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Substrate Specificity and Mechanism of Action of Acetoacetate Coenzyme A Transferase from Rat Heart†

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ABSTRACT: The specificity of succinyl-CoA:acetoacetate CoA transferase (as a partially purified preparation from rat heart) was examined by employing various analogs of succinate. Diacids with a connecting chain length of ≥3 methylene groups are inactive; oxalate and malonate are competitive inhibitors (K_i = 15 and 21 mM, respectively). Analogs with substitution into the ethylene group of succinate are generally inactive, except for inhibition by 2,2-difluorosuccinate and perfluorosuccinate (K_i = 6.4 and 18 mM, respectively). 3-Sulfopropanoate and compounds with substitution into one of the carboxyl groups (monomethyl succinate, succinamate, maleamate, and *N*-ethylmaleamate) also inhibit competitively (with K_i values from 11 to 33 mM), but do not serve as substrates. Only maleate proved to be a substrate (K_m = 35 mM *vs.* a K_m value for succinate of 28 mM) with a V_{max} one-ninth of that for succinate. Fumarate is ineffective; acetylenedicarboxylate weakly inhibits. The mechanistic implication of these observations is that a cis, coplanar relationship between two proximal carboxyl groups is essential in a substrate molecule in order to have nucleophilic attack by the reactive carboxyl group on the enzyme thiol ester intermediate (with coenzyme A). Finally, usage of acetoacetate is inhibited competitively by succinate and maleate; the other inhibitors to succinate display mixed-type inhibitions for acetoacetate, indicating their potential usefulness for studies on the metabolism of ketone bodies.

For the complete utilization of ketone bodies in mammalian tissues acetoacetate must be converted to the activated coenzyme A derivative. The enzyme succinyl-CoA:acetoacetate CoA transferase performs this function in nonhepatic tissues by means of catalyzing the reaction between acetoacetate and succinyl-CoA (Jencks, 1973). Jencks and his colleagues have unequivocally shown, using kinetic (Hersh and Jencks, 1967) and chemical (Solomon and Jencks, 1969) methods, that the reaction catalyzed by the purfied pig heart enzyme proceeds *via* an enzyme-CoA intermediate. Stern and coworkers have demonstrated the high degree of specificity of the pig heart enzyme with respect to the substrate succinate, finding that only malonate was able to serve as a substrate (Stern *et al.*, 1956; Menon and Stern, 1960). Starting with these observations we have extended the specificity studies using a greater number of succinate analogs with the CoA transferase isolated from rat heart mitochondria. The objectives of these studies have been to gain some added insight into the mechanistic details of the

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enzyme-catalyzed reaction and to obtain selective inhibitors of the transferase that can be employed in subsequent physiological studies examining ketone body comsumption and its regulation.

Experimental Section

Animals and Chemicals. Male adult Buffalo rats weighing 200–250 g were obtained from Simonsen Laboratories and fed *ad libitum*. Coenzyme A was purchased from P-L Biochemicals as the free acid and was converted to acetoacetyl-CoA from diketene (Lynen *et al.*, 1958) and to succinyl-CoA from succinic anhydride (Simon and Shemin, 1953). Lithium acetoacetate was obtained using the procedure of Hall (1962). 3-Sulfopropanoic acid was prepared by oxidation of 3-mercaptopropanoic acid with hydrogen peroxide (Hardell and Theander, 1971); maleamic acid was synthesized from maleic acid using a published procedure (Sauers and Cotter, 1961). The remaining succinate analogs were purchased from Aldrich Chemical Co., except for the fluorine-containing compounds (PCR, Inc.). Their identity and purity were determined by thin-layer chromatographic methods and melting point and infrared spectral data. In a few cases further purification was achieved by recrystallization or vacuum sublimation. All other chemicals and enzymes were purchased from Sigma Chemical Corp.

Isolation of Rat Heart CoA Transferase: The heart from a rat sacrificed by cervical fracture was removed quickly and placed in a chilled (4°) solution (pH 7.4) containing 10 mM Tris-HCl, 70 mM sucrose, 0.21 M mannitol, and 0.1 mM EDTA (Tris-sucrose solution). After cleaning and weighing the heart tissue, it was minced with scissors in the chilled Tris-sucrose solution (final volume 20 ml/g of tissue). This mince was incubated with Nagarse (Enzyme Development Corp.) using 4 mg/g of heart and homogenized according to the procedure of Pande and Blanchaer (1971). The homogenate was centrifuged at 10,000*g* for 15 min (4°); the supernatant, containing 15–20% of the total CoA transferase activity, was discarded. The pellet was suspended in 0.02 M potassium phosphate buffer at pH 7.0 (2 ml/original g of heart) and sonicated at 4° and 100 W for a total of 2 min (in 15-sec exposures followed by cooling for 15 sec). The supernatant after centrifugation at 47,000*g* for 30 min (4°) was placed upon a Bio-Rad P-150 or a Sephadex G-100 column (1.5 × 40 cm) and the transferase activity was eluted with phosphate buffer (0.02 M, pH 7.0). The pooled fractions, containing 80–90% of the activity placed upon the column, provided the enzyme preparations used for the following studies.

The specific activities at the different stages of purification (in a typical purification, 0.8 μmol of acetoacetate formed per min per mg of protein for the sonicated total homogenate, 1.41 for the particulate sonicate, and 3.23 for the Bio-Rad P-150 chromatographed material) indicate a fourfold purification can be achieved by these few steps carried out during 1 day. The phosphate solutions of enzyme were frozen and used over a period of 1–2 weeks, during which time less than 10% of the original activity was lost. No attempts were made to purify further this material. Isoelectric focusing experiments revealed the presence of only one active component with a p*I* value of 6.8. Polyacrylamide gel electrophoresis indicated the presence of several protein components in the chromatographed material. Only trace levels of acetoacetyl-CoA deacylase and thiolase activities could be normally detected; malate dehydrogenase and β-hydroxybutyrate dehydrogenase were not present in these preparations. Citrate synthase, a mitochondrial enzyme like CoA transferase (A. Fenselau and K. Wallis, submitted for publication) with a slightly lower molecular weight than the transferase, contaminated all these preparations.

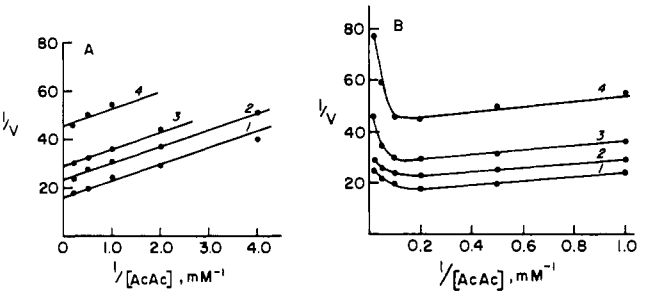

FIGURE 1: Initial velocity patterns for the forward reaction catalyzed by rat heart CoA transferase. Assay conditions are described in detail in the Experimental Section. For both A (parallel line plots) and B (substrate inhibition) the assays were carried out using 38 μg of a rat heart CoA transferase preparation (specific activity 1.58) and succinyl-CoA concentrations of (1) 0.40 mM; (2) 0.20 mM; (3) 0.15 mM; and (4) 0.080 mM.

Kinetic Studies. Spectrophotometric measurements at 25° were made involving the Mg^{2+}–acetoacetyl-CoA complex with a maximum of 310 nm and a millimolar extinction coefficient of 11.9 (Hersh and Jencks, 1967). For the determination of the rate of succinyl-CoA formation the following conditions were used (in order of addition): 0.067 M Tris-HCl (pH 8.1), 5 mM $MgCl_2$, 2 mM iodoacetamide, enzyme-containing solution, 0.16 mM acetoacetyl-CoA, and 50 mM succinate (to give a final volume of 1.0 ml). Assays in the reverse direction used identical conditions except for the substitution of 0.22 mM succinyl-CoA and 2.0 mM acetoacetate (last addition) for acetoacetyl-CoA and succinate. The enzyme was incubated 2 min before the addition of substrates with iodoacetamide, which selectively inactivates thiolase (Stewart and Rudney, 1966). All measurements represent initial velocities and were made in duplicate at 25° and 310 nm using a Gilford recording spectrophotometer (Model 222). The reaction in the direction producing acetoacetate was generally linear after the first 15 sec; in the reverse direction a linear plot pertained from 15 to 90 sec and was used to calculate enzyme activity. Corrections in the direction producing acetoacetate were made for losses of acetoacetyl-CoA due to spontaneous hydrolysis of the substrate and "acetoacetyl-CoA deacylase" activity.

The various kinetic parameters (K_m, V_{max}, and K_i values) were determined from double reciprocal plots of substrate data or Dixon plots of inhibitor data. The velocity, *v*, is expressed in terms of micromoles of product per min per mg of protein (I.U./mg).

Results

CoA transferase from a variety of sources [pig heart (Hersh and Jencks, 1967), rat heart (Tildon and Sevdalian, 1972), and cow skeletal muscle (Blair, 1969)] displays a parallel initial velocity pattern for each half-reaction involving two substrates, like that shown in Figure 1A for our rat heart preparation using acetoacetate and succinyl-CoA as substrates. The existence of an enzyme–CoA intermediate implied by these kinetic results has been confirmed by chemical modification studies on the pig heart enzyme (Solomon and Jencks, 1969). In addition, kinetic studies on substrate inhibition can provide further support for the "ping-pong" mechanism suggested by parallel line plots of the initial velocity data (Cleland, 1970). In Figure 1B is shown the complex, but characteristic pattern of substrate inhibition for acetoacetate, which reinforces the assignment of a ping-pong mechanism in the functioning of the rat heart transferase. A similar reciprocal plot was observed for acetoacetyl-CoA (using concentrations up to 0.45 mM), but neither

TABLE I: Comparison of Kinetic Parameters for Acetoacetate CoA Transferase from Rat and Pig Heart.

Kinetic Parameter	CoA Transferase	
	Rat[a] Heart	Pig[b] Heart
K_m, mM		
Acetoacetate	0.44 ± 0.09	0.20
Succinate	34 ± 8	36
Acetoacetyl-CoA	0.040	0.72
Succinyl-CoA	0.28	4.2
V_{max}[c]		
Forward	0.12 ± 0.02	28.3
Reverse	2.95 ± 0.70	716
K_i (product inhibition), mM		
Succinate	0.72	1.0
Acetoacetate	3.7	0.78

[a] Present study based on three different preparations (± S.E.M.). The assay medium contains 5 mM $MgCl_2$. [b] Hersh and Jencks (1967). These constants were determined in an assay medium lacking Mg^{2+}. [c] Activity is expressed in terms of μmoles of acetoacetate used (forward reaction) or formed (reverse reaction) per min per mg of protein. The impurity of the rat heart preparation accounts in part for the differences noted in this comparison with the purified pig heart enzyme.

TABLE II: Effects of Succinimide, Maleimide, and *N*-Ethylmaleimide on the Activity of Rat Heart CoA Transferase.[a]

	Relative Activity		
	No Added Substrates	Succinate, 50 mM	Acetoacetyl-CoA, 0.15 mM
Control	1.00	0.95	0.92
Succinimide	0.99	0.95	0.95
Maleimide	0.22	0.46	0.08
N-Ethylmaleimide	0.15	0.32	0.0

[a] Rat heart CoA transferase (specific activity 2.88, 0.63 mg/ml) in a solution of 0.07 M Tris-HCl (pH 8.1) containing 5 mM $MgCl_2$ and 2 mM iodoacetamide (with or without the substrates) was allowed to incubate for 5 min at 25° with succinimide, maleimide, or *N*-ethylmaleimide (all at 1 mM). The activity was measured before and after this incubation using assay conditions described in Materials and Methods; the relative activity is the ratio of these two measurements.

succinate nor succinyl-CoA produced such complications (presumably due to our experimental inability to employ concentrations of these substrates that are greater than two or three times their respective K_m values). Additional similarities in kinetic parameters for the pig heart and rat heart CoA transferases are assembled in Table I. These results, along with the observation on both enzymes that acetoacetate and succinate show a pattern of competitive product inhibition with respect to one another, indicate that these two enzymes are functionally quite similar.

Since pig heart CoA transferase shows considerable specificity for succinate as a substrate (Stern *et al.*, 1956), we decided to carry out a survey of succinate analogs as possible substrates or inhibitors of the rat heart enzyme. The specificity of this enzyme preparation was examined using succinate analogs in which the following parameters were varied: (1) distance separating the two carboxylic acid groups (type I), (2) substituents on the ethylene bridge between the carboxyl groups (type II), (3) substituents on one of the carboxyl groups (type III), and (4) orientation of the two carboxyl groups (type IV). The analogs were screened initially at a concentration of 50 mM for their ability to serve as substrates or to inhibit the reaction involving acetoacetyl-CoA (0.16 mM) and succinate (20 mM). Substances displaying some effect were then studied in greater detail in order to ascertain the specific nature of the effect. Under these conditions an inhibitor with a maximum value for K_i of 30–35 mM might be detected.

As regards type I analogs only two competitive inhibitors, oxalate (K_i = 15 mM) and malonate (K_i = 21 mM), were found; glutarate and adipate have no effect on transferase activity. Malonate has been reported by Menon and Stern (1960) to be a substrate for the purified pig heart CoA transferase; however, the high levels of enzyme (16 time greater than with succinate as substrate) required to detect this low activity for the pig heart enzyme (2% of the level of activity using succinate) could not be employed in the present study.

Type II analogs are generally inactive; no effects were noted with *cis*- and *trans*-cyclobutane-1,2-dicarboxylate, *cis*- and *trans*-cyclohexane-1,2-dicarboxylate, methylsuccinate, mercaptosuccinate, malate, and aspartate. This observation is of some significance for the metabolic intermediates, malate and aspartate, since they would appear to be without physiological effect in controlling transferase activity. Replacement of hydrogen atoms in the ethylene bridge with fluorine atoms, as in 2,2-difluorosuccinate and perfluorosuccinate, did provide strong competitive inhibitors to succinate utilization with K_i values of 6.4 and 18 mM, respectively.

Modification of one carboxyl group (Type III analogs) produces a class of competitive inhibitors with inhibition constants in the 10–30 mM range, but none of these substances is able to serve as a substrate. This group includes (with K_i value in parentheses) monomethyl succinate (21 mM), succinamate (27 mM), maleamate (33 mM), *N*-ethylmaleamate (11 mM), and 3-sulfopropanoate (22 mM). The inhibitions by maleamate and *N*-ethylmaleamate were independent of time or order of mixing. However, even though the inhibitions of these reagents appear to be reversible under the conditions employed in this study, it was felt that the effects of two structurally similar, well-known protein modification reagents, maleimide and *N*-ethylmaleimide, deserved investigation. The results of these studies (Table II) indicate that these two reagents at low concentrations (1 mM) inactivate the enzyme, whereas succinimide (a cyclic imide lacking the ring double bond) is without effect. These inactivations were time-dependent and could not be reversed. Protection against the action of the maleimides was afforded by succinate and acetoacetate, but the rates of inactivation appear to be increased in the presence of acetoacetyl-CoA and succinyl-CoA. Recently Jencks and coworkers (White *et al.*, 1973) have reported a similar result with pig heart CoA transferase, implicating a sulfhydryl group in the display of enzyme activity. The reactivity of this group is dependent not only on the reacting enzyme species (enzyme-CoA intermediate is preferentially inactivated) but also on the nature of the reagent (maleimides inactivate, but the iodoacetam-

TABLE III: Comparison of Kinetic Constants for Succinate and Maleate as Substrates for Rat Heart CoA Transferase.[a]

Substrate	K_m, mM	V_{max}[b]	K_i, mM
Succinate	28	3.77	0.42
Maleate	35	0.41	4.0

[a] The values for K_m, V_{max}, and product inhibition constant K_i for succinate and maleate were determined on the same enzyme preparation using methods described under Materials and Methods. [b] The maximal velocity is in terms of μmoles of acetoacetyl-CoA used per min per mg of protein.

ide present in the assay medium has little effect). This latter observation is not unexpected due to the reagent differences in size, hydrophobicity, and mechanism of the alkylation reaction shown by these reagents (Fenselau, 1970).

Finally in the series of succinate analogs in which the orientation of the two carboxyl groups is determined on multiply bonded carbon atoms (type IV analogs), one notable result was obtained. Only maleic acid with the two carboxyl groups cis to one another is utilized as a substrate by rat heart CoA transferase. No effect, inhibitory or otherwise, could be detected with the trans diacid fumarate; acetylenedicarboxylate is at best a weak competitive inhibitor. A comparison of the kinetic constants for maleate and succinate is included in Table III. The K_m values for the two are quite similar; however, the V_{max} with succinate is at least nine times greater than the V_{max} with maleate.

Having identified some succinate analogs that bind to CoA transferase (as inhibitors or substrate), we further examined their inhibitory properties in the utilization of acetoacetate. First, supporting our observation that maleate is a substrate for CoA transferase is the result that both succinate (Figure 2A) and maleate (Figure 2B) demonstrate the expected pattern of competitive product inhibition (Hersh and Jencks, 1967). On the other hand, the remaining analogs, all competitive inhibitors to succinate consumption, show a complex inhibitory pattern to acetoacetate usage, such as that shown by 2,2-difluorosuccinate (Figure 2C). Unlike succinate or maleate (which can be chemically altered by the enzyme), these analogs can only serve as dead-end inhibitors. The ability of these substances to inhibit acetoacetate utilization is indicated by the concentration of analog required to produce 50% inhibition of a given level of enzyme activity in the physiological forward reaction (Table IV).

Discussion

The results of this study point up the close similarities in functional properties of CoA transferase from pig heart and rat heart. The two are similar with regard to K_m values for substrates, K_i values for and type of product inhibition, the values of the ratio of V_{max} in the reverse reaction (acetoacetate formation) to the V_{max} in the forward reaction, substrate specificity for succinate, maleimide inhibition, and a ping-pong mechanism. The differences in the absolute values for the maximal velocity for each half-reaction might reflect species differences, but clearly the impurity of our transferase preparation accounts for some of the differences. However, the many similarities argue that meaningful conclusions can still be drawn from the studies using our enzyme preparations and the succinate analogs.

As was just noted, the rat heart CoA transferase shares with

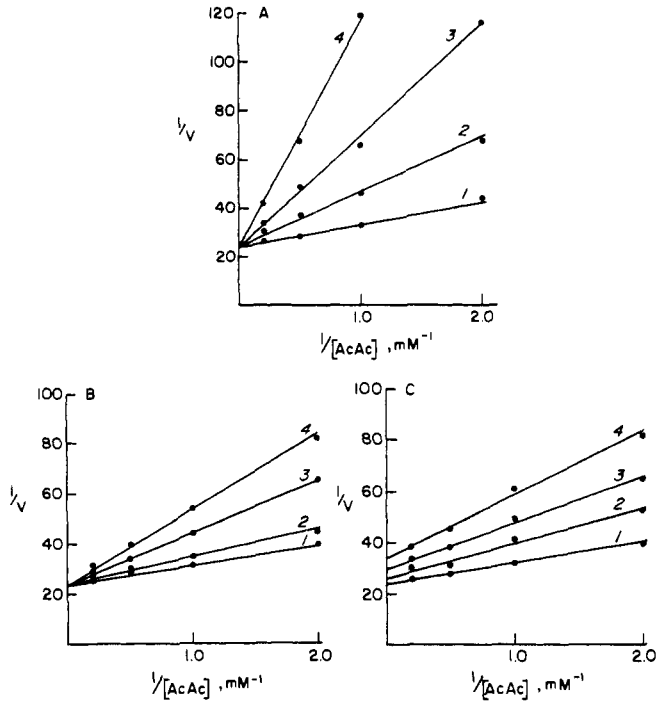

FIGURE 2: Effects of succinate, maleate, and difluorosuccinate on acetoacetate utilization by rat heart CoA transferase. All assays were carried out as described in the Experimental Section using 26 μg of a rat heart CoA transferase preparation (specific activity 1.17) and 0.19 mM succinyl-CoA. (A) Inhibition by succinate at concentrations of (1) 0 mM; (2) 0.50 mM; (3) 1.25 mM; and (4) 2.5 mM. (B) Inhibition by maleate at concentrations of (1) 0 mM; (2) 2.5 mM; (3) 5.0 mM; and (4) 10 mM. (C) Inhibition by 2,2-difluorosuccinate at concentrations of (1) 0 mM; (2) 2.5 mM; (3) 5.0 mM; and (4) 10 mM.

pig heart enzyme a high degree of specificity for succinate as a substrate. From the results with the various succinate analogs we conclude that the enzyme binds best a short chain acid bearing a terminal carboxylic acid group (or negatively charged group), no bulky substituents in the α position, and only one such substituent in the β position. Analogs containing these features act as competitive inhibitors of succinate utilization and mixed type inhibitors of acetoacetate utilization (Table IV). In this later capacity they may prove fruitful in

TABLE IV: Comparison of Inhibition of Acetoacetate Utilization for CoA Transferase by Succinate Analogs.[a]

Compound	ID_{50}, mM	Compound	ID_{50}, mM
Succinate	4.9	2,2-Difluorosuccinate	29
Oxalate	11	Maleamate	34
Maleate	15	Monomethyl succinate	36
Malonate	19	Sulfopropanoate	42
Perfluorosuccinate	25	Succinamate	47
N-Ethylmaleamate	27		

[a] The concentration of succinate or analog required for producing a 50% inhibition (ID_{50}) in the utilization of acetoacetate was measured using the same enzyme preparation for all determinations. The conditions used were: 13.7 μg of rat CoA transferase (specific activity 2.72) in the usual assay medium containing 0.15 mM succinyl-CoA, test substance, and 2 mM acetoacetate (last addition) (final volume, 1.0 ml).

metabolic studies probing the relationship between two mammalian enzymes that activate acetoacetate, namely acetoacetate CoA transferase and acetoacetate synthase (Buckley and Williamson, 1973; Stern, 1971). Since the synthase produces acetoacetyl-CoA from acetoacetate, ATP, and coenzyme A, succinate or its analogs should not affect the synthase activity.

Finally with respect to the structural properties that determine whether an analog will serve as a substrate, it appears that the active conformation of the bound substrate molecule must have two carboxyl groups in a cis, coplanar relationship to each other. One interpretation of the mechanistic significance of this structural limitation is that a specific binding (Coulombic) interaction can occur between one carboxyl group in the substrate molecule and a positively charged enzyme residue. Although substrate specificity can be partly accounted for in these terms, any explanation of substrate reactivity requires further elucidation of the enzyme active site.

On the other hand, substrate reactivity may be partially rationalized on the basis of novel structural features seen in the active substrate conformer. The cis, coplanar arrangement of two carboxyl groups places the two carbon atoms of these negatively charged groups within 3 Å of one another, creating an energetically unfavorable situation due to the presence of two like charges in such close proximity. Thus, there exists adequate driving force for a reaction between the diacid and the enzyme-CoA intermediate that eventually places an additional negative charge on the enzyme while forming the acyl-CoA derivative. Or stated another way, the reactive conformer of a substrate molecule contains a more nucleophilic (or basic) carboxyl group that more readily attacks the thiol ester enzyme intermediate. The basicity of this group is indicated by the magnitude of the second pK_a value (in water) of various diacids: fumarate, 4.4; succinate, 5.6; malonate, 5.7; and maleate, 6.1. Substitution of fluorine atoms (for the slightly smaller hydrogen atoms), therefore, probably leads to an increase in acidity of the carboxyl groups (and a decrease in their reactivity) due to the greater electronegativity of fluorine. These observations are in accord with those noted by White *et al.*, (1973), who found that the reaction rate of substituted acetates with the enzyme-CoA species increases with increasing basicity of the acid. Although our results do not eliminate a concerted four-center mechanism for the decomposition of enzyme-CoA to products (Benson and Boyer, 1969), they are quite consistent with the alternative mixed anhydride mechanism (involving the formation of a tetrahedral adduct of acid substrate and enzyme-CoA) that has received experimental support (White *et al.*, 1973).

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