Initial-velocity kinetics of succinoyl-coenzyme A–3-oxo acid coenzyme A-transferase from sheep kidney

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The initial-velocity kinetics of sheep kidney CoA-transferase are consistent with a Ping Pong mechanism. A $K_{\text{AcAc-CoA}}$ of $2.7 \times 10^{-3} \text{ M}$, $K_{\text{Succ-CoA}}$ of $1.6 \times 10^{-4} \text{ M}$, K_{Succ} of $5.6 \times 10^{-3} \text{ M}$ and K_{AcAc} of $6.7 \times 10^{-3} \text{ M}$ were determined by using a direct assay system that monitors the concentration of magnesium acetoacetyl-CoA enolate. However, product-inhibition kinetics of sheep kidney CoA-transferase are inconsistent with a Ping Pong mechanism. The possible involvement of separate binding sites for succinate and acetoacetate are discussed.

From all sources studied, CoA-transferases exhibit initial-velocity patterns consistent with a single-site Ping Pong mechanism (Hersh & Jencks, 1967; Fenselau *et al.*, 1975; Fenselau & Wallis, 1976; Sramek & Frerman, 1975, 1977; Schulman & Valentino, 1976). In the nomenclature of Cleland (1963a), the reaction for the succinyl-CoA–3-oxo acid CoA-transferase (CoA-transferase) (EC 2.8.3.5) would be written:

Sheep kidney CoA-transferase differs in its physical properties, particularly in its pI and hence amino acid composition, from other CoA-transferases (Sharp & Edwards, 1978). A kinetic study was undertaken to determine if this difference in its physical properties is also reflected in its kinetic properties. This study uses a direct assay method that allows a complete kinetic analysis to be performed. Although there is not doubt that CoA-transferase displays Ping Pong kinetics, there is evidence suggesting that the mechanism is not a simple Ping Pong, as follows. (1) The enzyme undergoes conformational changes during the reaction sequence (White *et al.*, 1976; Sramek *et al.*, 1977a,b), and these may result in non-classical

Abbreviations used: Succ, succinate; AcAc, acetoacetate; Succ-CoA, succinyl-CoA; AcAc-CoA, acetoacetyl-CoA.

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product-inhibition patterns (Fromm, 1975). (2) Its high degree of substrate-specificity suggests that CoA-transferase may have separate sites for its carboxylic acid substrates. The presence of separate binding sites may also result in non-classical product-inhibition patterns (e.g. see Northrop, 1969; Cleland, 1973).

Materials and methods

Purification of CoA-transferase

CoA-transferase was purified from sheep kidneys by the method previously described (Sharp & Edwards, 1978).

Preparation of acetoacetyl-CoA and succinyl-CoA

Acetoacetyl-CoA was prepared by the method described by Wieland & Rueff (1953), from CoA and redistilled diketene. Concentrations of acetoacetyl-CoA were determined spectrophotometrically by determining the absorbance of the magnesium acetoacetyl-CoA enolate at 303 nm. A molar absorption coefficient of $16.9 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was used (Middleton, 1973). Succinyl-CoA was prepared from succinic anhydride and CoA by the method of Simon & Shemin (1953). Concentrations of the succinyl-CoA were determined spectrophotometrically by determining the absorbance of the

thioester bond at 232 nm. A molar absorption coefficient of $4.5 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was used (Stadtman, 1957). The acetoacetyl-CoA and succinyl-CoA concentrations determined in this way were in good agreement with values obtained by reaction of the free CoA with 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959) after hydrolysis of the thioester bond at pH 12 for 30 min at 30°C. The amount of free CoA contaminating the synthesized acetoacetyl-CoA or succinyl-CoA, determined before hydrolysis by reaction with 5,5'-dithiobis-(2-nitrobenzoic acid), was typically less than 5% (a molar absorption coefficient at 412 nm of $13.6 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was used; Ellman, 1959).

Assay procedures

A Cary 118 recording spectrophotometer or a Cecil CE 272 linear-readout ultraviolet spectrophotometer, connected to a Rikadenki Kasset 1 mV potentiometric pen recorder, was used in all spectrophotometric assays. Additions of volumes ranging from 12 to 100 μl to reaction mixtures necessitated the use of micro-syringes.

The 1 ml reaction mixtures contained 100 mM-Tris/ H_2SO_4 buffer, pH 8.1, 25 mM- MgSO_4 , CoA-transferase and acetoacetate, succinyl-CoA, succinate and acetoacetyl-CoA as indicated. Reactions were initiated by the addition of acetoacetate or succinate, and the formation or utilization of acetoacetyl-CoA was monitored at 303 nm at 30°C. Under the reaction conditions used, rates remained linear for only approx. 30 s when acetoacetate was used as substrate. Reproducible rates could, however, be obtained when a full-scale deflexion on the chart recorder was set at 0.1 A. A molar absorption coefficient of $16.9 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for the magnesium acetoacetyl-CoA enolate was used to calculate initial velocities (Middleton, 1973).

CoA-transferase activities are quoted as units, where 1 unit of activity catalyses the conversion of 1 μmol of acetoacetyl-CoA into acetoacetate/min at 30°C at pH 8.1.

The equilibrium constant, K_{eq} , for the reaction was determined from reactions containing known concentrations of succinyl-CoA and acetoacetate and allowing the reaction to come to equilibrium. The concentration of the product acetoacetyl-CoA was determined spectrophotometrically. The equilibrium concentrations of both products are equal, and the equilibrium concentrations of the substrates were obtained by difference.

Analysis of kinetic data

Data for all initial-velocity studies were fitted to initial-velocity equations by using the programs of Cleland (1963b). The data are presented as computer-fitted lines with experimental points. Kinetic constants quoted were calculated in these

programs. Values for $K_{i,\text{intercept}}$ and $K_{i,\text{slope}}$ were determined by computer-fitting the experimental points to the equations for competitive or non-competitive inhibition (Cleland, 1963b).

Results

Initial-velocity studies

By using a direct assay method, that follows the appearance of magnesium acetoacetyl-CoA enolate at 303 nm, initial-velocity plots were obtained by varying acetoacetate over five concentrations (0.017–0.1 mM) with four concentrations of succinyl-CoA (0.067–1 mM) (Fig. 1) for the reaction in the direction of succinyl-CoA utilization. High substrate inhibition with acetoacetate was observed at concentrations above 25 mM. No substrate inhibition was observed with succinyl-CoA over the range studied (up to 1 mM).

The reaction in the direction of acetoacetyl-CoA utilization was followed by monitoring the disappearance of the magnesium acetoacetyl-CoA enolate. The initial-velocity plots were obtained with five acetoacetyl-CoA concentrations (2–20 μM) and five concentrations of succinate (1–7 mM) (Fig. 2). The range of acetoacetyl-CoA concentrations used was limited at the upper end of the range by substrate inhibition observed at concentrations above 100 μM . No substrate inhibition was observed with succinate at concentrations up to 10 mM.

Fig. 1. Double-reciprocal plots of initial velocity versus [acetoacetate] for the reaction catalysed by sheep kidney CoA-transferase

The Figure shows double-reciprocal plots of initial velocity with various concentrations of acetoacetate at different fixed concentrations of succinyl-CoA: $\blacktriangle$, 66 μM -succinyl-CoA; $\triangle$, 100 μM -succinyl-CoA; $\bullet$, 200 μM -succinyl-CoA; $\circ$, 800 μM -succinyl-CoA. Inset shows a secondary replot of intercept versus [succinyl-CoA].

Fig. 2. Double-reciprocal plots of initial velocity versus [acetoacetyl-CoA] for the reaction catalysed by sheep kidney CoA-transferase

The Figure shows double-reciprocal plots of initial velocity with various concentrations of acetoacetyl-CoA at different fixed concentrations of succinate: □, 1 mM-succinate; ▲, 2 mM-succinate; △, 3 mM-succinate; ●, 5 mM-succinate; ○, 7 mM-succinate. Inset shows a secondary replot of intercept versus [succinate].

The initial-velocity plots resulted in a series of parallel lines (Figs. 1 and 2). The plots of the kinetic data are computer-fitted lines with experimental points. Values of $K_{\text{Succ-CoA}}$ and K_{AcAc} of 1.6×10^{-4} M and 6.7×10^{-5} M respectively, and K_{Succ} and $K_{\text{AcAc-CoA}}$ of 5.6×10^{-3} M and 2.7×10^{-5} M respectively, were determined by using the computer programs. Secondary replots of intercept versus reciprocal of the substrate concentration were linear (insets in Figs. 1 and 2). An equilibrium constant (K_{eq}) of 3.2×10^{-3} was determined for the reaction.

Product inhibition

Product-inhibition studies showed that the inhibition by acetoacetyl-CoA and by succinate with respect to succinyl-CoA at fixed acetoacetate concentration (100 μM) is competitive and non-competitive respectively (Figs. 3 and 4). The secondary replots of slope versus acetoacetyl-CoA concentration and of intercept and slope versus succinate concentration are linear. At fixed succinyl-CoA concentration (100 μM) the inhibition by acetoacetyl-CoA with respect to acetoacetate was non-competitive (results not shown) and the inhibition by succinate with respect to acetoacetate was competitive (results not shown). Second-

Fig. 3. Product inhibition of sheep kidney CoA-transferase by acetoacetyl-CoA with succinyl-CoA. The Figure shows double-reciprocal plots of initial velocity with various concentrations of succinyl-CoA, at fixed acetoacetate concentration (100 μM) and at different fixed concentrations of acetoacetyl-CoA: ○, 0 μM-acetoacetyl-CoA; ●, 2 μM-acetoacetyl-CoA; △, 8 μM-acetoacetyl-CoA; ▲, 13 μM-acetoacetyl-CoA; □, 20 μM-acetoacetyl-CoA. Inset shows a secondary replot of slope versus [acetoacetyl-CoA].

Fig. 4. Product inhibition of sheep kidney CoA-transferase by succinate with succinyl-CoA. The Figure shows double-reciprocal plots of initial velocity with various concentrations of succinyl-CoA at fixed acetoacetate concentration (100 μM) and at different fixed concentrations of succinate: ○, 0 μM-succinate; ●, 20 μM-succinate; △, 60 μM-succinate; ▲, 80 μM-succinate. Inset shows secondary replots of slope (●) and intercept (○) versus [succinate].

Table 1. *Summary of product-inhibition data for sheep kidney CoA-transferase*

Fixed substrate	Variable substrate	Product	Inhibition pattern*	Dixon plot type	Intercept replot		Slope replot	
					K_{ii} (M)†	Type	K_{is} (M)†	Type
AcAc (100 μ M)	Succ-CoA	AcAc-CoA	Comp.	Linear	—	—	1.7×10^{-6}	Linear
		Succ	Non-comp.	Linear	1.2×10^{-4}	Linear	1.8×10^{-4}	Linear
Succ-CoA (100 μ M)	AcAc	AcAc-CoA	Non-comp.	Linear	2.3×10^{-5}	Linear	3.6×10^{-6}	Linear
		Succ	Comp.	Linear	—	—	1.2×10^{-5}	Linear
AcAc-CoA (20 μ M)	Succ	Succ-CoA	Non-comp.	Hyperbolic	2.6×10^{-3}	Hyperbolic	1.2×10^{-3}	Hyperbolic
		AcAc	Non-comp.	Linear		Linear		Linear
Succ (7 mM)	AcAc-CoA	AcAc	Comp.	Hyperbolic		Hyperbolic		Hyperbolic
		Succ-CoA	Non-comp.	Hyperbolic		Hyperbolic		Hyperbolic

* Comp., competitive; Non-comp., non-competitive.

† Values were not determined from hyperbolic plots.

any replots of slope and intercepts are linear. Kinetic data for the forward reaction are summarized in Table 1.

All the kinetic data obtained, initial-velocity plots and product inhibition patterns for the reaction:

are consistent with a single-site Ping Pong mechanism.

However, for the back-reaction:

the product-inhibition patterns obtained were not the expected patterns. Inhibition by succinyl-CoA with respect to acetoacetyl-CoA at fixed succinate concentration (7 mM) is non-competitive rather than the expected competitive pattern (Fig. 5). Secondary replots of slope and intercept and Dixon plots (results not shown), are curved downward, indicating hyperbolic inhibition (Cleland, 1970). The inhibition by acetoacetate with respect to acetoacetyl-CoA at fixed succinate concentration (7 mM) is competitive rather than non-competitive (Fig. 6). Replots of slope and Dixon plots (results not shown) are hyperbolic. Inhibition by succinyl-CoA with respect to succinate at fixed acetoacetyl-CoA concentration (20 μ M) is non-competitive (results not shown), and is the only inhibition pattern that is in agreement with the expected patterns for a classical Ping Pong mechanism for the back-reaction. However, the replots of slope and intercept and the Dixon plot (results not shown) are hyperbolic. At fixed acetoacetyl-CoA concentrations, the inhibition by acetoacetate with respect to succinate is non-competitive rather than competitive (results not shown). However, replots of slope and intercept and Dixon plots (results not shown) are linear. Product-inhibition data are summarized in Table 1.

Fig. 5. *Product inhibition of sheep kidney CoA-transferase by succinyl-CoA with acetoacetyl-CoA*. The Figure shows double-reciprocal plots of initial velocity with various concentrations of acetoacetyl-CoA at fixed succinate concentration (7 mM) and at different fixed concentrations of succinyl-CoA: O, 0 μ M-succinyl-CoA; ●, 10 μ M-succinyl-CoA; △, 20 μ M-succinyl-CoA; ▲, 40 μ M-succinyl-CoA; □, 80 μ M-succinyl-CoA. Inset shows secondary replots of slope (●) and intercept (O) versus [succinyl-CoA].

The inhibition patterns obtained for the back-reaction were repeated several times, with both purified and partially purified preparations (tissue

Fig. 6. Product inhibition of sheep kidney CoA-transferase by acetoacetate with acetoacetyl-CoA. The Figure shows double-reciprocal plots of initial velocity with various concentrations of acetoacetyl-CoA at fixed succinate concentration (7 mM) and at different fixed concentrations of acetoacetate: ○, 0 mM-acetoacetate; ●, 0.33 mM-acetoacetate; △, 0.67 mM-acetoacetate; ▲, 1.33 mM-acetoacetate; □, 2.67 mM-acetoacetate. Inset shows a secondary replot of slope versus [acetoacetate].

homogenized, centrifuged and dialysed into 10 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-EDTA) of CoA-transferase, and the described patterns were consistently observed.

A possible reason for the unusual product-inhibition patterns obtained for the back-reaction could be that the CoA-transferase preparation catalysed a thioesterase as well as a transferase reaction in the presence of succinate. To investigate this possibility, reaction mixtures were monitored for the appearance of free CoA. The method employed was quantitative spectrophotometric determination of substrate (acetoacetyl-CoA) and product (succinyl-CoA) concentrations during the course of a reaction. The procedure used was to scan standard assay mixtures in the range 310–200 nm. The concentrations of acetoacetyl-CoA were determined from the absorption maximum at 303 nm. The concentrations of succinyl-CoA were determined by the absorbance (at 232 nm) of the thioester bond. This procedure detected no significant change in the total concentration of acyl-CoA (i.e. acetoacetyl-

CoA + succinyl-CoA) during the course of the reaction.

Discussion

All CoA-transferases that have been studied display Ping Pong kinetics. Sheep kidney CoA-transferase also exhibits parallel initial-velocity plots with either acetoacetyl-CoA and succinate or succinyl-CoA and acetoacetate as substrates. Incomplete product-inhibition studies have been reported for pig heart CoA-transferase (Hersh & Jencks, 1967) and the *Escherichia coli* acetyl-CoA-acetoacetyl-CoA-transferase (Sramek & Frerman, 1977). These investigations indicate that these CoA-transferase catalyse simple, single-site, Ping Pong reactions.

For sheep kidney CoA-transferase, the product-inhibition patterns for the reaction:

are as predicted for a single-site Ping Pong mechanism. However, for the reaction:

the product-inhibition patterns are unusual. Only the pattern given for the inhibition by succinyl-CoA, with respect to succinate, is that expected for a Ping Pong mechanism. This is in contrast with the report by Hersh & Jencks (1967), who, using CoA-transferase from pig heart, obtained the expected competitive inhibition by acetoacetate with succinate and non-competitive inhibition by acetoacetate with acetoacetyl-CoA. Evidence to support the view that the sheep kidney CoA-transferase has a Ping Pong mechanism is that the covalent enzyme-CoA intermediate has been isolated for the sheep kidney CoA-transferase by the method described by Solomon & Jencks (1969). Furthermore, substitution of experimentally determined kinetic constants, into the Haldane relationship for a Ping Pong mechanism (Fromm, 1975):

$$K_{eq} = \frac{V_1^2 \cdot K_p \cdot K_q}{V_2^2 \cdot K_a \cdot K_b}$$

(by using values of $V_1 = 8 \times 10^{-7}$ units/min per mg and $V_2 = 5.3 \times 10^{-5}$ units/min per mg, where K_a , K_b , K_p and K_q are the Michaelis constants for succinyl-CoA, acetoacetate, succinate and acetoacetyl-CoA respectively) gives a calculated value for K_{eq} of 3.6×10^{-3} . This value is in good agreement with the experimentally determined value of 3.2×10^{-3} , and further supports the view that the kinetics are described by a Ping Pong mechanism.

For the reaction in the back direction with sheep

kidney CoA-transferase all the product-inhibition patterns, except for the inhibition by acetoacetate with succinate, gave downward-curving Dixon plots and secondary replots of slope and intercept. Cleland (1970) described this type of inhibition as hyperbolic inhibition, and stated that it is indicative of the reaction flux being diverted through an alternative pathway that is slower than the normal pathway. One possible alternative pathway may be that the magnesium acetoacetyl-CoA enolate used to follow the reactions is recognized by CoA-transferase as a substrate analogue of acetoacetyl-CoA. Whereas in the normal reaction pathway the Mg^{2+} would be dissociated from the magnesium acetoacetyl-CoA enolate, the alternative pathway would involve the magnesium acetoacetyl-CoA enolate, which may result in a pathway that is slower than normal. Although this could explain why only the reaction where acetoacetyl-CoA is used as a substrate results in hyperbolic inhibition plots, a similar method was used by Hersh & Jencks (1967) to determine the kinetics of the pig heart CoA-transferase, and only the expected inhibition patterns were observed.

A second possible alternative mechanism could be a result of the substrate inhibition by acetoacetyl-CoA. If the E·CoA-acetoacetyl-CoA form of the enzyme was not a dead-end product it might be capable of reacting with succinate to produce succinyl-CoA, but at a much lower rate than the normal pathway. However, this would require that CoA-transferase has separate binding sites for each of its substrate types (acetoacetate and succinate). The high degree of substrate-specificity that CoA-transferase exhibits for its substrate types suggests that CoA-transferase may have separate sites for its carboxylic acid substrates. Also, substrate inhibition by acetoacetyl-CoA may also be expected to result in a competitive inhibition pattern between acetoacetyl-CoA and acetoacetate.

A possible explanation of the unusual kinetic and hyperbolic patterns obtained for sheep kidney CoA-transferase may be that the patterns obtained are artifacts due to limited proteolysis of the CoA-transferase. During the purification of the CoA-transferase, proteolysis of the enzyme has been shown to occur (Sharp & Edwards, 1978). Even though CoA-transferase preparations had high specific activities (180 units/mg) it is possible that some degraded protein was present. With some enzymes, e.g. fructose biphosphatase, cleavage of the protein results in an alteration in some of the kinetic properties of the enzyme (Zalitis, 1969). However, this appears to be unlikely for CoA-transferase, since partially purified protein preparations, which would be expected to have little or no proteolysis, gave the same results as the purified preparations.

A non-classical Ping Pong mechanism has been reported for the Ping Pong Bi Bi mechanism of methylmalonyl-CoA carboxyltransferase (Northrop, 1969). The product-inhibition patterns obtained result from the carboxyltransferase having separate, non-interacting, binding sites for each of its substrate types. These multiple binding sites lead to a reversal of the expected inhibition patterns (i.e. expected 'competitive' inhibition patterns are non-competitive and 'non-competitive' patterns are competitive). The occurrence of multiple binding sites has also been reported for the Ping Pong Bi Bi Uni Uni mechanism of pyruvate dehydrogenase (Cleland, 1973).

A Ping Pong mechanism also results in non-classical inhibition patterns when an enzyme undergoes an isomerization during catalysis. For a mono-Iso Ping Pong Bi Bi mechanism all inhibition patterns, except that between product P (the first product to be released from the enzyme) and substrate B (the second substrate to bind to the enzyme), are non-competitive (Fromm, 1975). For a di-Iso Ping Pong Bi Bi mechanism all inhibition patterns are non-competitive (Fromm, 1975). It is very unusual for the inhibition pattern between substrate A (the first substrate to bind to the enzyme) and product P to be competitive for a Ping Pong mechanism. The only other Ping Pong mechanism where substrate A and product P are competitive is that reported for methylmalonyl-CoA carboxyltransferase (Northrop, 1969). This could indicate that the inhibition patterns obtained for sheep kidney CoA-transferase result from independent binding sites for each of the carboxylic acid substrates. The remaining patterns, all non-competitive, might result if the enzyme underwent an isomerization during catalysis. However, the occurrence of multiple binding sites would be expected to affect the inhibition patterns in the forward direction as well as the back direction. The most probable reason for the competitive inhibition between acetoacetate and acetoacetyl-CoA, as already discussed, is that it results from the substrate inhibition by acetoacetyl-CoA.

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