The Kinetics of Succinyl Coenzyme A Synthetase from Escherichia coli

A REACTION WITH A COVALENT ENZYME-SUBSTRATE INTERMEDIATE NOT EXHIBITING "PING-PONG" KINETICS*

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SUMMARY

Succinyl coenzyme A synthetase from Escherichia coli does not exhibit a steady state kinetic pattern indicative of "ping-pong" kinetics, despite the fact that catalytic participation of a phosphorylated enzyme covalent intermediate has been established. Instead, kinetic patterns are consistent with the sequential addition of all substrates, to form a quaternary complex, before the release of any product.

Double reciprocal plots are intersecting with ATP and succinate varied at nonsaturating concentration of the third substrate, CoA. These plots become parallel at higher CoA concentrations, suggesting that CoA can be the second substrate to add in the binding sequence. Analogous reciprocal plots with ATP and CoA varied at both nonsaturating and saturating succinate concentrations are also intersecting and parallel, respectively. The data may be reconciled with a binding sequence in which ATP binds to the enzyme first, followed by the random addition of CoA and succinate.

The finding of a sequential mechanism for the addition of substrates to this enzyme, whose catalysis is known to involve a covalent intermediate, serves to illustrate a possible danger in the use of initial rate kinetics. It is not possible to discount chemical mechanisms involving covalent intermediates by failure to observe the ping-pong kinetic pattern which may be anticipated for these catalytic routes.

During catalysis by succinyl coenzyme A synthetase (succinate:CoA ligase (ADP, EC 6.2.1.5)), a histidine residue of the enzyme becomes phosphorylated to form 3-phosphohistidine (1, 2). The intermediate participation of the phosphoenzyme in the catalysis may be formulated

\[ \text{Sum: ATP + succinate + CoA} \]

\[ \text{Mg}^{2+} \text{ADP + P_1 + succinyl-CoA} \] (3)

The obligatory participation of the phosphohistidine residue as an intermediate on the major catalytic pathway has been established by evaluation of the rate of appearance of the phosphoenzyme \((E-P)\) during the pre-steady state period (3). Thus, catalysis by this enzyme is known to involve turnover of a covalent enzyme-substrate intermediate.

In addition to the phosphoenzyme intermediate, it has been suggested (4-6) that Reaction 2 involves the participation of enzyme-bound succinyl phosphate. An alternative possibility, favored by Robinson, Benson, and Boyer (7), is that Reaction 2 may proceed in a concerted manner with formation of succinyl phosphate not an important step in the catalysis.

Despite these uncertainties in the overall chemical mechanism, there is agreement on the participation of the phosphoenzyme in the reaction. Therefore, without further consideration of the unusual properties of this enzyme, one might expect to observe a certain kinetic pattern (ping-pong kinetics (8)) generally seen for enzymes whose reactions involve covalent enzyme-substrate intermediates. This type of pattern (a series of parallel reciprocal plots when one substrate is varied at several fixed concentrations of another) arises when an irreversible step intervenes between the addition of these two substrates (8). This irreversible step could be release of a product, at zero concentration, before the addition of the second substrate in the case of reactions with covalent enzyme-substrate intermediates, such as seen with the amino acid transaminases (9). Another kind of irreversible step leading to parallel reciprocal plots could be the addition of a third substrate at saturating concentrations between the addition of the two varied substrates, as was first observed by Frieden (10) with glutamate dehydrogenase.

There may be a tendency to view ping-pong kinetics as an invariant characteristic of enzymes with covalent intermediates.
such kinetics only arise, however, when a product does dissociate before the addition of another substrate. For example, if we assume that the reaction catalyzed by succinyl-CoA synthetase involves the $E-P$ intermediate, with Reaction 1 occurring as a discrete step, the release of ADP prior to the addition of succinate or $P_i$ would lead to the ping-pong kinetic pattern when ATP was varied along with either of the other substrates. However, a complicating feature of this enzyme is the phenomenon which has been designated "substrate synergism" (3), whereby partial reactions such as the ADP-ATP exchange by Reaction 1 are markedly stimulated by the presence of the other substrates. This finding implies that in the overall catalysis of Reaction 1 may not proceed significantly as a separate step with dissociation of ADP preceding attachment of succinate or CoA, since the catalytic process is most rapid when all substrate-binding sites are filled. Because steady state kinetics can give information about the order of combination of enzyme with substrates and products, but can reveal nothing further about the sequence of covalent bonding of these reagents, a kinetic pattern indicative of sequential addition of substrates to form a quaternary complex might be expected in spite of the fact that the reaction involves the phosphohistidine intermediate. This report describes kinetic experiments which are consistent with the addition of all substrates prior to the release of a product, and serves to illustrate the possible danger in the use of steady state kinetics as a sole test for mechanism.

**Materials and Methods**

**Reagents**—Succinyl-CoA synthetase was prepared from *Escherichia coli* according to the method of Bridger, Ramaley, and Boyer (11) and was stored as a frozen solution in 0.05 M Tris-Cl pH 7.2, 10 mm MgCl$_2$, 1 mm dithiothreitol, 2.1 µM CoA$_s$, enzyme (0.003 unit), and succinate as follows: $\bigcirc\bigcirc$, 2.36 mm; $\Delta\Delta$, 0.48 mm; $\bullet\bullet$, 0.24 mm.

**Measurement of Enzyme Activity**—The reaction catalyzed by succinyl-CoA synthetase is accompanied by a change in absorbance near 230 nm resulting from thioester bond formation. In order to obtain accurate kinetic data, it was necessary to measure the rate of very small changes in absorbance at 230 nm, with substantial blanks. Accordingly, kinetic measurements were performed with a Cary model 15 recording spectrophotometer, with a 0 to 0.1 absorbance slide wire and, except where indicated, cells with a 4-cm light path and working volume of 3 ml (Thermal Syndicate, Inc., Wallsend, Northumberland, England, catalogue No. SMO/S/40). Under these conditions, even at the lowest substrate concentrations, the reaction was linear for several minutes and initial rates were readily estimable. In all experiments the temperature of the reaction was kept at 25°.

Rates are expressed as micromoles of succinyl-CoA formed per min per ml, with the value of 4.5 per cm for the increase in the millimolar extinction coefficient at 230 nm which accompanies the reaction (12). The concentration of MgCl$_2$ was 10 mm throughout. This is an optimal concentration even at the lowest substrate concentrations, and we have made the simplifying assumption that this concentration is sufficiently high that kinetic effects of Mg$^{2+}$ need not be considered.

**Analysis of Kinetic Data**—For all sets of data which appeared to give linear reciprocal plots, statistical analysis was carried out with a program$^1$ written for the Olivetti-Underwood Programma 101 which performs a weighted least squares fit according to the method of Wilkinson (13). The program also furnishes the standard errors of the apparent $K_m$ and $V_{max}$ for each set of data.

**Results and Discussion**

Fig. 1 shows the results of an experiment in which ATP is varied at several concentrations of succinate, while the concentration of CoA is held fixed at approximately 1 ½ times the $K_m$ value (see below). The results are a set of nonparallel lines, which may be drawn to a common point of intersection within the limits of the standard error of the coefficients. Fig. 2 shows replots of slope and intercept as a function of the reciprocal of the fixed succinate concentration. The fact that the slopes of the primary plots are significantly increased by lowering the succinate concentration indicates that the dissociation of ADP from the enzyme does not precede the attachment of succinate as was represented by Equations 1 and 2.

When the experiment was repeated, but with the concentration of CoA raised 5-fold, the kinetic pattern of Fig. 3 emerges.

$^1$ Copies of the program will be furnished on request.
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2 4 6 a

[SUCCINATE] -1 (m~l~

FIG. 3. Reciprocal plots showing effects of varying ATP and succinate at a fixed near-saturating concentration of CoA. The reaction mixtures contained, in a final volume of 3.15 ml, 0.1 M Tris-HCl, pH 7.2, 10 mM MgCl₂, 1 mM dithiothreitol, 10.9 μM CoA, enzyme (0.003 unit), and succinate as follows: C----C, 2.38 mM; A-A, 0.24 mM; O----O, 0.12 mM. The inset shows replots of slopes and intercepts as functions of the reciprocal of the succinate concentration.

[ATP] -1 (mM -1)

10 -2

10 -3

SLOPE

[ATP] -1 (mM -1)

10 -2

10 -3

INTERCEPT

[ATP] -1 (mM -1)

10 -2

10 -3

[COA] -1 (μM -1)

10 -2

10 -3

SLOPE

[COA] -1 (μM -1)

10 -2

10 -3

INTERCEPT

FIG. 4. Reciprocal plots showing effects of varying ATP and CoA at a fixed near-saturating concentration of succinate. The reaction mixtures contained, in a final volume of 3.09 ml, 0.1 M Tris-HCl, pH 7.2, 10 mM MgCl₂, 1 mM dithiothreitol, 0.5 mM succinate, enzyme (0.017 unit), and CoA as follows: O---C, 7.3 μM; O-O, 4.6 μM; A----A, 1.8 μM. The inset shows replots of slopes and intercepts as functions of the reciprocal of the CoA concentration.

[COA] -1 (μM -1)

10 -2

10 -3

INTERCEPT

[COA] -1 (μM -1)

10 -2

10 -3

SLOPE

FIG. 5. Reciprocal plots showing effects of varying ATP and CoA at a fixed near-saturating concentration of succinate. The reaction mixtures contained, in a final volume of 3.09 ml, 0.1 M Tris-HCl, pH 7.2, 10 mM MgCl₂, 1 mM dithiothreitol, 0.5 mM succinate, enzyme (0.017 unit), and CoA as follows: O---O, 7.3 μM; O-O, 4.6 μM; A----A, 1.8 μM. The inset shows replots of slopes and intercepts as functions of the reciprocal of the CoA concentration.

[ATP] -1 (mM -1)

10 -2

10 -3

SLOPE

[ATP] -1 (mM -1)

10 -2

10 -3

INTERCEPT

The primary plots are parallel with no significant change in slope as shown by the inset to this figure. Also shown is the linear dependence of the intercepts of the primary plots upon the reciprocal of the succinate concentration. The same experiment was repeated with a further increase in the CoA concentration to 87 μM, and again parallel plots clearly arose. In that case, the primary plots continued to move closer together, i.e. lower succinate concentrations (0.05 mM) were required in order to obtain a suitable vertical displacement. Thus, the experiments with ATP and succinate varied at high and low CoA concentrations suggest that CoA can be the second substrate to add in a sequential addition of substrate to form a ternary complex.

Further experiments, with ATP and CoA varied at fixed succinate concentrations, show that a simple ordered sequential scheme does not suffice to describe the kinetic mechanism. Fig. 4 shows the pattern obtained when ATP and CoA are varied at a concentration of succinate near \( K_m \). These lines are drawn by computer, but they can intersect at a common point within the standard errors of their coefficients. The inset to Fig. 4 shows that both slope and intercept of the primary plots are linear functions of the reciprocal of the succinate concentration. When succinate is made 5-fold more concentrated, however, a parallel pattern results as shown on Fig. 5. The inset shows the slope to be constant at all succinate concentrations. At this higher succinate level, the intercept is less strong a function of the CoA concentration, as was seen in the analogous case of Figs. 1 and 3. Thus, CoA and succinate appear to be kinetically equivalent since a high concentration of either produces parallel lines when the other two substrates are varied.

A model which we believe to be largely consistent with the above data is given by Mechanism I. Such a model predicts:

\[
\begin{align*}
E & \rightarrow (E\cdot ATP) \rightarrow (E\cdot ATP\cdot succinate\cdot CoA) \\
& \rightarrow \text{products} \\
\text{succinate} & \downarrow \\
\text{CoA} & \uparrow
\end{align*}
\]

MECHANISM I

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MECHANISM I
the observed equivalence of CoA and succinate; each can be the second substrate to add to the enzyme when present near saturating concentrations. A rate equation may be derived for Mechanism I with use of the method King and Altman (14). In the absence of products, the velocity is given by

\[ v = \frac{[E]_o (r_{1ABC} + r_{2ABCF} + r_{3ABC})}{r_1 + r_2 + r_3 + r_4 + r_5 + r_6 + r_7 + r_8 + r_9 + r_{10} + r_{11} + r_{12} + r_{13}} \]

where \( A \) is the first substrate to add, followed by random addition of \( B \) and \( C \), and \( r \) through \( r_{17} \) are collections of rate constants. This equation is of the second degree (15) in \( B \) and \( C \) and is not well suited to simplification to interpretable reciprocal equations. Equation 4 does predict, as expected, linear reciprocal plots when \( A \) is the variable substrate, and curved plots when \( B \) or \( C \) is varied unless \( C \) or \( B \), respectively, is saturating. Our failure to detect significant curvature suggests that the random loop may approximate a rapid equilibrium situation.

When either CoA or succinate is present at saturating concentrations, the kinetic mechanism would tend to follow either the top or bottom branch of the random loop in Mechanism I. Therefore the ordered ter rate equation can be applied to this system. This equation in the absence of products, as derived by Cleland (8), is as follows

\[ v = \frac{V_{1ABC}}{K_{a}K_{b}K_{c} + K_{a}K_{b}A + K_{a}K_{c} + K_{b}K_{c} + K_{a} + K_{b} + K_{c} + K_{bc}} \]

where \( A, B, \) and \( C \) are, respectively, the first, second, and third substrates to add to the enzyme. When this equation is converted to its reciprocal form with \( A \) the variable substrate, one obtains

\[ \frac{1}{v} = \frac{1}{V_1} \left( 1 + \frac{K_a}{B} + \frac{K_b}{C} + \frac{K_aK_b}{BC} \right) + \frac{K_a}{AV_1} \left( 1 + \frac{K_{ac}}{K_{b} + K_{bc}} \right) \]

As others have pointed out (8, 9) the slopes of reciprocal plots of \( 1/v \) versus \( 1/A \) of several fixed concentrations of \( C \) become constant and equal to \( K_a/V_1 \) when \( B \gg K_a \) and \( K_b \). Accordingly, we have calculated a value of 0.02 mm for \( K_{ATP} \) from the experiment in which ATP and succinate were varied at 87 µM CoA. Rearrangement of Equation 6 also shows that \( K_i \) may be calculated by tertiary plots of the \( y \) intercept of the secondary intercept replots against the reciprocal of the concentration of the second substrate to add, according to the relation

\[ \text{Intercept}_{2AB} = \frac{1}{V_1} + \frac{K_a}{BV_1} \]

An example of this is shown in Fig. 6, in which data from Figs. 2 and 3 and another similar experiment are plotted. From Fig. 6 we calculate \( K_{[ADP]} \) to be 1.5 µM. The same treatment of the intercept replots of Figs. 4 and 5 and other experiments gives a value of \( K_{[CoA]} \) to be 0.10 mm. The kinetic data reported are clearly not those expected for the simple scheme, given in Equations 1 and 2, with the release of ADP preceding binding of succinate and CoA. Further evidence against the operation of Equation 1 as a discrete step has been obtained from a study of product inhibition of ADP. In Equation 1, ATP is shown to combine with the free enzyme \( E \), whereas ADP combines with the phosphoenzyme form \( E-P \). According to the theory of Cleland (8), noncompetitive inhibition should result. However, when ADP is tested as an inhibitor with ATP the variable substrate, the data of Fig. 7 are obtained. There would appear to be little change in intercept, suggesting that ADP and ATP combine with the same enzyme form, presumably free enzyme. An unexpected finding was that the presence of remarkably low concentrations of ADP causes the reciprocal plots to become curved.

All the kinetic experiments are, therefore, in keeping with an order of substrate addition as shown in Mechanism I. While this must certainly be a major catalytic route, we do not feel that such initial rate kinetic experiments allow us to rule out the operation of alternative minor pathways. In fact, measurements of the effects of substrates on the rate of inactivation of the enzyme
by trypsin clearly show that succinate, at somewhat elevated concentrations, is able to combine with free succinyl-CoA synthetase and enhance its susceptibility to proteolytic inactivation. Furthermore, preliminary experiments studying the kinetics of isotope exchange at equilibrium are clearly incompatible with the exclusive operation of either a simple ping-pong mechanism (Equations 1 and 2), or of a sequential mechanism in which ATP and ADP are the first substrate to add and the last product to be released, respectively.

The major point which we wish to emphasize, however, is that the kinetic patterns cannot be reconciled with a simple ping-pong mechanism. Thus the reaction catalyzed by this enzyme, despite the obligatory participation of a phosphohistidine residue (3), still exhibits kinetics indicative of a sequential addition of substrates to form a quaternary complex. This illustrates a limitation of initial rate kinetics. Information may be provided about the order of substrate attachment and product release, but we can learn nothing about the chemical mechanism nor the order of covalent bond formation and breakage outside of the limitations imposed by the binding sequence. These experiments show the danger in ruling out the possibility of covalent participation of enzyme, in double displacement or similar mechanisms, by failure to observe ping-pong kinetics normally anticipated for these mechanisms.

While there are certain similarities in the kinetic patterns reported here to those of succinyl-CoA synthetase from pig heart (16), certain differences are apparent. For the latter enzyme, a sequence of substrate addition has been proposed involving the random combination of GTP and CoA with the enzyme, followed by subsequent addition of succinate. In that case also, the kinetics are inconsistent with a simple ping-pong mechanism, but the catalytic participation of the phosphoenzyme has yet to be established. A mechanism analogous to that which we suggest herein has been proposed for ATP-citrate lyase (17), an enzyme sharing many common features with succinyl-CoA synthetase, including the possible intermediate participation of a phosphohistidine residue (18).

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