Formation of Stable Anhydrides from CoA Transferase and Hydroxamic Acids*

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Acetoxyhydroxamic acid reacts with the enzyme-CoA form of succinyl-CoA:3-ketoacid coenzyme A transferase to give an inactive product with a rate constant of 860 m⁻¹ min⁻¹ at pH 8.1, 25°C. The reaction is reversible in the presence of coenzyme A and has an equilibrium constant of 0.040. The product is an anhydride that is an analog of the intermediate that has been postulated in the natural catalytic pathway; it is inactive because coenzyme A does not react with the acyl group of the hydroxamic acid. The equilibrium constant for formation of the anhydride from the thiol ester of enzyme and methyl 3-mercaptopropionate is 75 times larger than the equilibrium constant of 2.2 for the formation of N,O-diacetylhydroxylamine from acetohydroxamic acid and acetyl-CoA. This shows that the enzyme stabilizes the anhydride at the active site by at least -2.6 kcal mol⁻¹. Succinomonohydroxamic acid reacts with enzyme-CoA as both a substrate and an inactivator, with relative rate constants of 25:1. The inactivation is irreversible, indicating that the enzyme provides a larger stabilization of at least -5.9 kcal mol⁻¹ for the anhydride of an analog of the specific substrate, succinate. The results are consistent with the hypothesis that the enzyme stabilizes an anhydride that is formed at the active site during turnover of normal substrates through a stepwise reaction mechanism.

Succinyl-CoA:3-ketoacid coenzyme A transferase (CoA transferase, EC 2.8.3.5) catalyzes the transfer of coenzyme A between succinate and acetoacetate through two half-reactions, with the formation of an intermediate thiol ester of CoA and the γ-carboxyl group of a glutamate residue at the active site of the enzyme (Equations 1 to 3).

\[ E - CoA + R,CO = E - COA + R,CO \]

\[ R,CSCoA + ECO = R,CSCoA + ECO \] (2)

\[ R,CSCoA + R&O = RzCSCoA + RICO \] (3)

The transfer of the leaving thiol group of CoA from one carboxyl group to another in each half-reaction could occur through a concerted four-center mechanism or through the formation of an intermediate anhydride of the substrate and active site carboxylic acid groups (Equation 4). The large dependence of the reaction rate on the basicity of carboxylate substrates provides evidence supporting an anhydride mechanism for the reactions of a series of substituted acetates (1). The lifetime of any anhydride intermediate must be short, however, because it has not yet been possible to trap either an anhydride or free CoA and experiments with labeled CoA have shown no detectable exchange of CoA that is liberated upon anhydride formation, with CoA in the medium (1). This is not surprising because the intramolecular reaction of bound CoA with an anhydride is expected to be extremely fast. Thiol transfer from a thiol ester to a carboxylic acid through an anhydride intermediate has been demonstrated in nonenzymic model reactions (2, 3).

We describe here the formation of stable anhydrides between the active site carboxyl group and hydroxamic acids. These anhydrides are stable because the acyl group of the hydroxamic acid in the anhydride is unable to react with the thiol group of CoA because of its low chemical reactivity and because it is shifted by 1 atom from the normal position of an anhydride carbonyl group (Equation 5).

\[ E - COA + SCoA = E - COA + CoA + SCoA \] (5)

The experiments described here were carried out following the demonstration by Moore that the enzyme is inhibited in the presence of acyl-CoA substrates and hydroxylamine, or the nonenzymic reaction products of acyl-CoA substrates and...
hydroxylamine, and that this inhibition is reversed by CoA (3). It was shown by Gergen that ECoA is not inactivated by exposure to hydroxylamine alone (0.1 M for 15 min or 0.5 M for 5 min at pH 8.1).  

**EXPERIMENTAL PROCEDURES**

**Materials**—Tris (Schwarz/Mann ultrapure), magnesium sulfate (Schwarz/Mann enzyme grade), and inorganic salts were used without further purification. Acetohydroxamic acid, dithiothreitol, DTNB, iodoacetamide, N-hydroxysuccinimide, imidazole, and succinic acid were recrystallized, and methyl 3-mercaptopropionate (Aldrich) was redistilled. Coenzyme A was obtained from P.L. Biochemicals (Chromatopure) and acetyl-CoA was obtained from Sigma or P.L. Biochemicals. Glass-distilled water was used in all experiments.

AcetacCoA was synthesized and assayed as described previously (11). Sodium AcAc was synthesized as described by Seely (4) and assayed according to Walker (5). The monosodium salt of succinonohydrolactic acid (SHAl) was synthesized according to Bernheim (6). Solutions of SHA were usually prepared by base hydrolysis of N,O-diacetylhydroxylamine. In a typical reaction 0.5 M of the imide was added to 5 ml of water containing 1.5 M sodium hydroxide; hydroxamic acid formation was followed by the ferric chloride test (7) and was complete in 50 to 70 min. Following adjustment of the pH to about 8 with concentrated hydrochloric acid, the solution was frozen at -17°C. Such solutions were stable for at least 3 weeks at this temperature. The same rate constant for inactivation of ECOA by 5 mM SHA was obtained using both methods of preparation.

N,O-Diacetyl-N-methylhydroxylamine (8) and N,O-diacetylhydroxylamine (9) were synthesized and purified according to Ames and Grev (10). N,N-Diacetylbenzoxylamine from benzyloxylamine hydrochloride (O-benzylhydroxylamine hydrochloride, Aldrich) by a method similar to that used for N,O-diacetylhydroxylamine, except that a 10-fold molar excess of acetic anhydride was used; the product melted at 98-99°C (reported 101-102°C) and had the expected uv spectrum (10).

**Methods**—The pKₐ of O-methylacetohydroxamate was found to be 9.9 by titration at ionic strength 1.0 M (KCl). The reported pKₐ for N,N-diacetylacetohydroxamic acid of 8.8 (13) was confirmed by titration (ionic strength 1.0 M, KCl). Measured pKₐ values of 4.3 and 9.6 for succinomonoxydrolactic acid (ionic strength 0.2) are close to previously reported values of 4.5 and 9.6 (10). The pKₐ of N,O-diacetylhydroxylamine was found to be 6.95 ± 0.1 by carefully adding 0.5 M of sodium hydroxide to a cold aqueous solution of the anhydride and measuring the pH after warming to 25°C (ionic strength 0.00 and 0.1 M). Rate constants for the reactions of acetohydroxamic and succinomonoxydrolactamides with AcAcCoA were determined spectrophotometrically by following the decrease in absorbance of the enolate of AcAcCoA at 310 nm in the presence of 100 mM Tris/sulfate, 0.2 M EDTA, 11 mM iodoacetamide, 57 μM AcAcCoA, 4.6 μM CoA transferase, and 0.24 mM ["HJ]AHA in 0.27 mM at 25°C. The ["HJ]AHA was added last and 1-μl aliquots were assayed for enzyme activity. Inactivation occurred with a half-time of 4.7 min, close to the half-time of 3.4 min expected from kinetic studies with AHA. At 15 min after the addition of AHA, the enzyme could be reactivated with CoA to 80% of its initial activity. Control experiments showed losses of 10% and 6% enzyme activity in 20 min when AHA and AcAcCoA, respectively, were omitted. After 33 min the labeled enzyme was separated from free ["HJ]AHA as a column (15.5 × 0.4 cm) of Sephadex G-25 equilibrated with 25 mM Tris/sulfate, 1 mM EDTA, pH 8.1. The fraction containing protein was collected and made 0.1 M with sodium hydroxide. Free ["HJ]AHA peak. The position of the protein-containing fractions was estimated from the position of elution of blue dextran in a preliminary run.

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dissolving succinic anhydride in neutral aqueous hydroxylamine, as a standard. The same extinction coefficients were observed for succinonohydroxamic and acetohydroxamic acids in ferric chloride solution. The kinetics of the reactions of dicarboxyhydroxylamines with imidazole and ethylenediamine in 8 M urea were followed by measuring the absorbance at 540 nm of 2.0 ml aliquots of the reaction mixtures added to 1.0 ml of 20% FeCl₃·6H₂O in 3 M hydrochloric acid. Other reactions of dicarboxyhydroxylamines were followed by adding 0.5-ml aliquots to 9.5 ml of 8% FeCl₃·6H₂O in 0.5 M hydrochloric acid and measuring the absorbance within 2 min against a blank in which 0.5 ml of 8 M urea had been added to the assay. The zero time absorbance was determined by adding the acyl compound and nucleophile reagent separately to the assay. In some cases end points were verified by assaying an aliquot of dicarboxyhydroxylamine that had been incubated 20 min with 1 M hydroxylamine, 88% base. Pseudo first order rate constants were obtained from the relation \( k = 0.693/t_{1/2} \), and half-times that were obtained from linear semilogarithmic plots of \( A_{0} - A_{t} \) against time. Free thiol concentrations were determined spectrophotometrically with DTNB (16). Micromolar solutions of AcAcCoA for use in enzyme reactions were prepared by dilution with 5 mM sulfuric acid of a stock solution that was standardized spectrophotometrically at 310 nm, based on \( \epsilon = 9300 \) M⁻¹ cm⁻¹ at pH 8.1 (1).

Enzyme activity was measured spectrophotometrically by following the linear decrease in absorbance of the enolate of 50 to 100 μM AcAcCoA at 310 nm in 67 mM Tris/sulfate, pH 8.1, 5 mM magnesium sulfate, 10 mM potassium succinate, 25°C (1). Typically, an aliquot of a reaction mixture containing enzyme was diluted 150- to 600-fold, to a final enzyme concentration of <2 mM, into 3.0 ml of assay mixture lacking AcAcCoA. AcAcCoA, 5 to 10 μl, was then added to initiate the assay. Identical rates were found when both succinate and sulfate, 10 mM potassium succinate, 25°C (1). Typically, an aliquot of this solution was assayed for activity under standard conditions. The reaction rate with acetate was estimated by drawing tangents to the observed absorbance change and extrapolating the slopes of these tangents to zero time.

The slow reaction of acetate with ECoA was conveniently followed by utilizing the inactivation of ECoA by AHA to measure the amount of remaining ECoA. Each reaction mixture contained 100 mM Tris/sulfate buffer, 2 mM EDTA, 0.25 mM potassium acetate, 1.2 mM AcAcCoA, and 0.6 μM CoA transferase in a volume of 50 μl at 25°C. The appearance of free enzyme was measured with 10-μl aliquots that were added to 25 μl of 5 mM AHA in 100 mM Tris/sulfate at pH 8.1 to inactivate the remaining ECoA. After 2 to 5 min, 20 μl of this solution was assayed for activity under standard conditions. The reaction rate with acetate was found to follow first order kinetics in each run. Control experiments showed that the rate of hydrolysis of ECoA was <10% of \( k_{cat} \) at pH 8.2 and 8.8 and that the incubation of free enzyme with AHA under these conditions gave a 25% loss of enzyme activity. Since this loss was constant for every time point, it did not affect the half-time or first order rate constants.

The remaining tritium-labeled EAH in kinetic experiments was measured in a scintillation counter after precipitation of the protein with cold trichloroacetic acid. Each sample was centrifuged and the pellet was washed once with 3 ml of 15% trichloroacetic acid. The pellets were dissolved in 0.5 ml of 0.5 M sodium hydroxide and then neutralized with 0.05 ml of 5 M hydrochloric acid. The samples were counted in an 8.1 mixture of New England Nuclear formula 949 scintillation fluid/Biosolve. The zero time sample (prepared by adding labeled protein and reaction mixture separately to cold quenching solution) had 5300 cpm and the samples were corrected for background (28 cpm).

The pH values of small volumes of reaction mixtures containing enzyme were generally estimated from the measured pH of larger volumes of mock reaction mixtures lacking enzyme.

RESULTS

Reversible Inactivation of ECoA by Acetohydroxamic Acid—Acetohydroxamic acid, AHA, reacts with the ECoA form of CoA transferase, formed from enzyme and AcAcCoA (1), to give a catalytically inactive enzyme (open circles, Fig. 1). Addition of coenzyme A to a concentration of 43 μM causes reactivation to 70% of the original activity (it will be shown below that the absence of complete reactivation is the result of an equilibrium between the ECoA and the EAH forms of the enzyme). AHA has no effect on the free enzyme (closed circles, Fig. 1).

The inactivation reaction follows pseudo-first order kinetics for >90% reaction in the presence of a given concentration of AHA (Fig. 2). This is the behavior that is expected if the product of the reaction has no significant activity. The observed first order rate constants increase linearly with increasing AHA concentration up to \( 5 \times 10^{-4} \) M (Fig. 2, inset) and give a second order rate constant of \( k_{2} = 860 \) M⁻¹ min⁻¹ for the inactivation reaction at pH 8.1. No evidence of saturation was

![Fig. 1. Reversible inactivation of CoA transferase by AHA in the presence of AcAcCoA](http://www.jbc.org/content/journal/jbc/125/1/fig-f1)

![Fig. 2. Dependence of the rate of inactivation of ECoA on AHA concentration](http://www.jbc.org/content/journal/jbc/125/1/fig-f2)
observed at AHA concentrations up to 1.5 mM; this is not surprising in view of the $K_v$ value of 23 mM for acetate as a substrate (1).

The specific inactivation of the ECoA form of the enzyme can be utilized to titrate the number of active sites in a sample of enzyme by forming ECoA from known concentrations of AcAcCoA that are less than stoichiometric. The same technique has been used previously to titrate the enzyme by inactivating ECoA with a nonspecific thiol (3). The titration shows that complete inactivation requires 2 mol of AcAcCoA/mol of enzyme (Fig. 3), consistent with previous results with inactivating ECoA with a nonspecific thiol (3). The titration of other techniques and the dimeric structure of the enzyme (3, 14, 15).

Acetoacetate protects against inactivation of ECoA by AHA, presumably by binding to the active site and shielding against attack by AHA. Increasing the total concentration of AcAc and AcAcCoA from 0.095 to 9.5 mM results in a 6-fold decrease in the rate of inactivation (Table I). At the higher concentration, most of the enzyme exists as the Michaelis complex ECoA-AcAc (14).

The first order rate constants for the inactivation of ECoA by 0.25 mM AHA increase with increasing pH (Fig. 4, open circles). This behavior is qualitatively consistent with participation of the hydroxamate anion in the reaction; however, the dependence of $k_{obs}$ on pH is less steep than is predicted by the ionization constant of AHA (pK$_v$ = 9.4). If AHA reacts as a substrate analog, its reaction rate would be expected to be affected by the dependence of enzyme activity, as well as the concentration of AHA anion, on pH. The closed circles in Fig. 4 show that the activity of the enzyme toward acetate, a (poor) substrate that resembles AHA, decreases with increasing pH and follows an apparent pK$_a$ of 8.7 under these conditions (which correspond to $k_{act}/K_{act}$ for the acetate reaction). The solid line for the AHA reaction is calculated for the reaction of AHA (pK$_a$ = 9.4) as the anion with the active form of the enzyme (pK$_a$ = 8.7) and is consistent with the observed dependence of the rate of inactivation on pH.

The second order rate constant for the reaction of AHA with the active form of the enzyme is 21,000 M$^{-1}$ min$^{-1}$, which is larger than the corresponding second order rate constant of $k_{act}/K_{act} = 1800$ M$^{-1}$ min$^{-1}$ for acetate as a substrate. However, the rate constants for the reaction of nonspecific carboxylate substrates with ECoA increase with increasing basicity with a value of $pK_v = 1.0$ (1), and the observed rate constant with AHA anion is much smaller than the rate constant of 10$^5$ M$^{-1}$ min$^{-1}$ calculated for a carboxylate substrate of pK 9.4. Thus, the anion of AHA is a poor "substrate" compared with a substituted carboxylate substrate of the same pK.

Reactivation of enzyme in the presence of low concentrations of CoA follows pseudo-first order kinetics (Fig. 5). The observed rate constants increase linearly with increasing concentration of CoA over the range examined, up to 40 mM (inset, Fig. 5), giving a second order rate constant for the reactivation reaction at pH 8.1 of 2.1 X 10$^4$ M$^{-1}$ min$^{-1}$. The reactivated enzyme was found to be completely and irreversibly inactivated by 2 mM sodium borohydride. This is evidence for reactivation of the enzyme to ECoA, not free enzyme, because ECoA (but not free enzyme) is inactivated by reduction with 2 mM sodium borohydride (17). The amount of reactivation of EAHA that is obtainable with CoA was found to decrease gradually upon standing for several hours.

The reversible inactivation of ECoA by AHA was shown to be an equilibrium reaction that follows Equation 6 by measuring the amount of active enzyme in the presence of

$$\text{ESCoA} + \text{AHA} \rightleftharpoons \text{EAHA} + \text{CoASH}$$

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**Table I**

Protection of the Michaelis complex of CoA transferase, ECoA-AcAc, against inactivation by AHA

<table>
<thead>
<tr>
<th>[AcAc]</th>
<th>[AcAcCoA]</th>
<th>$k_{obs}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0845</td>
<td>0.01</td>
<td>0.38</td>
</tr>
<tr>
<td>8.45</td>
<td>1.0</td>
<td>0.064</td>
</tr>
</tbody>
</table>
"buffers" containing an excess of AHA and CoA and approaching equilibrium from both directions, as shown in Fig. 6. The equilibrium constant is then given by the ratio of the active to active enzyme and the concentrations of AHA and CoA according to Equation 7. The data in Fig. 6 give a value of $K_{eq} = 0.039$ (pH 8.1, 25°C), which agrees with the value obtained from the ratio of the rate constants in the forward and reverse directions of $K_{eq} = k_1/k_r = 0.041$. The amount of reactivation by CoA shown in Fig. 1 is also consistent with a value of $K_{eq} = 0.040$. Equilibrium constants of 0.026, 0.038, and 0.069 were obtained at pH 7.6, 8.0, and 8.6, respectively. The dependence of these apparent equilibrium constants on pH is consistent with an ionization of EAHA with $pK_a = 7.8$ and a pH-independent equilibrium constant for the uncharged reactant of $K = 0.018$.

Chemical Nature of the Inactive Enzyme—EAHA was shown to be an anhydride composed of the carboxyl group of the active site glutamyl residue that forms ECoA (17) and the terminal oxygen atom of AHA by comparing its chemical reactivity with the reactivity of model compounds in 8.5 M urea. Under these conditions, any special reactivity of the anhydride linkage in EAHA caused by its location at the active site of the enzyme should be lost and, since the $pK_a$ of the γ-carboxyl group of the active site glutamyl residue is close to that of acetic acid, the reactivity of EAHA should be close to that of the corresponding acetate derivative (17-19). The anhydride bond with AHA could be through either the terminal oxygen or the nitrogen atom of AHA, corresponding to $N,O$-diacylhydroxylamine, 1, or $N,N$-diacylhydroxylamine, 2, respectively. The reactivities of 1, 2, and labeled EAHA, prepared by the reaction of ECoA with tritiated AHA, were compared in the presence of 1.06 M imidazole (60% base), 0.106 M ethylenediamine to trap acetylimidazole, and 8.5 M urea (Fig. 7). The pseudo-first order rate constant of 0.27 min⁻¹ for the reaction of EAHA under these conditions is several times faster than the rate constant of 0.073 min⁻¹ for 2 and is close to the rate constant of 0.39 min⁻¹ for 1; the small difference might be caused by a small steric effect of the peptide chain.

An attempt was also made to determine the atom of AHA that reacts with ECoA by examining the reactions of ECoA with $N$-methylacetoxyhydroxamic acid, 3, and $O$ methylacetoxyhydroxamate, 4, in which the nitrogen or oxygen atom is blocked. These experiments were unsuccessful because neither 3 nor 4 gave a detectable reaction with ECoA at a concentration of 0.05 to 0.10 M, pH 8.1. This result is not surprising because the enzyme is known to have a low activity toward compounds with a methyl group near the reacting atom; for example, the value of $k_{cat}/K_m$ for methylsuccinate is 6100 times smaller than for succinate (1) and a similar rate decrease would make inactivation by methyl-AHA derivatives undetectable. Acylation on the carbonyl oxygen atom of AHA to give 5 is possible in principle, but the product of such acylation is not a known compound and it is not likely that it...
would have the same rate constant as 1 for reaction with imidazole. The hydroxylamine oxygen atom is the normal site of acylation of AHA (30).

The chemical reactivities of N,O-diacetylhydroxylamine and N,N-diacetylhydroxylamine were found to show only small differences from each other under a variety of conditions. Some representative results are shown in Table II.

EHA A was not reduced to irreversibly inactivated enzyme by 100 mM sodium borohydride at pH 8.2. Under the same conditions N,O-diacetylhydroxylamine was reduced about 15% to give AHA, as measured by the ferric chloride assay.

Reaction of a thiol group at the active site with DTNB is faster for EHA A than for free enzyme (ECOO'), but slower than for ECoA (Fig. 8). The first order rate constant for the reaction with EHA A was obtained from activity measurements after removal of the AHA by a preliminary incubation with CoA (the product of the reaction with DTNB is inactive (14)). The pseudo-first order rate constants for inactivation of ECOO', EHA A, and ECoA are 0.060, 0.19, and 0.38 min⁻¹, respectively, in the presence of 0.1 mM DTNB at pH 8.1.

Reactions of ECoA with Succinomono-hydroxamic Acid—Succinomono-hydroxamic acid (SHA) is an analog of the normal substrate, succinate, which contains both hydroxamic and carboxylic acid groups and has the potential to act as both an inhibitor and a substrate. This hydroxamic acid inactivates ECoA with pseudo-first order kinetics. The first order rate constants increase with increasing inhibitor concentration, but the dependence is neither linear nor hyperbolic (Fig. 9). The data are consistent with the model of Equation 8, in which one molecule of SHA inactivates ECoA and binds to form a complex,
ECoA-SHA, and a second molecule of SHA reacts with the ECoA-SHA complex to cause inactivation through a second order reaction at higher SHA concentrations. The solid line in Figure 9 is calculated from Equation 9 using values of 

\[ k_{obs} = \frac{[SHA]}{[SHA] + K_c [k_{cat} + k_{[SHA]}]} \]  

\[ K_c = 2 \text{ mM}, \quad k_{cat} = 0.17 \text{ min}^{-1}, \quad \text{and} \quad k_e = 7.0 \text{ M}^{-1} \text{ min}^{-1} \]  

consistent with the experimental results. The second order rate constant for the reaction of SHA with free ECoA at pH 8.1 is \( k_{cat} / K_c = 85 \text{ M}^{-1} \text{ min}^{-1} \). The model of Equation 8 is supported by the observation that the ECoA-SHA complex, formed in the presence of 40 mM SHA, undergoes inactivation by added 1.5 mM AHA; the products were distinguished by reactivation of ESHA, but not ESHA, in the presence of CoA (see below). This shows that the ECoA-SHA complex is susceptible to inactivation through a second order reaction with an added inhibitor.

SHA also reacts as a substrate with ECoA, causing an enzyme-catalyzed disappearance of AcAcCoA (Table III). The ratio of turnover, \( k_t \), to inhibition, \( k_i \), in the presence of SHA (Equation 10) was determined from the amount of inactive ESHA that was formed from the reaction of a known amount of AcAcCoA, \([\text{AcAcCoA}]_0\), with enzyme, after a small correction for the hydrolysis of ECoA (\( k_h \)). The nonenzymic reaction of SHA with AcAcCoA (\( k_h \), Table III, see “Experimental Procedures”) is relatively slow under these conditions and was neglected. The concentration of ESHA was determined from the initial activity and the activity at the end of the reaction, when all of the AcAcCoA has reacted and inactivation has stopped. The concentrations of AcAcCoA were chosen such that the final concentration of ESHA was near 50% of the total enzyme. The rate constant \( k_t \) was calculated from Equations 11 and 12 using known values of \( k_t \) (Fig. 9) and a value of \( k_h = 0.10 \text{ min}^{-1} \) (14).

The result (Table III) shows that the ratio \( k_t / k_i = 25 \) is constant over the range 2.5 to 9.1 mM SHA; i.e. 25 molecules of SHA react with ECoA at the carboxylate group as a substrate for every one that reacts at the hydroxamate group to give inactive ESHA.

The inactivation of ECoA by SHA is irreversible under the conditions of our experiments. No activity was regained up to 40 min after dilution of ESHA into solutions containing CoA at concentrations up to 1.0 mM and the presence of 1.0 mM CoA was found to have no effect on the rate of inactivation of ECoA by 10 mM SHA. Assuming that SHA reacts according to the mechanism of Equation 5, a limiting value of \( K < 2 \) for the formation of ESHA from SHA and ECoA at pH 8.1 is given by the ratio of rate constants of 85 M\(^{-1}\) min\(^{-1}\) for the forward reaction and <46 M\(^{-1}\) min\(^{-1}\) for the reactivation reaction.

**Other Reactions**—Rate constants for the reaction of 0.1 mM acetyl-CoA with a large excess of AHA were determined spectrophotometrically at 412 nm by trapping the thiol product with 0.4 mM DTNB. The observed pseudo-first order rate constants of 0.043 and 0.067 min\(^{-1}\) at 0.02 and 0.03 mM AHA, respectively, give a second order rate constant of 2.2 M\(^{-1}\) min\(^{-1}\), based on the total concentration of AHA at pH 8.0 (in 0.1 M Tris/sulfate and 2 mM EDTA), and 58 M\(^{-1}\) min\(^{-1}\) based on the concentration of the anion of AHA (\( pK_a = 9.4 \); Ref. 19).

Rate constants for the reaction of CoA with a large excess of N,O-diacetylhydroxylamine were determined at pH 6.95, using the hydroxylamine compound as a buffer, by analyzing the amount of remaining thiol in aliquots of the reaction mixture with DTNB (Table IV). The observed pseudo-first order rate constants at two concentrations of N,O-diacetylhydroxylamine give an apparent second order rate constant of \( k_2 = 0.53 \text{ M}^{-1} \text{ min}^{-1} \), based on the total concentrations of reactants at pH 6.95, 25°C. Correction for ionization of the reactants based on \( pK_a \) values of 6.95 for N,O-diacetylhydroxylamine and 9.6 for CoA (3) gives a value of \( k_2 = 480 \text{ M}^{-1} \text{ min}^{-1} \) for the reaction of the thiol anion of CoA with the uncharged anhydride according to the rate law of Equation 13. The apparent second order rate constant at pH 8.0 is then

\[ \text{Table III} \]

<table>
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<tr>
<th>Conditions</th>
<th>([\text{AcAcCoA}]_0) (\mu\text{M})</th>
<th>SHA (\mu\text{M})</th>
<th>ECoA (\mu\text{M})</th>
<th>ESHA (\mu\text{M})</th>
<th>(k_t) (\text{min}^{-1})</th>
<th>(k_i) (\text{min}^{-1})</th>
<th>(k_t / k_i)</th>
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Anhydride from CoA Transferase

TABLE IV
Rate of the reaction of N,O-diacetylhydroxylamine with 0.64 mM CoA in the presence of 1 mM EDTA at pH 6.95, 25°C

<table>
<thead>
<tr>
<th>N,O-Diacetylhydroxylamine</th>
<th>$k_{obs}$</th>
<th>$k_i$</th>
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<tr>
<td>M</td>
<td>min$^{-1}$</td>
<td>M$^{-1}$ min$^{-1}$</td>
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<tr>
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</tr>
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* $k_i = k_{obs}/[N,O-diacetylhydroxylamine].$

\[
u = k_2[\text{CH}_2\text{CONHOCOCH}_3][\text{CoAS}]
\] (13)

0.98 M$^{-1}$ min$^{-1}$, based on the total concentrations of reactants. The equilibrium constant for the reaction of Equation 14 is

\[
\text{CH}_2\text{COSCO}_A + \text{AHA} \leftrightarrow \text{CH}_2\text{CONHOCOCH}_3 + \text{CoASH}
\] (14)

$= 2.2/0.98 = 2.2$, based on the total concentrations of reactants at pH 8.0; for uncharged reactants the value of $K$ is 0.18. This value is close to the equilibrium constant of 0.14 for acetyl methyl transfer group from N-acetylglutathione to pyridinealdoxime methiodide at 37°C (21).

The thiol ester of methyl 3-mercaptopropionate, a short chain analog of CoA, and the carboxyl group at the active site of the enzyme, ESR, can be prepared by the reaction of ECoA with methyl 3-mercaptopropionate (3). The reaction of ESR with AHA was followed by taking advantage of the much faster reactivation of ESR than of EAHA by CoA, as described in the legend to Fig. 10. The reaction follows pseudo-first order kinetics and the rate constants at 1.8 and 4.5 mM AHA give a second order rate constant of 37 M$^{-1}$ min$^{-1}$ based on total AHA concentration at pH 8.1, 25°C (Fig. 10). The reverse reaction of RSH with ESR to give ESR could not definitely be detected in the presence of 0.1 or 0.2 M RSH; a slow, partial reactivation to 20% of the original activity with 0.1 M RSH gave an estimated upper limit for the rate constant of this reaction of $<=0.008$ min$^{-1}$. This gives an upper limit of $k < 0.08$ M$^{-1}$ min$^{-1}$ for the reverse reaction if it is second order under these conditions. The data of Moore (3) suggest that $K_n$ is $>0.05$ M for RSH and, if a value of 0.05 M is assumed for $K_n$, the second order rate constant for the reaction of RSH with free EAHA is $k_{obs}/K_n = 0.26$ M$^{-1}$ min$^{-1}$. The ratio of the second order rate constants for the forward and reverse reactions gives a limiting value of $K_R > 0.05$ for the equilibrium constant of the reaction (Equation 15). This limiting value $K_R = [\text{ESR}] + [\text{AHA}] = [\text{EAHA} + \text{RSH}]
\] (15)

may be compared with the value $K_n = 170$ that is obtained from the product of the equilibrium constants of $K = 0.040$ for the reaction of Equation 6 and $K = 4200$ for the reaction of Equation 16 (3).

\[
\text{ESR} + \text{CoASH} \leftrightarrow \text{ECoA} + \text{RSH}
\] (16)

DISCUSSION

Formation of a Stable Anhydride with CoA Transferase—The existence of a stable anhydride between acetoxyhydroxamic acid, AHA, and the γ-carboxyl group of a glutamate residue at the active site of CoA transferase is shown most directly by the demonstration that the reaction of ECoA with acetoxyhydroxamic acid is a reversible, stoichiometric chemical reaction that is described by Equation 6. It is known that ECoA is a thiol ester of the γ-carboxyl group of glutamate at the active site (17). Formation of the inactive EHA species requires ECoA and results in the release of 1 mol of CoA. This is demonstrated by the existence of an equilibrium reaction for which an equilibrium constant of $K = 0.040$ was determined directly and from the ratio of the rate constants for the forward and reverse reactions. The formation of ECoA upon the reaction of EHA with CoA was shown by the return of enzyme activity and by the irreversible inactivation of the product by borohydride; ECoA is reduced to an inactive form by borohydride but neither EHA nor free enzyme reacts with borohydride.

All of the possible structural elements of EHA are analogs of the anhydride intermediate that has been postulated for the normal pathway of catalysis (1). The structure involving a bond between the enzyme carboxyl group and the hydroxylamine oxygen atom of AHA (Equation 5) is supported by the closely similar rate constants for the reaction of EHA and of $N,O$-diacetylhydroxylamine with imidazole in 8.5 M urea; $N,N$-diacetylhydroxylamine has a smaller reaction rate. However, the initial formation of an unstable anhydride through the carbonyl oxygen atom of AHA followed by rearrangement to the isomeric oxygen anhydride in 8.5 M urea is not rigorously excluded.

The 400-fold larger rate constant for the reaction of AHA with ECoA compared with acetyl-CoA reflects a modest activation of the enzymic compared with the corresponding nonenzymic reaction. However, the rate constant of 21,000 M$^{-1}$ min$^{-1}$ for the reaction of the anion of AHA with ECoA is 5000 times smaller than the rate constant of 10$^6$ M$^{-1}$ min$^{-1}$ that is expected for a carboxylate substrate of pK 9.4, in spite of the fact that the anion of AHA is an “a-effect” nucleophile.

FIG. 10. Dependence of the rate of EAHA formation from ESR on the concentration of AHA at 25°C. ESR was prepared in reaction mixtures containing 100 mM Tris/sulfate, pH 8.1, 6.6 mM AcCoA, 2.4 μM enzyme, and 36 mM methyl 3-mercaptopropionate (added last) in 50 μl. After 3 min a 5-μl aliquot assayed by Method 2 showed ~50% activity, as expected from the equilibrium constant for ESR formation from ECoA (13). At 9 to 14 min after RSH addition, 30 μl of the first reaction mixture was diluted 9-fold into a second reaction mixture. Final concentrations were 100 mM Tris/sulfate, pH 8.1, 3.1 mM EDTA, 20 mM iodoacetamide, and 1 μM (C), 4.5 μM (A), or 50 mM (●) AHA. (The remaining RSH and CoA were alkylated by iodoacetamide with $t_1/2 < 2$ min under these conditions.) Aliquots of 25 μl were assayed at intervals by three methods. (a) Free enzyme was assayed by Method 2. (b) Free enzyme and ESR were assayed by Method 2 in the presence of 7 μM CoA. ESR and EAHA are reactivated with $t_1/2$ of 0.01 min and 5 min under these conditions. (c) Enzyme, ESR + EAHA were assayed by Method 2 after initial incubation for 4 min in 70 μM CoA, which reactivates both ESR and EAHA. The quantity $100[(rate(b) - rate(a))/(rate(c) - rate(a))]$ is plotted in the figure; the quantity [rate(c) − rate(a)] remained constant throughout each run.

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that has an enhanced chemical reactivity toward acyl compounds compared with carboxylate ions (22, 23). This provides another example of the importance of an exact fit of a substrate into the active site in order to obtain a large amount of catalysis for this enzyme: separation of the carbonyl group of the substrate from the reacting oxygen anion by an intervening nitrogen atom is sufficient to cause a decrease in rate of some 5000 fold. The ECoA species shows a high specificity for anionic nucleophiles that is physiologically advantageous, because most nucleophilic reactions would cause inactivation of the enzyme by forming an unreactive derivative of the active site carboxyl group. Only a few nucleophiles have been shown to react with ECoA and the only uncharged nucleophile that is known to react is water (24). Even the small, reactive hydroxylamine molecule shows no detectable reaction.  

The second order rate constant for the reaction of succinohydroxamic acid, SHA, with ECoA is some 10-fold smaller than that for AHA, although SHA is an analog of the normal substrate succinate and gives an inactive ESHA product, which is so stable that its formation cannot be reversed. This shows that SHA must overcome a barrier in order to react that is not present for the smaller AHA molecule. The chemical reactivity of the two hydroxamic acids with AAcO−CoA is essentially the same and they have similar pKₐ values of 9.6 and 9.4. There appears to be a similar barrier for the binding of succinate itself because the value of Kₐ > 23 mM for succinate is larger than the values of Kₐ ≈ 0.5, 23, and 14 mM for acetate, acetate, and propionate, respectively (1). This barrier appears to be associated with the additional carboxylate group of succinate and SHA, which provides no net binding energy. The carboxylate group does cause an increase in reaction rate of the specific substrate succinate, which has a value of kcat/Km that is 2000 times larger than that of acetate (1). Evidently, the binding energy of this carboxylate group is utilized to bring about an increase in reaction rate with succinate, but cannot do so with SHA, which has one additional atom separating the carboxylate group from the nucleophilic oxygen atom.

It is probable that the barrier for the binding of SHA in a productive mode is so severe that it is forced to bind in a nonproductive mode that does not block reactions of the thiol ester so that a second molecule of SHA can react with a smaller second order rate constant to cause inactivation (Fig. 9). Nonproductive binding is also supported by the relatively small dissociation constant of Kₐ = 2 mM for SHA and the second order rate constant of kcat/Km = 2100 M⁻¹ min⁻¹ for reaction of the carboxylate end of SHA as a substrate. This rate constant is within a factor of 2 of the value expected for a nonspecific substituted acetate (1), which suggests that SHA may react from solution as a nonspecific substrate.

**Stabilization of Intermediates and Transition States**—

The equilibrium constant for the formation of an anhydride from ESR and AHA, Kₑ = 170, is 75 times larger than the equilibrium constant of Kₑ = 2.2 for anhydride formation from acetyl CoA and AHA at pH 8.0. Since the chemical properties of thioesters of CoA and RSH are almost identical (3), this corresponds to a stabilization of the anhydride at the active site of the enzyme by −2.6 kcal mol⁻¹. However, this comparison is perturbed by the different pKₐ values for the ionization of N,O-diacetyldihydroxylamine (6.95) and ESHA (7.8). The pH-independent equilibrium constants of 75 and 0.18 for the enzymic and nonenzymic reactions, respectively, (based on the concentrations of uncharged reactants) give an advantage of 420 fold for the enzymic reaction, which corresponds to a stabilization of the anhydride by −3.6 kcal mol⁻¹ at the active site of the enzyme.

Although these equilibrium constants and stabilization energies are influenced by several factors, it appears probable that the stabilization energy of −3.6 kcal mol⁻¹ for the uncharged anhydride of AHA exists also for anhydrides formed from physiological substrates. Stabilization by the enzyme of the unstable anhydride intermediate will increase the equilibrium concentration of this intermediate during turnover and should make a significant contribution to the catalytic activity of the enzyme. This stabilization is observed in spite of the incorrect structure of the hydroxamic acids, as manifested in their slow reaction rate, and it is likely that there is a considerably larger stabilization for anhydrides of normal carboxylic acid substrates that lack the extra nitrogen atom of hydroxamic acids. Stabilization of such unstable intermediates is analogous to the stabilization of transition states and transition state analogs at the active site and probably is a characteristic property of enzymes, especially when the intermediate is sufficiently unstable that it resembles the transition state (25–29). An analogous stabilization of mixed anhydrides of carboxylic and adenyllic acids has been observed in luciferase and amino acid activating enzymes (30, 31).

The anhydride that is presumably formed from the specific substrate analog SHA is even more stable. It was not possible to estimate the amount of this additional stabilization because the anhydride is too stable to permit measurement of the equilibrium constant for its formation, but the limiting value of Kₑ ≈ 2 means that it is more stable than the anhydride with AHA by at least −2.3 kcal mol⁻¹, giving a total stabilization of more than −5.9 kcal mol⁻¹ at the active site. It is interesting that the ESHA anhydride is more stable than the anhydride with AHA, although the transition state for its formation is less stable than that for AHA. Evidently, the covalent radius of the additional nitrogen atom of SHA prevents a good fit of the transition state for attack of SHA in the active site, but the smaller anhydride is more nearly complementary and binds tightly in the active site.

**States of the Enzyme and Utilization of the Binding Energy of CoA**—

Several lines of evidence indicate that some barrier must be overcome in order that substrates and reagents can react at the active site of CoA transferase and that this barrier is reduced or removed in ECoA and ESR. This barrier and its removal have been interpreted as representing “closed” and “open” states of the enzyme, respectively (3, 14). The increase in the rate of inactivation by DTNB of ESHA compared with free enzyme, while not as large as that observed with ECoA (Fig. 8), suggests that ESHA is at least partly in the open state. It is interesting that even such a small molecule as SHA can hold the active site in a partly open state. However, the lower reactivity of RSH with ESHA (k蠹/Kₑ < 0.26 m⁻¹ min⁻¹, pH 8.1) than of CoA with N,O-diacetylhydroxylamine (k = 0.98 m⁻¹ min⁻¹, pH 8.0) suggests that ESHA may not be completely in the open state.

It has been suggested that the weak binding of CoA to the free enzyme is a consequence of the requirement that the intrinsic binding energy of CoA must be utilized in order to force the enzyme open, so that binding can occur, and that the large equilibrium constant for the formation of ECoA from ESR is observed because ESR is already partly in the open state so that more of the binding energy of CoA can appear directly as binding (3, 14). Moore has determined a value of −4.9 kcal mol⁻¹ for this binding energy from the equilibrium constant for the formation of ECoA from ESR (3). The equilibrium constant for the formation of ESHA from ESR is at least 3500 times larger than for its formation from ECoA. This reflects a stabilization of ECoA relative to ESR by at least −4.8 kcal mol⁻¹, which agrees with the value of −4.9 kcal mol⁻¹ determined by Moore.

Although ECoA is thermodynamically stabilized relative to
ESR, it also has an enhanced chemical reactivity relative to ESR (3). The rate of the reaction of AHA with ECoA is 23 times faster than with ESR; this represents a modest stabilization of the transition state leading to anhydride formation in the ECoA reaction. In the reverse direction, the reaction of EAHA with CoA is at least 95,000 times faster than with RSH, corresponding to a stabilization by CoA of the transition state leading to thiol ester formation by at least -6.8 kcal mol⁻¹. This stabilization represents utilization of the binding energy of CoA to increase a reaction rate.

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