NOTES

Steady-State Kinetic Analysis of Phosphotransacetylase from Methanosarcina thermophila

Sarah H. Lawrence† and James G. Ferry*
Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania 16802-4500

Received 15 September 2005/Accepted 14 November 2005

Phosphotransacetylase (EC 2.3.1.8) catalyzes the reversible transfer of the acetyl group from acetyl phosphate to coenzyme A (CoA), forming acetyl-CoA and inorganic phosphate. A steady-state kinetic analysis of the phosphotransacetylase from Methanosarcina thermophila indicated that there is a ternary complex kinetic mechanism rather than a ping-pong kinetic mechanism. Additionally, inhibition patterns of products and a nonreactive substrate analog suggested that the substrates bind to the enzyme in a random order. Dynamic light scattering revealed that the enzyme is dimeric in solution.

Together with acetate kinase (equation 2), phosphotransacetylase (Pta) plays an essential role (equation 1) in the conversion of acetyl coenzyme A (acetyl-CoA) to acetate and in the synthesis of ATP in fermentative anaerobes belonging to the domain Bacteria. Acetate kinase and Pta also activate acetate to acetyl-CoA (reverse of equations 1 and 2) for conversion to methane and carbon dioxide in the energy-yielding metabolism of Methanosarcina species belonging to the domain Archaea.

\[
\text{CH}_3\text{COS-CoA} + \text{HPO}_4^{2-} \rightarrow \text{CH}_3\text{CO}_2\text{PO}_4^{3-} + \text{CoA} \quad (1)
\]

\[
\text{CH}_3\text{CO}_2\text{PO}_4^{3-} + \text{ADP} \rightarrow \text{CH}_3\text{CO}_2^- + \text{ATP} \quad (2)
\]

Pta was first purified from a fermentative organism, Clostridium kluyveri, in the 1950s (13). Early kinetic analyses of the Ptas from C. kluyveri and Veillonella alcalescens belonging to the domain Bacteria are consistent with the presence of a ternary complex kinetic mechanism (2, 6, 10). Mechanistic analyses of the enzyme were abandoned until cloning and heterologous expression of Pta from Methanosarcina thermophila, a methane-producing organism belonging to the domain Archaea, which allowed application of Pta from Methanosarcina thermophila in order to elucidate the catalytic mechanism.

The Pta from M. thermophila was heterologously expressed and purified as described previously (5), and the preparation appeared to be homogeneous, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The homogeneity and approximate hydrodynamic radius of Pta were examined by dynamic light scattering (DLS) using a DynaPro-MS800 molecular sizing instrument (Protein Solutions, Lakewood, NJ) as follows. A 40-μl aliquot of Pta (2.5 mg/ml) in 25 mM Tris-HCl (pH 7.2) containing 180 mM KCl was centrifuged (10,000 × g, 10 min), and an aliquot was loaded into a 12-μl quartz cuvette. The hydrodynamic radius, molecular weight, and size distribution were determined by the means of at least 10 DLS measurements. Data analysis was performed using Dynamics 5.0 (Protein Solutions, Lakewood, NJ). A sample DLS data set is shown in Fig. 1. Although the enzyme was initially reported to exist in solution as a monomer (7), Pta was found to have a hydrodynamic radius of 3.7 ± 0.1 nm, corresponding to a molecular mass of 71 ± 3 kDa, which is twice the calculated molecular mass of a Pta monomer. The observed molecular mass indicated that Pta exists in solution as a dimer, which is consistent with the dimeric states observed for the crystal structures of Ptas from M. thermophila and Streptococcus pyogenes (4, 14).

The rates for both the forward (acetyl-CoA-forming) and reverse (acetyl phosphate-forming) directions of the reaction catalyzed by Pta were measured at 25°C by monitoring the change in absorbance at 233 nm concomitant with formation or hydrolysis of the thioester bond of acetyl-CoA (ε = 4,360 M⁻¹ cm⁻¹), using a 0.1-cm-path-length quartz cuvette in a Hewlett-Packard 8452A diode array spectrophotometer. The standard reaction mixture (200 μl) contained 50 mM Tris-HCl (pH 7.2), 20 mM NH₄Cl, 20 mM KCl, 2 mM dithiothreitol, the appropriate substrate for the experiment, and a concentration of Pta sufficient to yield a linear rate over at least 2 min (usually 0.05 μg/ml). Reactions were initiated by addition of the second

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‡ Present address: Fox Chase Cancer Center, Philadelphia, PA 19111-2497.

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA 16802-4500. Phone: (814) 863-5721. Fax: (814) 863-6217. E-mail: jgf3@psu.edu.
substrate. All components were maintained on ice and warmed to 25°C immediately prior to initiation of the reaction.

The initial velocity patterns of the two-substrate, two-product reaction catalyzed by Pta were investigated in order to differentiate between a ternary complex kinetic mechanism and a ping-pong kinetic mechanism. For each direction of the reaction catalyzed by Pta, the initial velocity of the reaction was measured by using a matrix of five different concentrations of each substrate. Rates were measured using the standard activity assay, and each reaction was initiated by addition of the varied substrate. Data were expressed as double-reciprocal plots and analyzed to determine the nature of the inhibition. Data were fitted using Grafit 5.0 to equations describing competitive (equation 4) or non-competitive (equation 5) inhibition using two-dimensional least-squares analysis (12).

\[
1/v = K_m/V(1 + I/K_i)(1/S) + 1/V(1 + I/K_i) \quad (4)
\]

\[
1/v = K_m/V(1 + I/K_i)(1/S) + 1/V \quad (5)
\]

where \( V \) is the maximal velocity, \( A \) and \( B \) are the concentrations of the varied and fixed substrates, respectively, \( K_m \) and \( K_a \) are the Michaelis constants for substrates \( A \) and \( B \), respectively, and \( K_{d(A)} \) is the dissociation constant for the varied substrate.

For both directions, the data yielded sets of intersecting lines fitted to equation 1 (Fig. 2). For the forward direction, Michaelis constants of 186 ± 6 and 65 ± 7 \( \mu \)M were obtained for acetyl phosphate and CoA, respectively, and the \( k_{cat} \) was 5,190 ± 30 s\(^{-1}\). For the reverse direction, Michaelis constants of 96 ± 13 and 742 ± 86 \( \mu \)M were obtained for acetyl-CoA and phosphate, respectively, and the \( k_{cat} \) was 1,500 ± 30 s\(^{-1}\). The initial velocity patterns were similar to those observed for the Ptas from \( V.\ alcalescens \) and \( C.\ kluyveri \) and are consistent with a kinetic mechanism that proceeds via formation of a ternary complex between Pta and both substrates prior to any chemical step, rather than via a ping-pong mechanism.

The product inhibition patterns of the reaction catalyzed by Pta were analyzed to determine if substrate binding and product release are random or ordered. Inhibition of the forward (acetyl-CoA-forming) reaction catalyzed by Pta by the product inhibitors, acetyl-CoA and inorganic phosphate, was analyzed with respect to various concentrations of the substrates, CoA and acetyl phosphate, using the standard activity assay. All four product-substrate pairs were analyzed at saturating and subsaturating conditions using a matrix of five concentrations of substrates and inhibitors for each experiment. Data were expressed as double-reciprocal plots and analyzed to determine the nature of the inhibition. Data were fitted using Grafit 5.0 to equations describing competitive (equation 4) or non-competitive (equation 5) inhibition using two-dimensional least-squares analysis (12).

\[
1/v = K_m/V(1 + I/K_i)(1/S) + 1/V(1 + I/K_i) \quad (6)
\]

\[
1/v = K_m/V(1 + I/K_i)(1/S) + 1/V \quad (7)
\]

where \( K_m \) is the Michaelis constant for the substrate, \( S \) is the concentration of the substrate, \( I \) is the concentration of the inhibitor, and \( K_i \) is the inhibition constant for the product inhibitor.

Phosphate was a competitive inhibitor versus acetyl phosphate when CoA was at saturating (600 \( \mu \)M) or subsaturating (60 \( \mu \)M) levels (Fig. 3A and B). Phosphate was a noncompetitive inhibitor versus CoA when acetyl phosphate was at a
subsaturating level (150 μM) (Fig. 3C), but it did not inhibit versus CoA when acetyl phosphate was at a saturating level (4 mM). Acetyl-CoA was a competitive inhibitor versus CoA when acetyl phosphate was at subsaturating levels (Fig. 3D), but it did not inhibit versus CoA when acetyl phosphate was at saturating levels. Similarly, acetyl-CoA was a competitive inhibitor versus acetyl phosphate when CoA was at subsaturating levels (Fig. 3E), but it did not inhibit versus acetyl phosphate when CoA was at saturating levels. This pattern of inhibition is diagnostic for a kinetic mechanism that proceeds through formation of a ternary complex in which the substrates can bind to the enzyme in random order (1, 12).

The inhibition patterns of the nonreactive CoA analogue desulfo-CoA were analyzed with respect to CoA and acetyl phosphate, and the results further supported the random binding suggested by the product inhibition patterns. Data were expressed as double-reciprocal plots and fitted to equations 4 and 5 describing competitive and noncompetitive inhibition, respectively. Desulfo-CoA was a competitive inhibitor with respect to CoA (Fig. 4A), with a $K_i$ of $1.3 \pm 0.1 \mu M$, confirming
2.8 /H11006 Ki inhibitor with respect to acetyl phosphate (Fig. 4 B), with a previous report (3). Desulfo-CoA was a noncompetitive inhibitor at 4 mM, and the desulfo-CoA concentration was kept constant versus CoA. The acetyl phosphate concentration was kept constant at 400 /H9262 M( ), while the CoA concentration was varied. (B) Desulfo-CoA versus acetyl phosphate. The CoA concentration was kept constant at 4 /H17040 M( ), or 50 /H9262 M( ) while the acetyl phosphate concentration was varied.


