Mechanism and Specificity of Succinyl-CoA:3-Ketoacid Coenzyme A Transferase*

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(a) The reactivity of substituted acetates as substrates for CoA transferase increases sharply with increasing basicity and exhibits a slope of 1.0 in a plot of either log $k_{cat}$ or log ($k_{cat}/K_m$) against p$K_a$ ($\beta_{nucl} = 1.0$). This result shows that the catalyzed reaction, which involves both carboxylate activation and leaving group transfer, does not proceed through a fully concerted reaction mechanism in the rate-determining step. The result is consistent with a stepwise reaction mechanism that proceeds through an anhydride intermediate. (b) Equilibrium constants for thiol ester formation, either bound to the enzyme or free in solution, show the same dependence on the basicity of carboxylate ions ($\beta_{nucl} = 1.0$) and are independent of acidity when expressed in terms of the carboxylic acid. Thus, the polar environment around substituents on the acyl group is the same for carboxylic acids, thiol esters, and oxygen esters. (c) The interaction of the terminal CH$_2$CO group of acetoacetate with the active site causes a 200,000-fold increase in $k_{cat}/K_m$, corresponding to a decrease in $\Delta G^*$ of 7.2 kcal/mol compared with an unsubstituted acid of the same pK. The binding energy of the coenzyme A moiety of the substrate is utilized to interact with the active site and cause a $10^5$ to $10^6$-fold increase in $k_{cat}$, corresponding to a decrease in $\Delta G^*$ of 6 to 9 kcal/mol, compared with fragments of the coenzyme A moiety added separately or together. (d) The exchange of labeled coenzyme A into acyl-CoA substrates was found to be $\geq 10^9$ slower than substrate turnover.

We describe here a series of experiments directed toward a better understanding of the mechanism of the rate acceleration brought about by CoA transferase from pig heart, which catalyzes the reaction shown in Equation 1 with R$_1$COO$^-$ as acetoacetate and R$_2$COO$^-$ as succinate.

$$R_1C=O + R_2COO^- \rightleftharpoons R_1C-SCoA + R_2CO^-$$

The mechanism of this reaction is of special interest because it involves two complex chemical processes in a single reaction—the activation of an unreactive carboxylate ion to a “high energy” thiol ester, and the transfer of the coenzyme A leaving group from a thiol ester to a carboxylic acid.

There has been considerable interest in recent years in the question of whether acyl group activation reactions of the kind shown in Equation 2 proceed through a stepwise (upper pathway) or a concerted (lower pathway) mechanism (1–3). These reactions generally utilize ATP or a similar phosphohydride as the dehydrating agent, X $\sim$ Y, which extracts oxygen from the carboxylate ion and makes possible its replacement by an acyl group acceptor Z, such as CoA-SH, RNH$_2$, or a ribose hydroxyl group of tRNA. The stepwise mechanism involves nucleophilic attack of the carboxylate ion on X $\sim$ Y, with the displacement of Y$^-$ and the formation of a mixed anhydride, RCO $\sim$ X, which may be an acyl phosphate or an acyl adenylate. This is followed by the displacement of XO$^-$ by the nucleophile Z$^-$ to form the product. In the concerted mechanism these processes occur simultaneously through a transition state in which four bonds are formed or broken at once. It is difficult to identify the chemical driving force for such a concerted reaction; it is also difficult either to demonstrate or to exclude it experimentally.

The reaction catalyzed by CoA transferase is also an acyl group activation reaction of this kind, but has the novel feature that the dehydrating agent, X $\sim$ Y, is a thiol ester which...
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provides a leaving group, Y, that also serves as the nucleophile to accept the acyl group and form a new thiol ester (Equation 3).

\[
\begin{align*}
R-C-O^- & \quad \text{Enzyme-COO}^- \\
Y^- & \quad \text{RCOSCoA} \\
\end{align*}
\]

Although similar leaving group transfer reactions occur in nonaqueous solvents at high temperature, there has been no detailed study of a model for the enzymic reaction, and the chemical mechanism of the enzymic reaction itself has not been elucidated by previous experiments (4). As in the case of other acyl group activation reactions, leaving group transfer can occur either through a stepwise mechanism with the intermediate formation of a mixed anhydride of the two acyl substrates (upper pathway) or through a concerted mechanism with a four-center transition state (lower pathway, Equation 3) (5-7). There is chemical precedent for acyl group transfer through a four-center transition state in the rearrangements of isoximides and S-benzyloisothioureas (Equation 4, X = O or S) (8, 9).

\[
\begin{align*}
Ar-C=X & \quad Ar-C=X \\
R-N=C & \quad R-N=C \\
Ar-HC=O & \quad Ar-HC=O \\
Ar-N=P-Ar_3 & \quad Ar-N=P-Ar_3 \\
\end{align*}
\]

The reaction shown in Equation 5 and related reactions of ylids provide precedent for leaving group transfer through a four-membered ring transition state; these reactions are thought to proceed by a stepwise reaction mechanism with the intermediatem formation of an addition compound (10-12).

It has been demonstrated that the reaction catalyzed by CoA transferase proceeds through the initial formation of an enzyme-CoA intermediate (Equation 6) in which the coenzyme A is bound to the enzyme as a thiol ester of the y-carboxyl group of glutamate (13-16).

\[
\text{RCOSCoA} + \text{Enzyme-COO}^- \rightarrow \text{RCOO}^- + \text{Enzyme-COSCoA}
\]

However, this information does not help to elucidate the chemical mechanism of the reaction, since the chemical problem for the transfer of coenzyme A to a carboxylate group is the same regardless of whether the carboxylate group is on the enzyme or on a bound substrate.

The examination of substituent effects on reaction rates provides a method for mapping out the charge distribution of the transition state—its "electronic anatomy"—relative to the ground state. We report here a study of substituent effects on the reactions catalyzed by CoA transferase which provides results that are inconsistent with the symmetrical charge distribution expected for the transition state of a concerted reaction in the rate-determining step, but are consistent with the expected transition state for a stepwise reaction mechanism. The data also provide information on the charge distribution and equilibria of thiol esters themselves. Finally, the results provide an interesting example of the utilization of the binding energy between specific substrates and the active site to provide the driving force for rate acceleration. Preliminary reports of this work have appeared (4, 17).

**EXPERIMENTAL PROCEDURE**

Materials—Reagent grade oxalic, formic, acetic, and citric acids, inorganic salts, and N-tris (Mann Ultrapure) were used without further purification. Purified sodium pyruvate and sodium lactate were kindly provided by Dr. Robert Ables; perfluorocarboxylic acid, succinic monomethyl ester, and L- and D-methylisocitrate by Dr. Thomas Hollocher; desulfococenzyme A by Dr. Jack Henkin (all of Brandeis University); and oxycocenzyme A by Dr. C. J. Stewart (San Diego State University). Malonyl-CoA, acetyl-CoA, and 2,5-difluorocarboxylic acid were obtained from Sigma Chemical Co., and dihydroxyacetone-CoA dehydrogenase was obtained from Boehringer Co. Coenzyme A was obtained from Sigma Chemical Co. (grade I, 85 to 90% pure) and from P-L Laboratories (Chromatopure, 90 to 95% pure); the latter material was used for all kinetic experiments.

Acetyl-CoA was prepared by the following modification of the method of Simon and Shemin (13, 18). All syntheses of thiol esters were carried out in solutions through which argon had been bubbled and a gentle stream of argon was blown over the surface during additions. A 1.7-fold molar excess of solid succinic anhydride (usually 10 mg) was added to the coenzyme A (usually 50 mg) in 2.0 ml of 0.1 M sodium bicarbonate at 0°. The solution was stirred until the succinic anhydride dissolved and was then either continuously extracted with ether at pH 1.0 for 6 to 8 hours, or chromatographed on a column of Sephadex G-10 in 0.01 M sulfuric acid to remove succinic acid. The concentration was measured by the hydroxamic acid method (19, 20), using a weighed sample of succinic anhydride dissolved in 2 M hydroxylamine buffer, 87% base, as a standard. Solutions of succinyl-CoA were stable at pH 1.5 and 17° for at least 6 months.

Acetoacetyl-CoA was prepared by a modification (13) of the method of Luxon et al. (21), using a 1.2-fold molar excess of freshly distilled and purified (b.p. 27-29°, 3 mm) diketene. The diketene (usually 5 µl) was added to the coenzyme A (usually 40 mg) in 0.1 M sodium bicarbonate. The solution was stirred under argon for 20 min. The minimum amount of diketene required to convert all of the coenzyme A to acetoacetyl-CoA was estimated by adding 3 µl of diketene to 40 mg of coenzyme A, which gave approximately 75% of the maximum observed yield of acetoacetyl-CoA (measured by A_335, as described below), 85 to 90% theoretical (based upon the weight of coenzyme A). Addition of more than 5 µl of diketene did not increase the yield, and higher concentrations of diketene gave an enzymically inactive contaminant with absorbance at 310 nm. The product contained an amount of acetoacetate that was insignificant for most purposes and was generally used directly; some preparations were extracted continuously for 6 hours with ether at pH 1.5, 0°. Acetoacetylthio thrine and S-acetoacetyl-N-acetylcysteamine were prepared in the same manner. The thiol compounds were prepared by adding portions of solid sodium borohydride to the corresponding disulfides (Sigma Chemical Co.) in 0.1 M sodium borate buffer, pH 8.5, over 1 hour until reduction was complete, as measured with DTNB (22). Acetoacetyl-CoA was found to be stable at pH 2 to 3 and 17° for at least 6 months.

Sodium acetate (23) was determined by the method of Walker (24). Solutions were stored at −17° and were discarded when bubbles appeared; the concentration of acetate was found to remain constant on storage. Potassium malonate monomethyl ester was prepared by adding 100 ml of 1 M potassium hydroxide in absolute ethanol.

"The abbreviation used is DTNB, 5,5'-dithiobis(2-nitrobenzoic) acid."
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Dropwise to 0.1 mol of redistilled diethyl malonate in ethanol, and was recrystallized from water.

Butyryl-CoA, chloroacetyl-CoA, methoxyacetyl-CoA, and N-acetyl- 
glycine-CoA were prepared from the corresponding ethyl carbonate 
mixed anhydrides by the method of Kass et al. (25). The reaction of 
ethyl chlorofominate with butyrate was found to be instantaneous, 
but N-acetylglycine and methoxyacetate required 10 to 30 min, and 
chloroacetate 12 hours. A 1.5- to 4-fold excess of the mixed anhy-
drides was added, in 50-μl aliquots, to 1 mg of coenzyme A in 1.0 ml 
of 0.05 M potassium bicarbonate at pH 7.5, 0°. The reaction was 
followed by measuring the disappearance of thiol with DTNB, using 
10-μl aliquots (22). The product was brought to pH 1.0 with dilute 
sulfuric acid and continuously extracted with ether for 4 to 6 hours 
at 0° to remove free carboxylic acids. The concentration of thiol ester was determined with DTNB after alkaline hydrolysis, as described 
below for acetooctyl-CoA.

Succinyl-CoA-3-ketoacid coenzyme A transferase was prepared as 
described in a subsequent paper (26). Other organic reagents were 
recrystallized or redistilled. Glass-distributed water was used throughout.

Extinction Coefficient of Acetoxyl-CoA—The results of this work 
are based on a molar extinction coefficient of 9,300 for acetoxyl-CoA 
in 0.067 M Tris sulfate, pH 8.10, and 5 mM magnesium sulfate at 25°. 
This is significantly smaller than a previously reported value of 11,900 
(27); however, later work has shown that acetoxyl-CoA prepared 
from comparable materials contains approximately 35% of enzymically 
inactive acetoxylacetylglutathione and other contaminants (28). A value of ε = 9350 was obtained by measuring the decrease in NADH 
absorbance at 340 nm in the presence of 0.1 MM NADH, 20 μg/ml of β-
hydroxyacyl-CoA dehydrogenase and 7.2 units of succinyl-CoA:3-keto-
acid coenzyme A transferase. A less accurate value of ε = 9,400 to 9,800 was obtained 
by measuring the amount of thiol released upon hydrolysis of acetoxyl-
CoA in 0.02 M sodium carbonate under argon for 10 min, by reaction with DTNB (22). The maximum yields of acetoxyl-CoA 
prepared from thiolate and CoA were 85 to 90%, based on ε = 9,300; the same yields were found for the synthesis of succinyl-CoA. The thiol 
content of the starting CoA (P-L Chromatopure) was found to be 88%, 
and the product thiol esters were found to contain roughly 10% CoA 
disulfide, as judged visually after chromatography. The absorbance at 
310 nm of the acetoxyl-CoA preparations used in the earlier work. Table I 
also includes a comparison of [Kj values measured by product 
inhition of the reaction rate (13) and by an inactivation method (32).

The second column of Table I contains the composition of the 
experimentally measured constants in terms of the micro-
scopic equilibrium and rate constants in Equation 7, in which 
AcAc = acetoxylacetate and Succ = succinate. These constants 
were derived by Hersh (33), based on the assumption that the 
binding and dissociation steps of the reaction are fast relative 
to [k1 and k−1. The simple combination of the kcat/Km terms makes 
possible the direct calculation of the equilibrium 
constants for the formation of E-SCoA in the twofold reactions 
from the experimental kinetic constants, as shown in Equation 
8 (R represents the acyl compound). The results are

\[ \frac{[RCOO]^{-}[E]}{[RSCoA][E]} = \frac{k_{\text{cat}}}{K_{\text{m}}K_{\text{n}}} \]

RESULTS

Kinetics—The reaction catalyzed by CoA transferase was 
found to follow ping-pong kinetics, with parallel lines in double 
reciprocal plots, in both directions, and gave kinetic constants 
(Table I) that generally agree with those obtained previously 
(13) within the large estimated experimental error of up to 
±50% for constants that are based on multiple extrapolations.

A significant difference was found for the value of KmAcAcCoA, 
which is 6-fold smaller than the earlier value, and there is a 
corresponding difference for constants that are derived from 
this value. This difference presumably reflects a difference 
in the enzyme preparations or, more likely, an impurity in the 
acetoxyl-CoA preparations used in the earlier work. Table I 
also includes a comparison of Kj values measured by product 
imhibition of the reaction rate (13) and by an inactivation method (32).
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**TABLE I**
Summary of kinetic constants for CoA transferase at pH 8.10, ionic strength 1.0, 25°C

<table>
<thead>
<tr>
<th>Constant</th>
<th>Microscopic composition</th>
<th>Data of Hersh</th>
<th>Present study</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_m Ac</td>
<td>K_m Sc / (K_a + K_4s)</td>
<td>0.20</td>
<td>0.16</td>
</tr>
<tr>
<td>K_m AC</td>
<td>K_m S / (K_a + K_4s)</td>
<td>0.93</td>
<td>0.19</td>
</tr>
<tr>
<td>K_m S</td>
<td>K_a / (K_a + K_4s)</td>
<td>36</td>
<td>23</td>
</tr>
<tr>
<td>K_4s AC</td>
<td>K_4s Sc / (K_a + K_4s)</td>
<td>4.2</td>
<td>4.2</td>
</tr>
<tr>
<td>K_4s Sc</td>
<td>K_4s S / (K_a + K_4s)</td>
<td>0.78</td>
<td>0.29d</td>
</tr>
<tr>
<td>K_4s S</td>
<td>K_4s / (K_a + K_4s)</td>
<td>1.0</td>
<td>0.54d</td>
</tr>
<tr>
<td>K_0Ac</td>
<td>K_0Sc / (K_a + K_0s)</td>
<td>1.9</td>
<td>2.4d</td>
</tr>
<tr>
<td>K_0Sc</td>
<td>K_0S / (K_a + K_0s)</td>
<td>4.2</td>
<td>2.5</td>
</tr>
<tr>
<td>k_cat x 10^-4 min^-1</td>
<td>k_cat x 10^-4 Sc / Sc</td>
<td>1.3</td>
<td>1.8</td>
</tr>
<tr>
<td>k_a x 10^-4 min^-1</td>
<td>K_a x 10^-4 Ac</td>
<td>4.5</td>
<td>13</td>
</tr>
<tr>
<td>k_a x 10^-6 min^-1</td>
<td>K_a x 10^-6 Ac</td>
<td>6.4</td>
<td>13</td>
</tr>
<tr>
<td>k_cat x 10^-5 min^-1</td>
<td>K_cat x 10^-5 Sc</td>
<td>3.0</td>
<td>4.3</td>
</tr>
<tr>
<td>k_cat x 10^-6 min^-1</td>
<td>K_cat x 10^-6 Sc</td>
<td>1.2</td>
<td>1.1</td>
</tr>
</tbody>
</table>

**Definition**
- A, acetoacetate; S, succinate; C, CoA. The microscopic constants are defined in Equation 7.
- *From previous data (13) and the extinction coefficient reported here.
- *Conditions were 0.32 M sodium sulfate/5 mM magnesium sulfate 0.08 M Tris sulfate, pH 8.10, 25°C. Determined from kinetics except where noted.
- *Conditions identical with (b) but determined by inactivation (32).
- *For the reaction of acetoacetyl-CoA and succinate.
- *For the reaction of succinyl-CoA and acetoacetate.
- Defined in Equation 7.
- Calculated from the data of Hersh and Jencks (13).
- Derived from the measured kinetic constants of Table I. A, acetoacetate; S, succinate; C, CoA.

shown in Table II, along with values or limits for the other kinetic constants of Equation 7. There is satisfactory agreement between the equilibrium constants for the two half-reactions calculated from the present and earlier kinetic results, as shown in the first two entries in Table II, and the third entry shows that the agreement for the overall equilibrium constant is considerably closer than expected. These values of 30 and 27 agree, within the probable experimental error, with the value of 40 ± 5 that was determined more directly, as described in the following paper (32).

A number of the relationships between the experimental and microscopic constants in Table I may be simplified to provide numerical values or limits for the constants in the mechanism of Equation 7, as summarized in Table II. An example is shown in Equation 9. Since k_cat, for the half-reaction with acetoacetyl-CoA, must be

at least as large as the observed rate constant for the overall reaction, k_cat = 2.5 x 10^4 min^-1, Equation 9 requires that the value of k_cat be between 3.7 x 10^3 min^-1 (if k_cat = 2.5 x 10^4 min^-1) and 3.2 x 10^2 min^-1 (if k_cat = 8). The ratio of microscopic rate constants in Equation 9 may be calculated independently from different experimental constants, as shown in Equation 10. The agreement

between the results of the two calculations (Table II, fourth and fifth lines) provides a check on the internal consistency of
the data. An analogous treatment for the second half-reaction gives the rate constant ratios shown in the sixth and seventh lines of Table II. The requirement that $k_r > k_{cat} = 2.5 \times 10^4$ min$^{-1}$ gives a value of $k_r$ in the range 500 to 1100 min$^{-1}$; the latter value must be more nearly correct since $k_r$ must be at least as large as the overall observed maximum rate constant, $k_{sat} = 1800$ min$^{-1}$. These values show that the formation of enzyme-CoA from bound succinyl-CoA is largely, but not entirely rate determining in the reaction of succinyl-CoA with acetoacetate at saturation.

The dissociation constant for acetoacetate, $K_{ac}$, was estimated by the two methods shown in Equations 11 and 12 with comparable results. The value of $K_{ac}$ was calculated similarly, but only lower limits could be estimated for $K_{ac}$ and $K_{ac}$ (Table II).

The ratios of $k_r$ and $k_f$ for the two half-reactions provide limits for the internal equilibrium constants for the transfer of CoA between bound thiol ester substrate and the enzyme (last two lines, Table II). These values are within 1 order of magnitude of the overall equilibrium constants for the two half-reactions involving unbound substrates (first two lines, Table II), and consequently provide no indication for any large change in binding interactions with the enzyme during the transfer of CoA from the bound acyl-CoA substrate.

Reactivity of Nonspecific Substrates—Although mammalian CoA transferase has generally been considered to be specific for succinate, $\beta$-ketoacids, and malonate (4), it catalyzes the transfer of CoA to a number of other carboxylate substrates at rates $10^{-1}$ to $10^{4}$ as large (Table III). The reactions were measured using acetoacetyl-CoA as the CoA donor and, since the formation of enzyme-CoA from acetoacetyl-CoA is much faster than the reaction with the carboxyate substrate, the second half-reaction is rate-determining with these substrates. Because it is unlikely that rate determining dissociation of a small, nonspecific substrate such as acetyl-CoA would be $10^{4}$ to $10^{5}$ slower than the dissociation of the specific substrate succinyl-CoA, the observed values of $k_{cat}$ and $K_{ac}$ may be assigned to the internal rate constant, $k_r$, and the substrate dissociation constant, $K_{ac}$, for the second half-reaction. Fig. 1 shows representative experimental data for a substrate that shows saturation (butyrate, Fig. 1A) and for a substrate that shows no saturation, for which only a second order rate constant is reported (formate, Fig. 1B). No reaction was observed, and a limit for the second order rate constant $k_{cat}/K_{ac}$ of $<10^3$ min$^{-1}$ was obtained for the following acids and related compounds: lactic, pyruvic, isovaleric, pivalic, tetrafluorosuccinic, 2,2-difluorosuccinic, oxalic, aspartic, citric, fumaric, glutaric; and hexafluoroacetone hydrate, trifluoroethanol, azide, and bicarbonate. Thiolactic acid is the only noncarboxylate nucleophile that was found to react at a significant rate with enzyme-CoA.

The reactions of acetoacetyl-CoA with maleate and of succinyl-CoA with oxalacetate are fast enough to give classical ping-pong kinetics, as illustrated in Fig. 2A for the latter reaction. The intercepts were replotted, as shown in Fig. 2, to obtain $k_{cat}$ and $K_m$ values. The rate of the reaction with oxalacetate was followed by measuring the rate of appearance of a product that absorbs at 310 nm, presumably the enolate form of oxalacetyl-CoA (Scheme I).

\begin{align*}
\text{Thiolacetic} & \quad 2.12 \quad 7 \quad 0.74 \quad 63 \quad 2.4 \times 10^3 \\
\text{Cyanacetic} & \quad 2.33 \quad 15 \quad 0.74 \quad 9 \quad 3.9 \times 10^4 \\
\text{Chloroacetic} & \quad 2.72 \quad 7 \quad 4.5 \quad 2.6 \quad 87 \quad 2.0 \times 10^4 \\
\text{Mechoxyacetic} & \quad 3.43 \quad 30 \quad 0.4 \quad 3 \quad 3 \times 10^4 \\
\text{Malonic, monoethyl ester} & \quad 3.5 \quad 56 \quad 440 \quad 7.9 \times 10^4 \\
\text{Formic} & \quad 3.56 \quad 150 \quad 3 \quad 3 \times 10^4 \\
\text{N-Acetylglycine} & \quad 3.87 \quad 3.5 \quad 1.2 \quad 35.0 \quad 1.1 \times 10^4 \\
\text{Glycolic} & \quad 3.82 \quad 25 \quad 0.6 \quad 8 \quad 76 \\
\text{Succinic, monoethyl ester} & \quad 4.35 \quad 55 \quad 89 \quad 3.9 \times 10^4 \\
\text{Oxalacetic} & \quad 4.37 \quad 2 \quad 2 \quad 9 \quad 9 \times 10^4 \\
\text{Acetic} & \quad 4.61 \quad 23 \quad 1.7 \quad 3 \quad 14 \quad 500 \quad 1.9 \times 10^4 \\
\text{Malonic} & \quad 4.7 \quad 260 \quad 2.6 \quad 3 \quad 2 \quad 4 \quad 6 \quad 6 \times 10^4 \\
\text{Butyric} & \quad 4.78 \quad 15 \quad 0.55 \quad 3 \quad 5 \quad 2 \quad 6 \quad 3 \times 10^4 \\
\text{Propionic} & \quad 4.78 \quad 14 \quad 7 \quad 3 \quad 1 \quad 7 \quad 1 \quad 5 \quad 10 \\
\text{Valeric} & \quad 4.78 \quad 12 \quad 83 \quad 6 \quad 9 \quad 10 \\
\text{Maleic} & \quad 5.2 \quad 33 \quad 50 \quad 2 \quad 1 \quad 7 \quad 3 \quad 1 \quad 5 \\
\text{Malic} & \quad 5.2 \quad 50 \quad 7 \quad 4 \quad 3 \quad 5 \\
\text{L-Methylsuccinic} & \quad 5.6 \quad 100 \quad 18 \quad 100 \quad 100 \\
\text{d-Methylsuccinic} & \quad 5.6 \quad 100 \quad 18 \quad 100 \quad 100 \\
\text{Thiolactic} & \quad 3.20 \quad 2.0 \quad 6.7 \quad 3.3 \times 10^3 \\
\end{align*}
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**FIG. 1.** Dependence on substrate concentration of the reaction of acetoacetyl-CoA with butyrate (A) and formate (B) and of butyryl-CoA with acetoacetate (C) catalyzed by CoA transferase at pH 8.1, ionic strength 1.0, 25°C.

**FIG. 2.** A, double reciprocal plot for the reaction of acetoacetyl-CoA with maleate catalyzed by CoA transferase at pH 8.1, ionic strength 1.0, and 25°C. B and C, reciprocal plot of the intercepts of A and the corresponding plot for acetoacetyl-CoA.

Rate constants for the reverse reactions of several nonspecific acyl-CoA substrates with acetoacetate were also determined, using the coupled assay system with NADH and β-hydroxyacyl CoA dehydrogenase (Table III). These reactions are sufficiently slow that the first half-reaction is rate-determining, and the observed constants K_{mACo}, and k_{cat} may be assigned to the substrate dissociation constant K_{RCoA} and the catalytic constant for the formation of enzyme-CoA k_{c}, respectively. Data for the reaction with butyryl-CoA are illustrated in Fig. 1C. The reported constants for the nonspecific substrates are estimated to be accurate within ±20%.

No increase in the rate of disappearance of 0.2 mM acetoacetyl-pantetheine or S-acetoacetyl-N-acetylcysteamine was brought about by the addition of 2.2 × 10^{-1} M CoA transferase in the presence of 0.01 M succinate under the standard reaction conditions. The addition of 1.0 mM 5'-AMP, ADP, adenosine 2',5'-diphosphate, desulfoCoA, or CoA did not increase the rate of disappearance of either of these analogs for acetoacetyl-CoA above the background from hydrolysis under the same conditions. The rate of absorbance change for all runs in the presence or absence of enzyme was (2.0 ± 0.5) × 10^{-2} at 310 nm, which corresponds to a maximum second order rate constant for reaction with the enzyme of ≤ 4.5 × 10^{4} M^{-1} min^{-1}. Thus, these compounds are less reactive than acetoacetyl-CoA by a factor of ≥ 3 × 10^{4}. Enzyme which had been incubated with these compounds retained full activity and exhibited no induction period upon reaction with normal substrates; i.e. there is no evidence for the formation of an unreactive enzyme-SH intermediate from these thiol esters.

The same compounds, in the presence or absence of added nucleotides, were found to cause no increase in the rate of inactivation of CoA transferase by 0.4 mM DTNB (32). Incubations were carried out for 10 min under the same conditions as the turnover experiments, and four 10-μl aliquots were found to be inactivated with a rate constant of 0.072 ± 0.005 min^{-1}, identical to that for the free enzyme. Assuming that formation of an enzyme-thiol ester intermediate would cause the same increase in inactivation rate with these compounds as with CoA (20-fold), less than 10% of the enzyme was converted to the thiol ester intermediate, and the second order rate constant for reaction with the enzyme is ≤ 0.5 × 10^{4} M^{-1} min^{-1}, which is smaller than the rate constant for reaction with acetoacetyl-CoA by a factor of ≥ 3 × 10^{4}. Under the same conditions 1 mM desulfoenzyme A was found to give a 2-fold increase in the inactivation rate and, in one experiment, 0.7 mM oxycoenzyme A was found to decrease the inactivation rate by 4-fold.

**Search for Exchange of Labeled Coenzyme A**—The demonstration of exchange of labeled CoA into a thiol ester substrate would provide strong evidence for an anhydride intermediate in the catalytic mechanism of CoA transferase. If an anhydride intermediate is formed with the expulsion of CoA (Y, Equation 3), this CoA will exchange with labeled CoA in the medium and result in the formation of labeled acyl-CoA.
provided that the release of bound CoA from the enzyme is sufficiently fast relative to its reaction with the anhydride to regenerate thiol ester, so that exchange will be detectable.

Incubation of tritiated CoA with 1.5 μM CoA transferase in the presence of acetoacetyl-CoA, succinyl-CoA, or butyryl-CoA gave no detectable exchange of label into the acyl-CoA above controls lacking enzyme or acyl-CoA (Table IV). If it is assumed that 10% exchange could have been detected, the first order rate constant for turnover of the enzyme to give exchange, based on the rate law \( v = k_{\text{cat}} [E_{\text{tot}}] \), is \( k_{\text{cat}} = 0.2 \) min\(^{-1}\). This is slower by a factor of 10\(^6\) than the turnover of the enzyme, \( k_{\text{cat}} = 2.5 \times 10^4 \) min\(^{-1}\). The absence of detectable exchange is consistent with the observation that CoA is not trapped by thiol reagents during enzyme-catalyzed turnover of substrates (32). We conclude that CoA is not a free intermediate in the reaction, and that if an anhydride intermediate is formed, the rate and equilibrium constants for its reaction with bound CoA to regenerate thiol ester are too large to permit significant dissociation of the bound CoA.

**Effect of Ionic Strength**—Increasing the ionic strength to 1.0 with sodium sulfate (solid symbols, Fig. 3) causes either an increase or a decrease in the observed rate depending on the concentration of the two substrates. At the higher ionic strength the \( K_m \) for acetoacetyl-CoA is increased and that for succinate is decreased. The result can be analyzed in terms of the effect of salt on the rates of the two half-reactions—at low acetoacetyl-CoA and high succinate concentrations the first half-reaction is rate-determining and the observed rate is decreased by salt, whereas at high acetoacetyl-CoA and low succinate concentrations the second half-reaction is rate-determining and the observed rate is increased by salt. The effect of salt was shown to be completely reversible.

**DISCUSSION**

**Mechanism**—The reactivity of a series of substituted acetates as substrates for CoA transferase was examined in order to determine the effect of polar substituents on reactivity. These substrate molecules are generally similar in size and should provide a measure of polar substituent effects with relatively little contribution from steric and specific binding interactions with the active site. The reactivity of substituted acetates as CoA acceptors in the leaving group transfer reaction catalyzed by CoA transferase increases sharply with increasing basicity (Table III). The second order rate constants, \( k_{\text{cat}}/K_m = k_{\text{cat}}/K_{\text{eq}} \), for the reactions with butyrate (pK 4.8) and acetate (pK 4.6) are 10\(^{1.9} \) to 10\(^{2.7} \) larger than for cyanoacetate (pK 2.3). Plots of log (\( k_{\text{cat}}/K_{\text{eq}} \)) against pK\(_a\) for the parent acids are shown for a series of substituted acetates in Fig. 4. The data for both plots illustrate a regular increase in reactivity with increasing basicity and fall close to lines of slope 1.0 (\( k_{\text{cat}}/K_{\text{eq}} = k_{\text{cat}}/K_{\text{eq}} \)). There appears to be a tendency for the larger substrates with alkyl or carbonyl substituents to exhibit positive deviations from the lines, especially for the correlation with pK\(_a\) against pK\(_a\) for the parent acids as shown for a series of substituted acetates in Fig. 4. The data for both plots illustrate a regular increase in reactivity with increasing basicity and fall close to lines of slope 1.0 (\( k_{\text{cat}}/K_{\text{eq}} = k_{\text{cat}}/K_{\text{eq}} \)).

**Table IV**

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<tr>
<th>Substrate</th>
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<tr>
<td>Acetoacetate</td>
<td>Butyrate</td>
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<td>RCS-CoA, RCOO-</td>
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<tr>
<td>CoA-SH</td>
<td>81</td>
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</table>

**Experimental**

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<th>Total Tritium %</th>
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</thead>
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<tr>
<td>RCS-CoA</td>
<td>17</td>
</tr>
<tr>
<td>CoA-SH</td>
<td>83</td>
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</table>
The results rule out a concerted mechanism for CoA transferase with a cyclic transition state in which all of the reacting bonds are forming and breaking at the same time in the rate-determining step of the reaction (Scheme II). Cyclic concerted reactions characteristically exhibit small or negligible effects of polar substituents because the concertedness of the reaction leads to a cancellation of net charge development as bonds to a given atom are formed and broken. In the CoA transferase reaction, for example, attack of the carboxylate ion on the thiol ester of the enzyme-CoA intermediate would be aided by electron donation which increases the nucleophilicity of the attacking oxygen atom, but the concerted attack of the thiol on the carboxylate ion would be aided by electron withdrawal which increases the susceptibility of the carbonyl group to nucleophilic attack. The large rate acceleration produced by electron-donating substituents means that there must be little or no nucleophilic attack on this carbonyl group in the transition state for its reaction with the thiol ester.

The simplest interpretation of the data, which is also attractive on chemical grounds, is that the reaction proceeds through a stepwise mechanism with the formation of an anhydride intermediate, and that the negative charge on the attacking carboxylate ion is lost in the transition state because a bond is fully formed to the carbonyl carbon atom of the thiol ester. Plausible transition states that are consistent with the data include that shown in Scheme III, in which the rate-determining step involves expulsion of thiol anion from the tetrahedral intermediate which is formed by attack of carboxylate ion on the thiol ester, and that in Scheme IV, which is essentially the mirror image of III and involves attack of thiol anion on the mixed anhydride. These transition states are chemically reasonable because the expulsion of a carboxylate group with a pK of 2 to 5 ($k_-$, and $k_h$, Equation 15) would be expected to be faster than the expulsion of a thiol with a pK of about 10 ($k_-'$, and $k_--$), Equation 15), so that thiol expulsion (Scheme III) or attack (Scheme IV) would be expected to be the rate-determining step. The stepwise mechanism is attractive on chemical grounds because there is chemical precedent and a readily identifiable driving force for nucleophilic reactions of carboxylate ions and for the reaction of a thiol anion with an anhydride, whereas it is difficult to identify either a precedent or driving force for the attack of a partially formed thiol anion on a carboxylate group to expel an oxygen atom that has not
Mechanism of CoA Transferase

yet fully formed a bond to another carbon atom. Further, the stepwise mechanism is consistent with the "principle of least motion" in the transition state (37).

Other transition states in which the charge on the attacking carboxylate group is effectively lost in the transition state are also consistent with the data. For example, it is conceivable that a thiol anion that is attacking or leaving one carbonyl group could have some interaction with the other carbonyl group of the anhydride (6), although such a transition state is likely to be geometrically unfavorable. A limiting interpretation is that thiol ester formation is complete, and that the rate-determining step of the reaction in both directions involves a change in the conformation of the enzyme in the transition state ($k_r$ and $k_-$, Equation 15). There is evidence that the formation and cleavage of the enzyme-CoA intermediate is accompanied by a conformation change of the enzyme, although the model proposed for this conformation change differs from Equation 16 (32). The mechanism of Equation 16 is consistent with the observed effects of polar substituents in substituted acetates on the rate, but it is more difficult to explain the faster rate of the reaction in both directions with specific substrates such as acetoacetyl-CoA. For example, the mechanism requires that substitution of a carbonyl oxygen atom for two hydrogen atoms must increase the rate of the conformation change driven by acetoacetyl-CoA by a factor of about $10^{10}$ compared with that for butyryl-CoA (Tables II and III); furthermore, the rate of the conformation change in the reverse direction, which is determined by $k_r$ and $K_r$, must be increased by a comparable amount when acetocaceted rather than butyrate is the substrate (see below).

Substituent Effects on Thiol Ester Equilibria and the Reverse Reaction—The ability of CoA transferase to utilize a series of substituted acetyl-CoA substrates, XCH₂CO-S-CoA, provides a tool to measure the sensitivity of the rate of the reverse reaction and the equilibrium constants for the hydrolysis of thiol esters to polar substituents in the acyl group. The equilibrium constants for the formation of $E$-SCoA from a series of acyl-substituted thiol esters of CoA were determined from the ratios of the second order rate constants for each reaction in the two directions (Equation 17, Table V). The equilibrium constants increase sharply with increasing acidity of the acid and fit a line of slope ~1.0 in a logarithmic plot of $K_{eq}$ against the $pK_a$ of the parent acid (Fig. 5). Maleic acid shows a negative deviation of 50-fold from the correlation that may be attributed to resonance stabilization of the thiol ester of this $\alpha,\beta$-unsaturated acid. The internal equilibrium constants for the formation of bound products from bound acyl-CoA ($k_r/k_-$, Equation 18, Table V) show essentially the same dependence.

TABLE V

<table>
<thead>
<tr>
<th>RCOOH</th>
<th>$K_{eq}^a$</th>
<th>$k_r/k_-^b$</th>
<th>$G^c_{H,25^o}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroacetic</td>
<td>140</td>
<td>15.0</td>
<td>-9.9</td>
</tr>
<tr>
<td>Methoxyacetic</td>
<td>23</td>
<td>3.5</td>
<td>-8.9</td>
</tr>
<tr>
<td>Acetoacetic</td>
<td>12.2 (9.2)$^d$</td>
<td>&gt;4.5</td>
<td>-8.5 (-8.3)$^d$</td>
</tr>
<tr>
<td>$N'$-Acetylglutamic acid</td>
<td>26</td>
<td>9.0</td>
<td>-9.0</td>
</tr>
<tr>
<td>Acetic</td>
<td>3.1</td>
<td>0.23</td>
<td>-7.7</td>
</tr>
<tr>
<td>Malonic</td>
<td>2.3 (4.6)$^d$</td>
<td>≤0.03</td>
<td>-7.6 (-8.0)$^d$</td>
</tr>
<tr>
<td>Butyric</td>
<td>1.7</td>
<td>0.062</td>
<td>7.4</td>
</tr>
<tr>
<td>Succinic</td>
<td>0.22 (0.44)$^d$</td>
<td>≤0.002</td>
<td>-6.2 (-6.6)$^d$</td>
</tr>
<tr>
<td>Maleic</td>
<td>0.00067 (0.013)$^d$</td>
<td>≥0.001</td>
<td>-4.2 (-4.6)$^d$</td>
</tr>
</tbody>
</table>

* $K_{eq} = (E \cdot S-CoA) \cdot (RCOO\cdot) / (RCOSCoA) \cdot (E) = (K_m \cdot c^2) / (K_c \cdot E)$.

$^a$ Values corrected for the two carboxylate groups; these values are plotted in Fig. 5.

$^b$ $E \cdot RCoS=\cdot CoA \rightleftharpoons E \cdot RCoS \cdot CoA$. $^c$ $E \cdot RCoS=\cdot CoA \rightleftharpoons E \cdot RCoS + RCOO^-$. $^d$ Statistically corrected for the enolization of acetoacetyl-CoA.

Fig. 5. The effect of polar substituents upon the equilibrium constant for the reactions of thiol esters with CoA transferase to form enzyme-CoA and carboxylate ion (left abscissa) and the Gibbs energy of hydrolysis, $\Delta G^\circ_{H,25^o}$, for the hydrolysis of thiol esters of coenzyme A (right abscissa).
Mechanism of CoA Transferase

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upon polar substituents as those for reaction of the unbound reactants. This shows that there is no significant differential effect of polar substituents on the binding of thiol esters and carbonylate ions and suggests that the transfer of the negative charge of the carbonylate group from water to the active site does not involve a change in its environment that is "seen" by polar substituents. It can easily be shown from Equations 17 and 19 that the standard Gibbs free energy of hydrolysis of the thiol esters at pH 7.0, \( \Delta G^\circ_{\text{H2O}} \), is given by Equation 20,

\[
\Delta G^\circ_{\text{H2O}} (R-SCoA) = K_m (R) + \Delta G^\circ_{\text{H2O}} (Ac-SCoA) (19)
\]

in which \( R \) and \( Ac \) represent a substituted carbonylate and acetate, respectively. The results in Table V, based on a value of \( \Delta G^\circ_{\text{H2O}} = -7.7 \text{ kcal/mol} \) for acetyl-CoA at 25°C (38, see also Ref. 39) also illustrate the large dependence of these equilibria on the acidity of the parent acid when the product is a carbonylate ion.

These results show that the effects of acyl substituents on the equilibria for the formation of a thiol ester from a carbonylate ion are the same as for the protonation of the carbonylate ion. If the equilibrium constants are expressed in terms of hydrolysis to the free acid (\( K_{\text{RCOOH}} \), Equation 19) rather than to the carbonylate ion they are independent of acyl substituents and have the constant value \( K_{\text{RCOOH}} = (2.0 \pm 1.0) \times 10^3 \text{ M} \) for all of the thiol esters examined except maleyl-CoA. The difference between this value and the corresponding value of \( K_{\text{RCOOH}} \approx 20 \text{ M} \) for the hydrolysis of simple oxygen esters reflects the more "energy rich" character of thiol esters of 2.7 kcal/mol (38, 40). The free energies and equilibria for the hydrolysis of oxygen esters to the free acids are also independent of the \( pK_a \) of the acid (40). This leads to the somewhat unexpected conclusion that a polar substituent on the acyl group "sees" the same electronic environment in carboxylic acids, oxygen esters, and thiol esters, in spite of the different electronegativity and resonance effects of the thiol group; evidently the effective charge on the carbonyl carbon atom adjacent to the substituent is the same in all of these compounds.

In marked contrast to the reactions with carbonylate ions, the rate constants \( k_r \) and \( k_c/k_m \) for the reactions of substituted thiol esters of CoA with CoA transferase show no significant dependence on the nature of polar substituents (Table III). The N-acetylglucine and butyric acid substrates exhibit small rate increases that are more marked for the free than for the bound reactants and presumably represent more favorable binding to the enzyme, as was noted previously for the reaction in the opposite direction. Thus, there appears to be no significant change in the charge on the acyl group in going from the thiol ester to the transition state, in agreement with the earlier conclusion that the reactions of carbonylate ions in the reverse direction behave as if there is a loss of 1.0 negative charge on going from the reactant to the transition state, and in accord with the mechanism of Equation 15 and the transition states shown in Scheme III or IV. This result unequivocally rules out the unlikely possibility that \( k_r \) represents a rate-determining dissociation of carbonylate ion from the enzyme-CoA intermediate, because a full negative charge would be developed on the acyl group in the transition state for this reaction.

Substrate Specificity and Kinetics—The specificity of substituted carbonylate substrates reveals some of the properties of the acyl binding site of CoA transferase. The rate enhancements in \( k_{cat} \) and \( k_{cat}/K_m \), relative to butyrate and corrected for the effect of varying basicity (Fig. 4), are shown in Table VI for acetoacetate, succinate, and a series of related substrates. In most instances the greater part of the rate increase observed upon adding specific substituents results from an increase in the value of \( k_{cat} \). This means that most of the Gibbs energy of interaction of these specific groups with the active site of the enzyme is utilized to bring about an increase in the catalytic rate of the reaction rather than an increase in binding to the active site. The substitution of a carbonyl oxygen atom for two hydrogen atoms in the \( \beta \) position of butyrate causes an increase of 45,000-fold in \( k_{cat}/K_m \), which means that the more favorable interaction with the active site brought about by this substitution reduces the Gibbs energy of activation required to reach the transition state from unbound substrates by \( -RT \ln 45,000 = 6.5 \text{ kcal/mol} \). Compared to acetate the difference is \( -RT \ln 2 \times 10^4 = 7.2 \text{ kcal/mol} \), which shows that the favorable interaction of the CH2CO-group of

<table>
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<tr>
<th>Substrate</th>
<th>Rate enhancement</th>
<th>( k_{cat} )</th>
<th>( k_{cat}/K_m )</th>
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<tr>
<td>O</td>
<td>1.0</td>
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<td>1.0</td>
</tr>
<tr>
<td>CH3-C=CH3-C-O</td>
<td>2.000</td>
<td>45,000</td>
<td></td>
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<td>0.9</td>
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<td>O</td>
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TABLE VI
Rate enhancement of substrates with functional groups in register with succinate or acetoacetate

Rate enhancement was calculated relative to butyrate and corrected for differences in basicity of the substrate, assuming that \( k_{cat} \) and \( k_{cat}/K_m \) increase with increasing basicity of \( RCOO^- \) with a value of \( \beta = 1.0 \) (Fig. 4).
acetooacetate with the enzyme in the transition state is stronger than might have been expected for a substituent of this size.

The high activity of maleate and the undetectable activity of fumarate suggest that succinate binds to the active site in an $s$-cis conformation; the same result and conclusion have been reported for the enzyme from rat heart by Fenselau and Wallis (41). There is a strong specificity for straight chain carboxylic acids. Acids with substituents on the $\alpha$ carbon atom, such as lactic and isovaleric acids, react at rates at least 10 to 50 times slower than unsubstituted compounds. Carboxylic acids with branched chains, such as methylisocarric and malic acid, may bind to the enzyme with the substituent $\beta$ to the carboxylate group. Oxalacetic acid, which contains the functional groups of both acetoacetic and succinic acids, exhibits an enhancement of $k_{cat}/K_m$ that is not very much less than that of succinate but has a relatively small reactivity of the bound substrate, $k_{cat}$, suggesting that this substrate may be bound nonproductively. The low reactivity relative to acetooacetate raises the possibility that both the polar carbonyl group of acetooacetate and the $\beta$-carboxylate group of succinate must be cis to the reacting carboxylate group for productive binding, and that oxalacetic binds in a conformation resembling succinate.

The kinetic data reported here (Tables I and II) confirm the earlier conclusion (13) that formation of the enzyme-CoA intermediate in the first half-reaction is the rate-determining step for the enzyme-catalyzed reaction of succinyl-CoA with acetooacetate; however, the fact that the rate constants $k_{cat}$ and $k_{cat}$ differ by less than 1 order of magnitude means that the second half-reaction of bound acetooacetate with enzyme-CoA will make an appreciable contribution to the observed rate at saturation with both substrates. It was shown previously that at a concentration of acetooacetyl-CoA that is 0.15 $K_{MAC}$ the rate of succinyl-CoA formation at saturating succinate is equal to the rate of exchange of acetooacetate into acetooacetyl-CoA at saturating acetooacetate concentration, suggesting that both of these reactions proceed through the rate-determining formation of a common enzyme-CoA intermediate (13). However, at saturation with acetooacetyl-CoA the overall rate of succinyl-CoA formation will be faster than acetooacetate exchange because $k_{cat}$ is 4 to 6 times larger than $k_{cat}$, the rate constant for the back-reaction required for exchange (Equation 7, Tables I and II), and enzyme-CoA formation is not necessarily rate-determining. The values of $k_{cat}$ and $k_{cat}$ can only be set as limits $\geq k_{cat}$ from the available data, but it is probable that they do not differ greatly from these limiting values. The ratios of the rate constants for the reactions of a series of nonspecific substrates in both directions show a simple relationship to the basicity of the carboxylate ion substrate (Table V and Fig. 5) and provide no indication of an effect of substituents that causes preferential binding of the carboxylate or thiol ester substrate. If the correct values of $k_{cat}$ and $k_{cat}$ were very different from the limiting values, it would be expected that the ratios of $k_{cat}/k_{cat}$ for these half-reactions based on the limiting values would deviate from this correlation. It is probable that specific catalytic effects that influence the rate of the reaction in the two directions will largely cancel out in these rate constant ratios, which are measures of the equilibrium constant of the reaction. The fact that the values of $k_{cat}/k_{cat}$ for succinate and acetooacetate based on these limiting values for $k_{cat}$ are not greatly different from those observed for other acids of comparable strength (Table V) suggests that the correct values are close to the limiting values. Thus, it is probable that both the first and second half-reactions are partially rate-determining in the reaction of acetooacetyl-CoA with succinate at saturation.

The assumption that binding and dissociation steps are not rate-determining has been implicit in this discussion of the kinetic data. It has been noted already that this assumption is very probably correct for substituted acetates and other relatively poor substrates. The fact that the ratios of $k_{cat}/k_{cat}$ are not grossly different for acetooacetate and succinate compared with other substrates supports this assumption for specific substrates as well because it suggests that this ratio is determined by chemical rather than by physical factors. For example, if the rate-determining step in the reaction of succinyl-CoA with acetooacetate at saturation ($k_{cat} = k_{cat}$) was the dissociation of succinate, the ratio $k_{cat}/k_{cat}$ for succinate would not represent the equilibrium constant for the reaction of bound succinyl-CoA with the enzyme to form bound products and would be unlikely to exhibit a value close to that expected for the chemical equilibrium for thiol ester interconversion.

The interrelationships of the various rate and equilibrium constants for the reactions of CoA transferase with its specific substrates may be conveniently summarized and visualized in the form of a reaction coordinate diagram as shown in Fig. 6. The solid lines in the figure are based on a standard state of 1.0 M, well below the $K_m$ values of the substrates, and describe the reaction under conditions in which the enzyme is not saturated with substrate and the reaction is described by the second order rate constants $k_{cat}/K_m$. The barriers for the association and dissociation rates are unknown and are drawn arbitrarily in the diagram. The composition of the central complexes is different for the two half-reactions, with the enzyme-SCoA-RCOO$^-$ form favored for acetooacetate and the enzyme-RCoS-CoA form favored for succinate. This is in accord with
the more negative Gibbs energy of hydrolysis $\Delta G^\circ$ for the thiol ester of acetoacetate compared with succinate. The activation barriers for the two half-reactions are similar, and each half-reaction makes a significant contribution to the observed rate at substrate saturation.

The most surprising fact that is illustrated by this diagram is that the binding of the acyl-CoA substrates is hardly any stronger than the binding of the carboxylate substrates. The equilibrium constant for the formation of enzyme-CoA from unbound succinyl-CoA is close to 1.0 in spite of the fact that the non-covalent binding interactions between the large CoA molecule and the active site would be expected to stabilize the enzyme-CoA intermediate. Furthermore, the binding of acyl-CoA substrates is weak in absolute terms ($K_a \approx 0.2$ to 5 mM), and CoA itself is only a weak inhibitor of the enzyme ($K_i = 1$ mm, (42)). This apparently weak interaction with the enzyme must be reconciled with the extreme specificity of the enzyme for the intact CoA substrate—there is no detectable activity with smaller substrates, such as thiol esters of pantothenic and $N$-acetylcycteamine, and no activity appears when portions of the remainder of the complete substrate, such as adenosine 3',5'-diphosphate, are added at the same time. These results require the conclusion that the enzyme interacts strongly and specifically with the coenzyme A moiety of specific substrates, but that this interaction is not manifested in the observed Gibbs binding energy. The binding energy must be utilized for some process that increases the catalytic rate, with little left over for binding. The large reaction rate of intact compared with nonspecific thiol ester substrates by factors of $10^{-4}$ to $10^{-7}$ requires the utilization of at least 6 to 9 kcal/mol of binding energy in such a way as to increase the reaction rate. This binding energy could be used to provide the driving force for a destabilization of the bound substrate relative to the transition state, to decrease its freedom of motion and entropy, and/or to change the conformation of the enzyme (43). The large increase in the rate constant for reaction with thiol reagents of the enzyme-CoA intermediate, relative to the free enzyme, provides evidence for such a conformation change (32). We might speculate that the binding energy of the coenzyme A group is utilized to force open the active site, which has been described in terms of an "alligator"-type model (32), and that the thiol ester group of the bound acyl-CoA is then clamped against the enzyme carboxylate group in a viselike grip that causes an increase in rate by decreasing the low frequency motions and entropy that must be lost to form a new covalent bond, and by destabilizing the bound thiol ester group relative to the transition state.

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H White and W P Jencks


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