two products of the ornithine carbamoyltransferase reaction in a single molecule, was expected to be a powerful inhibitor of this enzyme. This

Abbreviations: carbamoyl-P, carbamoyl-phosphate; PALO, N^{δ}-(phosphonacetyl)-L-ornithine; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

ISSN 0006-291X

compound may offer an effective reagent for studies on carbamoyl-P metabolism and its regulation in the liver of ureotelic animals where two pathways involving carbamoyl-P as an intermediate, i.e., urea and pyrimidine biosynthetic pathways, operate.

We report here the synthesis of PALO, a stable transition state analogue for the ornithine carbamoyltransferase reaction, and its inhibition of the enzyme from bovine liver.

MATERIALS AND METHODS

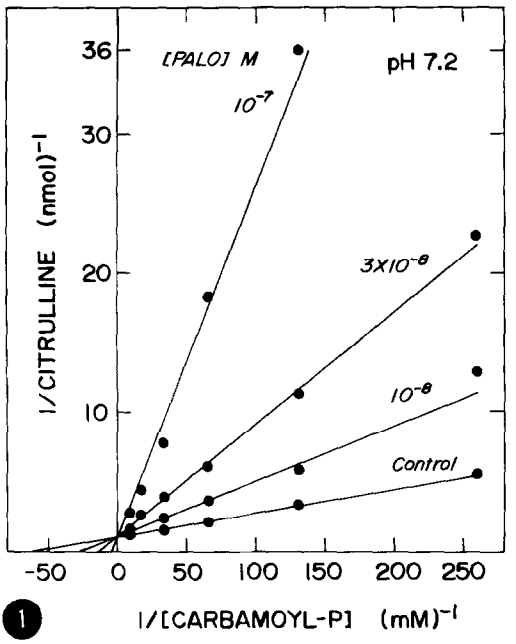
Preparations —— [^{14}C]Carbamoyl-P (1,000 cpm/nmol) was prepared from [^{14}C]cyanate (Radiochemical Centre, Amersham) by a microscale modification of the method of Spector et al. (9), and recrystallized from ethanol-H_2O (2:1, by volume) to remove radioactive impurities (10). Ornithine carbamoyltransferase was purified to near homogeneity from bovine liver according to the method of Marshall and Cohen (11).

Synthesis of PALO —— PALO was synthesized from phosphonacetic acid and the copper complex of L-ornithine essentially according to the method used by Swyryd et al. (7) for the synthesis of N-(phosphonacetyl)-L-aspartate, a transition state analogue for the aspartate carbamoyltransferase reaction. The preparation was identified by its nuclear magnetic spectrum. Details will appear elsewhere.

Assay of Ornithine Carbamoyltransferase —— The enzyme activity was measured either colorimetrically or isotopically using [^{14}C]carbamoyl-P essentially according to the method of Marshall and Cohen (8). One unit of enzyme activity is defined as that amount of activity which produces one μmol of citrulline in one min at 37° and pH 8.5. Details are indicated in the legends for Figures.

RESULTS AND DISCUSSION

Effect of Carbamoyl-P on Inhibition of Ornithine Carbamoyltransferase by PALO —— Marshall and Cohen (8) showed that bovine liver ornithine carbamoyltransferase had multiple binding sites for carbamoyl-P and that a double reciprocal plot of activity against the concentration of carbamoyl-P was concave downward. An apparent K_m for carbamoyl-P can be estimated from the linear portion of the curve at low concentrations of carbamoyl-P. In the present case a linear plot was obtained from 4 to 100 μM at pH 7.2 (Fig. 1) from which an apparent K_m for carbamoyl-P of 15 μM was calculated. The data in Fig. 1 also showed that PALO is an effective competitive inhibitor of the enzyme with respect to carbamoyl-P and the apparent inhibition constant (K_i) for PALO was calculated to



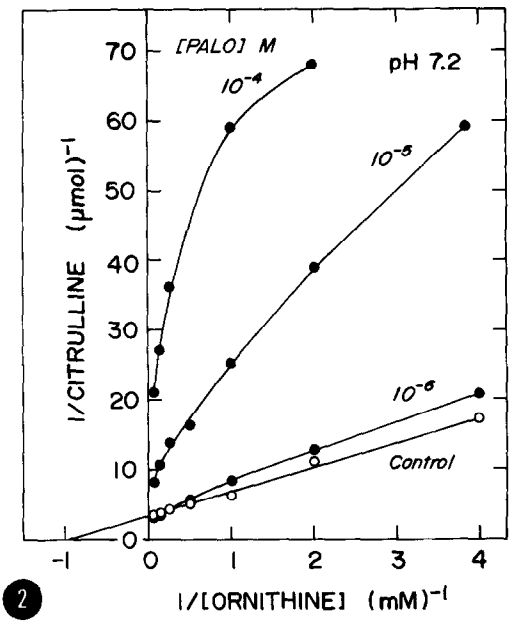


Fig. 1. Rate of citrulline synthesis as a function of carbamoyl-P concentration at varying concentrations of PALO at pH 7.2. The reaction mixture (0.2 ml) contained 0.05 M Hepes (pH 7.2), 5 mM L-ornithine, [^{14}C]carbamoyl-P and PALO as indicated, and 0.1 milliunit of enzyme and incubation was for 10 min at 37°. The maximal conversion of carbamoyl-P into citrulline was 18%.

Fig. 2. Rate of citrulline synthesis as a function of ornithine concentration at varying concentrations of PALO at pH 7.2. The reaction mixture (1.0 ml) contained 0.05 M Hepes (pH 7.2), 5 mM dilithium carbamoyl-P, ornithine and PALO as indicated, and 27 milliunits of enzyme and incubation was for 10 min at 37°. The maximal conversion of ornithine into citrulline was 23%.

be 7.1 nM. The apparent inhibition constant for PALO (K_i = 7.1 nM) is 2,100 times lower than the K_m for carbamoyl-P.

When the assay was carried out at pH 8.5 in 0.1 M Tris-HCl buffer, the inhibition by PALO was again competitive. The apparent K_m for carbamoyl-P and the apparent K_i for PALO at pH 8.5 were 33 μM and 4.7 nM, respectively.

Effect of Ornithine on Inhibition of Ornithine Carbamoyltransferase by PALO —— At pH 7.2 reciprocal plots of activity against the concentration of ornithine (K_m = 1.1 mM), which were linear in the absence of PALO, became concave in the presence of PALO (Fig. 2), and concentration of PALO lower than 1 μM actually stimulated the enzyme at higher concentrations of ornithine (about 30% at 16 mM ornithine).

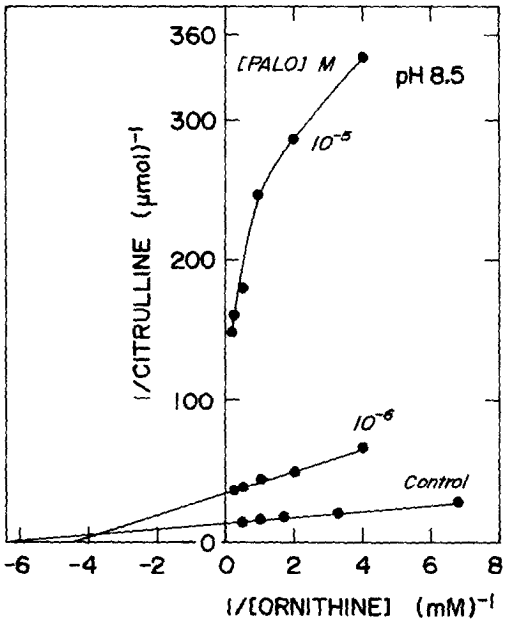


Fig. 3. Rate of citrulline synthesis as a function of ornithine concentration at varying concentrations of PALO at pH 8.5. The reaction mixture and incubation were the same as in Fig. 3 except that the mixture contained 0.1 M Tris-HCl (pH 8.5) and that the amount of enzyme was 8 milliunits. The maximal conversion of ornithine into citrulline was 24%.

At pH 8.5, where K_m for ornithine was 0.16 mM, the double reciprocal plot was linear at 1 μM PALO, and the inhibition was non-competitive (Fig. 3). However, the plot was concave at 10 μM PALO. Stimulation by PALO was not observed at any concentration of PALO and ornithine at this pH.

The inhibition of ornithine carbamoyltransferase by PALO, which is competitive with carbamoyl-P and non-competitive with ornithine, is consistent with the ordered mechanism proposed by Marshall and Cohen (8) in which carbamoyl-P binds first. Since our preliminary experiments showed that the administration of PALO to mice decreased their tolerance to ammonia, this transition state analogue probably also inhibits ornithine carbamoyltransferase in vivo. Experiments using isolated rat hepatocytes and whole animals are now in progress. The results to date with PALO indicate that this compound will be a useful reagent for studies on the regulation of carbamoyl-P metabolism in liver of ureotelic animals, particularly as related to the role of ornithine carbamoyltransferase in conditions

of hyperammonemia.

After the completion of this manuscript, we learned the report by Howell et al. (Fed. Proc. 36, 716 (1977); Abstract No. 2337) of similar findings.

ACKNOWLEDGMENTS

We are grateful to Drs. M. Marshall and P. P. Cohen, University of Wisconsin, for critically reading the manuscript. This work was supported in part by the Scientific Research Fund (D067020) from the Ministry of Education, Science and Culture, of Japan.

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