ORNITHINE TRANSCARBAMYLASE: STEADY-STATE KINETIC PROPERTIES

William E. Kurtin*, Stephen H. Bishop and Albert Himoe

Department of Biochemistry
Baylor College of Medicine
Houston, Texas 77025

Received August 23, 1971

SUMMARY

A steady-state kinetic study of Streptococcus faecalis ornithine transcarbamylase has been carried out. A ping-pong kinetic pattern was observed. Phosphate acted as a competitive inhibitor of carbamyl phosphate, δ-hydroxy-α-aminovaleric acid and norvaline were competitive inhibitors of ornithine. Citrulline did not inhibit the reaction even at a 0.1 M concentration level. The formation of a carbamyl-enzyme intermediate is suggested as a likely possibility.

The mechanism of enzymatic carbamyl transfer is largely unknown (1). As part of an effort to learn more about this reaction, we have undertaken an extensive kinetic study of ornithine transcarbamylase, isolated from Streptococcus faecalis.

Limited kinetic studies of beef liver ornithine transcarbamylase (2) and the catalytic subunit of E. coli aspartate transcarbamylase (3) have been carried out. The kinetic data of Joseph et al. (2) indicate a random substrate addition to beef liver ornithine transcarbamylase. The kinetic data of Porter et al. (3) was interpreted by the authors as indicating an ordered mechanism of substrate addition and product release.

We wish to report at this time preliminary kinetic results for the reaction catalyzed by Streptococcus faecalis ornithine transcarbamylase.

EXPERIMENTAL

Ornithine transcarbamylase was isolated from Streptococcus faecalis D_{10} essentially by the method of Bishop and Grisolia (4). It was shown to be

---

* Present address: Department of Chemistry, Trinity University, San Antonio, Texas.

homogeneous by the criterion of polyacrylamide disc gel electrophoresis. The preparation used had a specific activity of 150 μmoles citrulline produced per mg protein per minute at pH 8.0, 15° in 0.01 M Tris buffer, 0.1 M NaCl, 1.0 mM carbamyl phosphate, 0.5 mM ornithine.

Ornithine transcarbamylase was partially purified from rat liver mitochondria by the following procedure. Twice-washed rat liver mitochondria, isolated by the method of Schneider (5), were homogenized in 0.1 percent Lubrol-WX in a buffer consisting of 20 mM Tris-HCl in 20 percent glycerol, 5 mM mercaptoethanol, 5 mM KCN, pH 7.2. The homogenate was centrifuged at 15,000 x G for 20 minutes. After a second Lubrol extraction and centrifugation, the 15,000 x G supernatants were pooled, the 2.9 M through 3.1 M ammonium sulfate pellets were resuspended in the Tris-glycerol buffer. The suspension was applied to a 2 x 22 cm Sephadex G-150 column previously equilibrated with the Tris-glycerol buffer. Ornithine transcarbamylase activity was present in the second protein peak. The solution containing ornithine transcarbamylase activity was precipitated with 80 percent ammonium sulfate, centrifuged and redissolved in 0.01 M phosphate buffer, pH 7.5. This solution was diluted 1260-fold in the reaction mixtures used in the kinetic studies.

Automated assay procedures, following the appearance of citrulline (6) or phosphate, were used. Initial rates were determined by continuous sampling of the reaction mixture over a period of about 10 minutes. In a few experiments the automated citrulline assay procedure was modified to incorporate a substrate gradient (7).

RESULTS

The initial velocities were determined as a function of the concentrations of carbamyl phosphate (0.05 to 1.0 mM) and L-ornithine (0.05 to 1.0 mM). Additionally, inhibition by phosphate (0.5 to 5.0 mM) and by the ornithine analogs, DL-δ-hydroxy-α-aminovaleric acid (0.5 to 5.0 mM) and L-norvaline (0.01 to 0.2 mM) were investigated.

The results may be summarized as follows: (a) A ping-pong kinetic pattern (8) was observed under all conditions investigated. (b) Phosphate acted as a carbamyl phosphate analog in that it was a competitive inhibitor versus carbamyl phosphate and an uncompetitive inhibitor versus ornithine. (c) Norvaline and δ-hydroxy-α-aminovaleric acid were competitive inhibitors versus ornithine and uncompetitive inhibitors versus carbamyl phosphate. (d) Citrulline, 0.1 M, failed to affect the rate of the reaction. (e) There was a pH-dependence of K_m, as well as V_{max} values. The K_m (app) for ornithine increased while the K_m (app) for carbamyl phosphate decreased at lower pH values.

The principal results are summarized in Table I. The constants were obtained by computer fit by multiple regression analysis (9) to equation (1), omitting the inhibition term AIK_b/K_I.

TABLE I

Steady-State Kinetic Parameters for Ornithine Transcarbamylase

pH	temp °C	K_m (CP_i), mM	K_m (Orn), mM	V_{max}[d], μmoles product / mg enzyme, min
6.0[a]	15	0.030 ± 0.003	8.0 ± 0.8	11
7.0[b]	15	0.14 ± 0.01	1.4 ± 0.2	150
7.0[b]	37	0.11 ± 0.02	1.6 ± 0.3	385
7.5[b]	15	0.31 ± 0.03	1.7 ± 0.2	470
8.0[b]	15	0.53 ± 0.06	0.78 ± 0.09	463
8.0[b,c]	37	0.18 ± 0.01	0.74 ± 0.04	1125
8.0[b,c]	37	0.20 ± 0.01	0.82 ± 0.04	
8.4[b]	15	0.44 ± 0.09	0.45 ± 0.12	394

[a] Buffer composition: 0.01 M maleate, 0.1 M NaCl.

[b] Buffer composition: 0.01 M Tris, 0.1 M NaCl.

[c] These two lines contain the same data, the lower line also includes data for inhibition by 5-hydroxy-α-aminovaleric acid.

[d] V_{max} values were calculated from the Michaelis constants, using rate data obtained in one day with 1.0 mM ornithine, 0.4 mM carbamyl phosphate.

$$(1) \qquad V = \frac{V_{max} \cdot AB}{AB + AK_b + BK_a + \frac{AIK_b}{K_I}}$$

Where A = concentration of carbamyl phosphate

B = concentration of ornithine

I = concentration of a competitive inhibitor of ornithine

K_a = Michaelis constant for carbamyl phosphate

K_b = Michaelis constant for ornithine

Kinetic data in the presence of DL-δ-hydroxy-α-aminovaleric acid were fitted to equation (1) by multiple regression analysis in order to estimate K_I. At pH 8.0, 37°, a value of K_b/K_I of 0.53 ± 0.02 was obtained. Assuming that only the L-isomer binds, this result implies that this inhibitor binds to the enzyme just as strongly as ornithine. Similarly, at pH 8.0, 15°, K_I values of 0.05 m<u>M</u> for L-norvaline and 2 m<u>M</u> for phosphate were obtained. In an experiment carried out at pH 8.0, 15°, with 0.4 m<u>M</u> carbamyl phosphate and 1.0 m<u>M</u> ornithine, 100 m<u>M</u> citrulline failed to inhibit the reaction.

DISCUSSION

A chemically plausible pathway for the ornithine transcarbamylase reaction consistent with the steady-state kinetics is given in Scheme I.

<u>Scheme I</u>

$$\begin{array}{ccccccc} & & K_{ia} & & k_3 & & K_{ib}' & & k_5 \\ E & \rightleftarrows & E\cdot CP_i & \longrightarrow & E\text{-}Cb & \rightleftarrows & E\text{-}Cb\cdot Orn & \longrightarrow & E + \text{products} \\ & & \Updownarrow K_I & & & & \Updownarrow K'_I & & \\ & & E\cdot P_i & & & & E\text{-}Cb\cdot Norv & & \end{array}$$

Scheme I incorporates an enzyme-bound carbamyl intermediate. To be consistent with Scheme I, it is necessary to postulate that ornithine or its analogs cannot bind to the free enzyme but only to the intermediate complex represented as E-Cb.

The kinetic parameters of equation (1) in terms of Scheme I are: $V_{max} = k_3k_5/(k_3 + k_5)$, $K_a = K_{ia}k_5/(k_3 + k_5)$, $K_b = K_{ib}'k_3/(k_3 + k_5)$.

The ping-pong kinetic pattern displayed by <u>Streptococcus faecalis</u>

ornithine transcarbamylase differs from that previously reported for the beef liver enzyme (2). Because of this, a brief examination of the kinetic properties of rat liver ornithine transcarbamylase was undertaken in the expectation that it should resemble the beef liver enzyme. At pH 8.0, 15°, 0.01 M Tris buffer, 0.1 M NaCl, this enzyme also displayed a ping-pong kinetic pattern with K_m values of 0.2 mM for ornithine and carbamyl phosphate. Norvaline was also a competitive inhibitor of ornithine in the rat liver enzyme with a K_I = 0.03 mM.

Indirect evidence for a ping-pong kinetic pattern of Neurospora ornithine transcarbamylase can be inferred from an observation in the literature. Davis (10) reported that, as compared to the wild-type enzyme, a mutant ornithine transcarbamylase had a higher K_m value for ornithine (14 mM versus 1.9 mM) but a lower K_m value for carbamyl phosphate (< 0.1 mM versus 0.3 mM). Since the assay was carried out at an intermediate (6 mM) concentration of ornithine, a simple interpretation of the observation would be that the enzyme follows a ping-pong kinetic pattern, and that the mutant enzyme is defective in its ability to bind ornithine.

Aspartate transcarbamylase catalyzes a similar reaction, and it might be expected that the mechanism and kinetics of this enzyme would be similar to ornithine transcarbamylase. Although it has been suggested (3) that an ordered sequential mechanism involving a ternary aspartate-carbamyl phosphate-enzyme applies to the catalytic subunit of aspartate transcarbamylase complex, the authors failed to carry out the crucial step in determining the kinetic mechanism for the enzyme, namely the variation of concentration of both substrates. The K_m for aspartate was determined only at a fixed low concentration of carbamyl phosphate, while K_m for carbamyl phosphate was determined only at a fixed high concentration of aspartate. It is possible that aspartate transcarbamylase actually follows a ping-pong kinetic pattern. The observed carbamyl phosphate requirement for succinate binding fits well with this kinetic pattern (11).

The suggested carbamyl-enzyme intermediate cannot be proved or disproved by steady-state kinetic experiments alone. However, other types of experiments such as isotopic exchange, should answer this question.

ACKNOWLEDGMENTS

The authors wish to acknowledge the excellent technical assistance of Miss Gloria Catledge, assistance with the statistical treatment of the data by Dr. Bert Siebert of the Department of Biomathematics, and Mr. Raymond D. Potterf for the preparation of partially purified ornithine transcarbamylase from rat liver. This investigation was supported by grants Q-317 and Q-294 from The Robert A. Welch Foundation and by grant AM-12924 of the U.S. Public Health Service. One of us (W.E.K.) is a recipient of an NIH Postdoctoral Fellowship.

References

1. Bruice, T. C. and Benkovic, S. J., Bioorganic Mechanisms, Vol. II, W. A. Benjamin, Inc., p. 88 (1966).

2. Joseph, R. L., Baldwin, E. and Watts, D. C., Biochem. J. 87,409 (1963).

3. Porter, R. W., Modebe, M. O. and Stark, G. R., J. Biol. Chem. 244,1846 (1969).

4. Bishop, S. H. and Grisolia, S., Biochem. Biophys. Acta 139,344 (1967).

5. Schneider, W. C., J. Biol. Chem. 176,259 (1948).

6. Strandjord, P. E. and Clayson, K. J., J. Lab. Clin. Med. 67,154 (1966).

7. Himoe, A., Catledge, G. L. and Kurtin, W. E., submitted to Anal. Chem. (1971).

8. Cleland, W. W., Biochem. Biophys. Acta 67,104 (1963).

9. Cleland, W. W., Advan. Enzymol. 29,1 (1967).

10. Davis, R. H., Arch. Biochem. Biophys. 97,185 (1961).

11. Changeux, J.-P., Gerhart, J. C. and Schachman, H. K., Biochem. 7,531 (1968).