Role of Binding Energy with Coenzyme A in Catalysis by 3-Oxoacid Coenzyme A Transferase†

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ABSTRACT: Succinyl-CoA:3-oxoacid coenzyme A transferase (EC 2.8.3.5), which catalyzes the reversible conversion of succinyl-CoA and acetoacetate into acetoacetyl-CoA and succinate through a covalent enzyme thiol ester intermediate, E-CoA, utilizes binding energy from noncovalent interactions with CoA to bring about an increase in k_{cat}/K_M of $\approx 10^{10}$-fold. The ≈40-fold stronger binding of desulfo-CoA ($K_I = 2.7 \pm 0.7$ mM) compared to desulfopantetheine ($K_I = 110 \pm 15$ mM), both of which inhibit competitively with respect to acetoacetyl-CoA, shows that binding to the nucleotide domain of CoA at the active site provides ca. −2.2 kcal/mol of binding energy to stabilize noncovalent complexes with the enzyme. This is much smaller than the ca. −8.9 kcal/mol that the nucleotide domain contributes to the stabilization of the transition state and the ca. −7.2 kcal/mol that it contributes to stabilizing the E-CoA intermediate [Fierke, C. A., & Jencks, W. P. (1986) *J. Biol. Chem. 261,* 7603−7606]. This shows that most of the $\approx 10^6$-fold increase in k_{cat}/K_M that is brought about by binding to this domain is in k_{cat}, which is increased by a factor of about 10^5. Binding to the central pantoic acid domain of CoA is stronger in the transition state than in the Michaelis complex by ca. −3.4 kcal/mol; this corresponds to an additional increase in k_{cat} of ≈350-fold. Covalent enzyme thiol esters analogous to E-CoA but containing the short-chain CoA analogues *N*-acetylaletheine (NAA) and *N*-acetylcysteamine (NAC) are more stable than the enzyme thiol ester containing pantetheine (E-Pant) by ≈3.5 and ≈4.8 kcal/mol, respectively. Thus, interactions between the pantoic acid domain of CoA and the active site *destabilize* E-CoA by ≈4.8 kcal/mol, ≈1.3 kcal/mol of which arises from interaction with the amide group of the pantoic acid domain and ≈3.5 kcal/mol of which arises from interaction with other portions of the pantoic acid domain. E-Pant is more reactive toward acetoacetate and succinate by a factor of $\approx 10^7$ than E-NAA and E-NAC. This shows that the destabilization caused by these interactions in E-CoA is relieved in the transition state, in which binding to the pantoic acid moiety is strongly favorable with $\Delta\Delta G \approx -5.2$ kcal/mol. Thus, interactions with the nucleotide and pantoic acid domains of CoA play distinct roles in catalysis by CoA transferase: Binding to the nucleotide domain accelerates the formation of E-CoA and stabilizes it relative to the Michaelis complex, but does not significantly contribute to catalysis of the second half-reaction. Binding to the pantoic acid domain also contributes to accelerating the formation of E-CoA; however, binding to this domain *destabilizes* the E-CoA intermediate by ≈4.8 kcal/mol. Relief of this destabilization, coupled with strongly favorable binding to the pantoic acid domain in the transition state, lowers the activation barrier for the second half-reaction by ≈10 kcal/mol, which corresponds to an increase in k_{cat} for the reaction of E-CoA with acetoacetate or succinate by a factor of $\approx 10^7$. This large and critical change in the binding interactions between the enzyme and CoA involves only the small region of the CoA molecule containing the α, β, and γ carbon atoms of the pantoic acid domain and their substituents.

An exceptional feature of enzymes is that, in addition to binding substrates in an appropriate position relative to reactive groups on the enzyme, they also have the ability to utilize binding energy gained from noncovalent interactions with nonreacting portions of the substrate to stabilize the transition state and further increase the rate constant for the reaction (Pauling, 1946). However, in order to achieve efficient catalysis, it is not sufficient to stabilize the transition state; it is also necessary to decrease the activation energy required for reaching the transition state from the ground state Michaelis complex, thus ensuring rapid turnover of the enzyme and a large k_{cat} (Wolfenden, 1972; Lienhard, 1973; Fersht, 1974; Jencks, 1965, 1966, 1975). To achieve rapid turnover and to avoid inhibition of the reaction by excessively strong binding to substrates or products, binding energy must be expressed selectively: strongly in the transition state and only weakly in the ground state complexes with substrate and product. This differential expression of binding energy can be achieved by a combination of stabilizing interactions that exist only in the transition state, or are stronger in the transition state than in the Michaelis complex, and destabilizing interactions that exist in the ground state and are partly or fully relieved in the transition state.

Although there are numerous enzymes for which the operation of one or more of these principles has been

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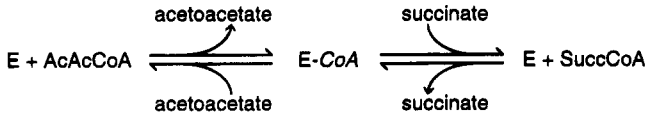
Scheme 1



demonstrated, there are few examples in which the role played by the differential expression of binding energy has been characterized in detail. The enzyme succinyl-CoA:3-oxoacid coenzyme A transferase (CoA transferase,[1] EC 2.8.3.5) provides one example for which a considerable amount of information exists on the utilization of binding energy for catalysis. This enzyme, isolated from pig heart, catalyzes the transfer of CoA between succinate and acetoacetate through a covalent E-CoA thiol ester intermediate involving the carboxyl group of glutamate 344 (Hersh & Jencks, 1967a; Solomon & Jencks, 1969; Rochet & Bridger, 1994). The overall reaction occurs in two steps (Scheme 1): the first half-reaction involves the formation of E-CoA from reaction of the enzyme with a thiol ester substrate, acetoacetyl-CoA or succinyl-CoA, and the second half-reaction comprises the reaction of E-CoA with a carboxylic acid substrate, succinate or acetoacetate, to regenerate the free enzyme (Hersh & Jencks, 1967a; White & Jencks, 1976a).

Truncation of CoA to a smaller thiol, methyl mercaptopropionate (MMP), decreases k_{cat}/K_M for reaction with thiol ester substrates by a factor of 10^{12} (Moore & Jencks, 1982a). This shows that interactions with nonreacting portions of the CoA molecule that are absent from E-MMP are responsible for a major fraction of the total rate acceleration of 10^{16}-fold that is provided by the enzyme (Moore & Jencks, 1982b; Fierke & Jencks, 1986). Moreover, interaction between the enzyme and the pantetheine moiety *destabilizes* the E-CoA intermediate and accelerates the rate of its reaction with carboxylate substrates (Fierke & Jencks, 1986). We report here that the majority of this destabilization is localized to interactions between the enzyme and part of the small pantoic acid domain of CoA that destabilize E-CoA by ≈3.5 kcal/mol, but stabilize the transition states for the formation of E-CoA and for its reaction with carboxylate ions by ca. −6.6 kcal/mol. We also show that the binding energy that is gained from interactions with the nucleotide domain of CoA is expressed strongly in the transition states and the E-CoA ground state, but only weakly in the Michaelis complexes. These and other results permit a detailed characterization of the different roles that the nucleotide and pantoic acid domains of CoA play in contributing to catalysis by CoA transferase.

MATERIALS AND METHODS

Materials. Succinic acid, potassium acetate and disodium ethylenediaminetetraacetate (EDTA) were obtained from Fisher or Fluka, and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and iodoacetamide were from Aldrich and were recrystallized before use. Methyl mercaptopropionate (MMP, Aldrich), diketene (Aldrich), and ethyl acetoacetate (Fluka) were purified by vacuum distillation. Other commercial products include the following: coenzyme A dilithium salt (P. L. Biochemicals or CalBiochem, 94−95% pure); deamino-CoA and dephospho-CoA (P. L. Biochemicals, 93 ± 5% pure); desulfo-CoA (Sigma, 90−95% pure); disodium adenosine 3′,5′-diphosphate (3′,5′-ADP, Sigma, 98% pure); disodium adenosine 5′-monophosphate (5′-ADP, Sigma, 99% pure); carbobenzoxy-β-alanine (Vega); cystamine dihydrochloride (Aldrich); sodium pantothenate (Sigma); and ethylamine (70 wt % solution in water, Aldrich).

The concentrations of stock solutions of desulfo-CoA were determined spectrophotometrically (ϵ_{260} = 15 000; Stewart et al., 1968). Potassium acetoacetate was prepared from ethyl acetoacetate by the method of Seeley (1955), and its concentration was determined by the method of Walker (1954). Acetoacetyl-CoA (AcAc-CoA), acetoacetyldeamino-CoA, and acetoacetyldephospho-CoA were prepared from the reaction of CoA or the appropriate CoA derivative with diketene, as described previously for CoA (Moore & Jencks, 1982a); excess free CoA was removed by treatment with iodoacetamide when necessary. Succinyl-CoA was prepared by the reaction of CoA with succinic anhydride (Moore & Jencks, 1982a). *N,S*-Diacetylcysteamine was prepared by the method of Gerstein and Jencks (1964). Aletheine was prepared from the reaction of carbobenzoxy-β-alanine and cystamine (King et al., 1953) and was converted to *N,S*-diacetylaletheine by heating with acetic anhydride, as described by Baddiley and Thain (1952). The product was purified on a silica gel column, and its identity was verified by its melting point (143 °C) (Tarbell & Cameron, 1956) and by NMR [^{1}H NMR ($CDCl_3$) δ 1.97 (s, 3 H), 2.36−2.46 (m, 5 H), 2.9−3.1 (t, 3 H), 3.3−3.6 (m, 4 H), 6.4 (s, 2 H)]. *N,S*-Diacetylcysteamine and *N,S*-diacetylaletheine were converted to *N*-acetylcysteamine (NAC) and *N*-acetylaletheine (NAA), respectively, with aqueous potassium hydroxide (Gerstein & Jencks, 1964) and purified on a Dowex-1-Cl column. The concentration of free thiol was measured by the DTNB assay of Ellman (1959), using a value of ϵ_{412} = 14 150 M^{-1} cm^{-1} for the extinction coefficient of the 2-nitro-5-thiobenzoic acid dianion (Riddle et al., 1979). The concentrations of thiol esters of acetoacetic acid were determined from their absorbance at 310 nm in 5 mM $MgSO_4$/67 mM Tris−SO_4 (pH 8.1) at 25 °C (ϵ_{310} = 9300 M^{-1} cm^{-1}; White & Jencks, 1976a). Pantoamide was prepared by a minor modification of method B of Sakuragi and Kummerow (1956).

Desulfopantetheine was prepared by couping pantothenic acid, obtained by extracting 20 mL of an acidified solution of sodium pantothenate into 300 mL of ethyl acetate, and 1.5 equiv of ethylamine (70 wt % in water) with 1.3 equiv each of dicyclohexylcarbodiimide and 1-hydroxybenzotriazole in tetrahydrofuran. The crude product was precipitated with hexanes (Fisher), the solid was separated by filtration and dissolved in 50 mL of water, and the pH was adjusted to 8.5 with 1 M NaOH. This solution was extracted three times with 300 mL of ethyl acetate, the extracts were dried over magnesium sulfate and evaporated, and the resulting clear oil was purified on a silica gel column by flash chromatography (5:1 methylene chloride/methanol) [^{1}H

[1] Abbreviations: CoA transferase or E, succinyl-CoA:3-oxoacid coenzyme A transferase (EC 2.8.3.5); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); 3′,5′-ADP, disodium adenosine 3′,5′-diphosphate; 5′-ADP, disodium adenosine 5′-monophosphate; AcAc-CoA, acetoacetyl-CoA; Succ-CoA, succinyl-CoA; AcAc, acetoacetic acid; AcAc-, acetoacetyl; Succ, succinic acid; Succ-, succinyl; NAC, *N*-acetylcysteamine; NAA, *N*-acetylaletheine; MMP, methyl mercaptopropionate; Pant, pantetheine; RSH, any of these thiols; E-CoA, E-NAC, E-NAA, E-MMP, E-Pant, and E-SR, enzyme thiol esters containing, respectively, CoA, *N*-acetylcysteamine, *N*-acetylaletheine, methyl mercaptopropionate, pantetheine, or any of the above thiols covalently bound in the active site.

NMR ($CDCl_3$) δ 0.77 (d, 6 H), 1.13 (t, 3 H), 2.28 (t, 2 H), 2.90 (q, 2 H), 3.2−3.4 (m, 4 H), 3.84 (s, 1 H)].

CoA transferase was purified from pig hearts to a specific activity of 285 μmol of AcAc-CoA/min/mg (Moore & Jencks, 1982a). The concentration of the pure protein was calculated from its absorbance at 280 nm (ϵ_{280} = 0.962 mL/mg; White & Jencks, 1976b). CoA transferase slowly loses activity upon prolonged storage at −70 °C; enzyme that was found to have decayed to a specific activity of less than ≈170 μmol of AcAc-CoA/min/mg was repurified on a blue dextran affinity column to remove inactive protein (Moore & Jencks, 1982a). The enzyme used in these experiments was the native form containing two intact subunits, not the proteolytically clipped, but still active form of the enzyme that has sometimes been observed (Moore & Jencks, 1982a; Lin & Bridger, 1992).

Preparation of Enzyme Thiol Esters. The enzyme-CoA thiol ester, E-CoA, was formed by reaction of ca. 1.2 molar equiv of AcAc-CoA with each active site of CoA transferase at room temperature in 0.1 M Tris−SO_4 (pH 8.1) (White et al., 1976). E-MMP was prepared by successive additions of MMP and iodoacetamide to E-CoA, as described by Fierke and Jencks (1986), and was purified on a gel filtration centrifuge column (Penefsky, 1979). E-NAC and E-NAA were prepared similarly by substituting NAC or NAA for MMP. By using this procedure, ≥90% of the CoA transferase was routinely converted to these enzyme forms. Tests for the presence of iodide ion in the effluent from the column, and for the presence of free thiol when the preparation was carried out without iodoacetamide treatment, showed that the centrifuge column was >99.99% effective in removing small molecule contaminants present in the buffer.

Kinetics. Steady state kinetic measurements were carried out by following the initial linear decrease in the absorbance of AcAc-CoA at 310 nm in the presence of 2.4 nM CoA transferase, 8.3 mM succinate, 45−190 μM AcAc-CoA, 0.1 M Tris−SO_4, and 2 mM EDTA at pH 8.1 and 25 °C, with the ionic strength maintained at μ = 0.3 or 1.0 M with Na_2SO_4 (White & Jencks, 1976a). Under these conditions, the extinction coefficient for acetoacetyl-CoA was determined to be ϵ_{310} = 4850 M^{-1} cm^{-1} at μ = 0.3 M and ϵ_{310} = 5200 M^{-1} cm^{-1} at μ = 1.0 M for solutions that were standardized using an extinction coefficient measured under similar conditions by White and Jencks (1976a).

The formation and reactions of the inactive enzyme thiol esters E-MMP, E-NAC, and E-NAA were monitored by following changes in the activity of CoA transferase in aliquots of the reaction mixtures. The activity was measured in assays containing 50 μM iodoacetamide-treated AcAc-CoA, 10 mM potassium succinate, 0.1 M Tris−sulfate, 0.5 mM EDTA, 0.2 mM iodoacetamide, and 5 mM magnesium sulfate at pH 8.1 and 25 °C (Hersh & Jencks, 1967b; Moore & Jencks, 1982a); under these conditions, the extinction coefficient for acetoacetyl-CoA is ϵ_{310} = 9300 M^{-1} cm^{-1} (White & Jencks, 1976a). To measure first-order rate constants for enzyme inactivation or reactivation, the appropriate form of CoA transferase was diluted into the reaction mixture, aliquots were removed at various times, and the enzyme activity was assayed as described earlier. To measure total enzyme activity, the inactivated enzyme was incubated with assay buffer containing 50 μM CoA for 4 min to convert it to an active form, E-CoA, before initiating turnover by adding AcAc-CoA. E-MMP was specifically reactivated by adding 1 μM CoA to the assay. Some reaction mixtures contained components that, after dilution into the assay solution, were found to inhibit the turnover of active enzyme by a small amount; in such cases the initial velocities were corrected for this inhibition by an appropriate factor, which was determined in a separate experiment. First-order rate constants were calculated by fitting the data to an exponential equation by nonlinear regression analysis using Enzfitter (Leatherbarrow, 1987) or Kaleidagaph (Synergy Software). In all cases the reactions followed first-order kinetics for ≥3 half-lives. Second-order rate constants were calculated from the slope of a plot of k_{obs} against the concentration of inhibitor or reactivator.

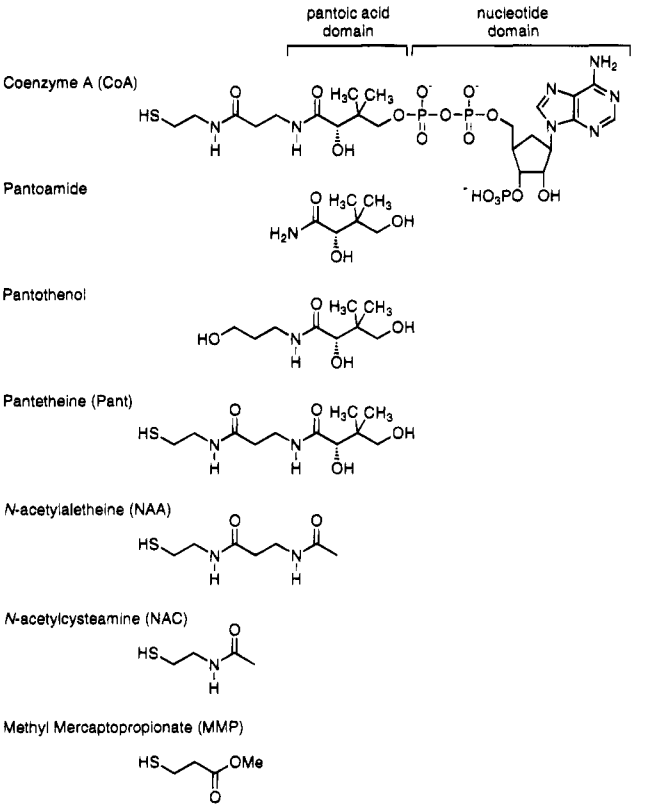
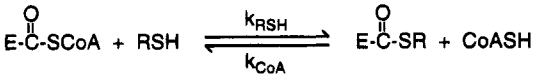


FIGURE 1: Structures of coenzyme A and of the truncated analogues of CoA used in these experiments.

Scheme 2



RESULTS

Reversible Inactivation of E-CoA by Thiols. *N*-Acetylcysteamine (NAC) and *N*-acetylaletheine (NAA), short-chain analogues of coenzyme A (Figure 1), react with the enzyme thiol ester E-CoA to form a catalytically inactive enzyme, E-SR (40−200 nM enzyme, 0−45 mM RSH, 0.1 M Tris−sulfate buffer, and 0.5 mM EDTA at pH 8.1 and 25 °C; Scheme 2). Similar inactivation has been observed previously with methyl mercaptopropionate (MMP) (Moore & Jencks, 1982a). No inactivation was observed when the free enzyme was incubated with either NAC (0.31 mM) or NAA (0.5 mM) in 0.1 M Tris−sulfate (pH 8.1, 25 °C) until a low concentration of acetoacetyl-CoA (AcAc-CoA) was added to convert the enzyme to the E-CoA thiol ester (Hersh &

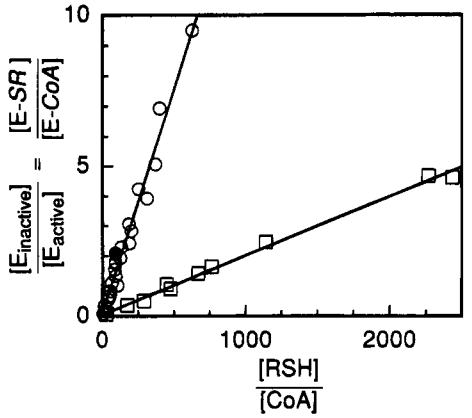


FIGURE 2: Formation of inactive E-NAC or E-NAA from E-CoA at equilibrium. A 2.5−5-fold molar excess of AcAc-CoA was added to CoA transferase (0.04−0.2 μM), followed immediately by the addition of CoA (0−500 μM) and either 0−45 mM NAC (○, ●) or 0−24 mM NAA (□), in 0.1 M Tris−sulfate/0.5 mM EDTA, pH 8.1, 25 °C. $[E]_{active}$ was measured by diluting a 10 μL aliquot of the reaction mixture into an assay solution and measuring the initial rate of the absorbance decrease at 310 nm, as described in Materials and Methods (final conditions: 55 μM AcAc-CoA, 10 mM potassium succinate, 0.2 mM IAA, 5.5 mM $MgSO_4$, 0.5 mM EDTA, and 0.1 M Tris−sulfate, pH 8.1, 25 °C). This measurement was repeated at intervals, up to 60 min after the addition of thiols, to show that the activity was stable. The total enzyme activity was measured by diluting enzyme into assay buffer containing 50 μM CoA and incubating for 4 min before assaying activity. $[E]_{inactive}$ was calculated from $[E]_{TOT} - [E]_{active}$. The solid circle denotes a reaction containing 24 mM 3′,5′-ADP in addition to the other components.

Jencks, 1967a). The fraction of enzyme that is inactivated is linearly dependent on the concentration of added AcAc-CoA; complete inactivation is observed with 2 mol of AcAc-CoA/mol of enzyme, consistent with the dimeric structure of CoA transferase (White & Jencks, 1976b). This inactivation is stable for several hours and requires a free sulfhydryl group on the inactivating reagent; incubation of E-CoA with the same concentration of either *N,S*-diacetylcysteamine or *N,S*-diacetylaletheine has no effect on enzyme activity. CoA transferase that was inactivated by incubation with NAC or NAA is rapidly reactivated upon the addition of CoA, and the extent of reactivation depends on the ratio of the concentrations of CoA and added thiol (NAC or NAA). Thus, all of the data are consistent with the reaction of the thiol group of NAC or NAA with the E-CoA thiol ester to form a new, inactive E-NAC or E-NAA thiol ester at the active site of the enzyme (Scheme 2).

Figure 2 shows that, upon incubation of E-CoA with NAC or NAA, the ratio of inactive to active enzyme reached at equilibrium is linearly dependent on the ratio of the concentrations of RSH and CoA. When the total concentration of thiol is varied at a constant radio of [RSH]:[CoA] (0.4−46 mM RSH, 4−500 μM CoA), the ratio of inactive to active enzyme remains constant. This shows that there is no significant noncovalent binding of RSH or CoA to the enzyme that affects the activity. The slopes of the lines in Figure 2 define the equilibrium constants for the formation of E-SR from E-CoA and RSH (Scheme 2), $K_{Eq}^{E\text{-}NAC} = (1.6 \pm 0.1) \times 10^{-2}$ and $K_{Eq}^{E\text{-}NAA} = (2.0 \pm 0.1) \times 10^{-3}$, and show that the stability of the enzyme thiol esters decreases in the order E-CoA > E-NAC > E-NAA. The equilibrium constants for the formation of a series of enzyme thiol esters are given in Table 1. Addition of 24 mM 3′,5′-adenosine diphosphate to an equilibrium mixture of E-NAC and E-CoA (10 mM NAC and 0.1 mM CoA) did not affect the ratio of inactive to active enzyme (Figure 2). This suggests that there is no significant binding of 3′,5′-ADP to either E-NAC or E-CoA ($K_D \geq 50$ mM), although it does not exclude the unlikely possibility that it binds to both enzyme species with equal affinity.

Table 1: Reactivity of Thiol Esters of CoA Transferase with Thiols[a]

RSH[b]	$K_{Eq}^{E\text{-}SR}$ [c]	k_{RSH}[d] (M^{-1} s^{-1})	k_{CoA}[e] (M^{-1} s^{-1})
CoA	1		
Pant	8×10^{-6} [f]	0.027 ± 0.007[g]	3.4×10^3 [h]
NAA	$(2.0 \pm 0.1) \times 10^{-3}$	8.2 ± 1.5	$(4.5 \pm 0.3) \times 10^3$ [i] ($(4.2 \pm 0.2) \times 10^3$)[j]
NAC	$(1.6 \pm 0.1) \times 10^{-2}$	67 ± 10	$(3.3 \pm 0.3) \times 10^3$ [i] ($(3.3 \pm 1.7) \times 10^3$)[j]
MMP	2.4×10^{-4} [k]	330[l]	$(1.3 \pm 0.1) \times 10^6$ [m]

[a] 0.1 M Tris−sulfate/0.5 mM EDTA, pH 8.1, 25 °C. [b] Thiols are coenzyme A (CoA), pantetheine (Pant), *N*-acetylaletheine (NAA), *N*-acetylcysteamine (NAC), and methyl mercaptopropionate (MMP). [c] Equilibrium constant for the formation of E-SR from the reaction of E-CoA and RSH, measured as described in the legend to Figure 2. [d] Second-order rate constant for the reaction of E-CoA with RSH, measured as described in the legend to Figure 3. [e] Second-order rate constant for the reaction of E-SR with CoA. [f] Determined from $9(k_{(E+AcAcCoA)}/k_{(E\text{-}Pant+AcAc)})$, from Fierke and Jencks (1986). [g] Fierke, 1984. [h] Calculated from $k_{CoA} = k_{RSH}/K_{Eq}^{E\text{-}Pant}$. [i] Measured as described in the legend to Figure 4. [j] Measured as described in the legend to Figure 3. [k] Moore & Jencks, 1982a. [l] Calculated from $k_{RSH} = (K_{Eq}^{E\text{-}MMP})(k_{CoA})$. [m] Calculated as described in the text.

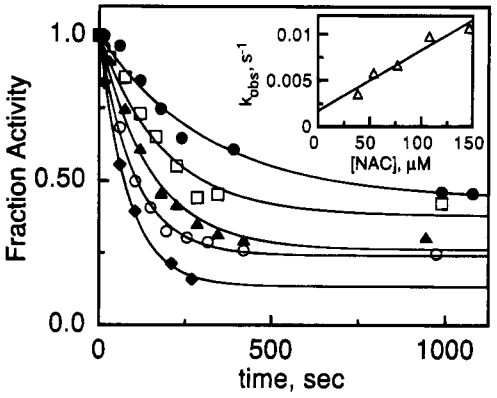


FIGURE 3: Inactivation of E-CoA by NAC. E-CoA was formed by adding AcAc-CoA (0.117 μM) to CoA transferase (0.038 μM) in 0.1 M Tris−sulfate (pH 8.1) and 0.5 mM EDTA, 25 °C in the presence of 0.46 μM CoA. Inactivation was initiated by the addition of NAC: 39 (●), 54 (□), 77 (▲), 108 (○), or 146 μM (◆). At various times, aliquots of the reaction mixture were assayed for activity as described in the legend of Figure 1. Pseudo-first-order rate constants for inactivation were calculated by fitting the data to the equation: $A_t = A_{eq} + (1 - A_{eq})e^{-k_{obs}}$, in which A_t is the activity observed for an aliquot taken at time t, and A_{eq} is the activity observed at the end point of the reaction. Note that as [NAC] increases, k_{obs} increases and A_{eq} decreases. The second-order rate constants for the reaction in each direction, k_{NAC} and k_{CoA}, were calculated from the slope and intercept/[CoA], respectively, of the data shown in the inset plot.

Kinetics of Inactivation and Reactivation. Figure 3 shows that both the observed first-order rate constant for inactivation of E-CoA by thiols and the fraction of inactive enzyme formed at equilibrium increase with increasing concentrations of NAC. Similar behavior was observed for reaction with NAA (data not shown). The second-order rate constants for inactivation, k_{RSH}, and for reactivation by CoA, k_{CoA}, were

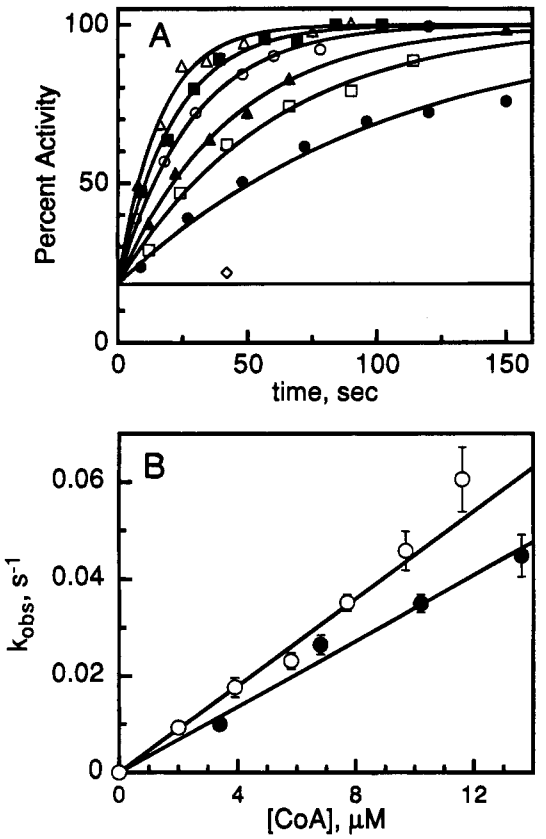


FIGURE 4: Reactivation of E-NAA and E-NAC by CoA. (A) E-NAA was prepared as described in Materials and Methods to give 3.4 μM CoA transferase (80% E-NAA, 20% E; 0.5 mM EDTA/0.1 M Tris−sulfate, pH 8.1) and was used immediately. Aliquots of this solution were diluted 15-fold into 0.1 M Tris−sulfate/0.5 mM EDTA (pH 8.1, 25 °C) with the following concentrations of CoA: 0 (◇), 2.0 (●), 3.9 (□), 5.8 (▲), 7.7 (○), 9.7 (■), or 11.6 μM (△). Aliquots of the reaction mixture were diluted 500-fold into assay buffer and assayed as described for Figure 2. The data were fit to first-order exponentials. (B) Second-order rate constants for reactivation of E-NAA (○) and E-NAC (●) were calculated from the slopes of plots of the pseudo-first-order rate constants for reactivation against the concentration of CoA.

determined from the dependence of the observed first-order rate constants for inactivation of E-CoA on the concentration of RSH (NAC or NAA) in the presence of a fixed concentration of CoA. Under pseudo-first-order conditions (i.e., [RSH], [CoA] ≫ [E]), the observed rate constant for the approach to equilibrium concentrations of E-SR and E-CoA (Scheme 2) is given by $k_{obs} = k_{CoA}[CoA] + k_{RSH}[RSH]$. Under these conditions, k_{obs} is linearly dependent on the concentration of RSH. The slope of this plot is equal to the second-order rate constant k_{RSH}, and the intercept corresponds to $k_{CoA}[CoA]$ (Figure 3, inset; Table 1). The values of k_{RSH} and k_{CoA} are given in Table 1 and are consistent with the values of $K_{Eq}^{E\text{-}SR}$ that were measured directly, as described earlier.

To confirm these rate constants, we also directly measured the rate constants for this reaction in the reverse direction, i.e., the reactivation of E-SR by CoA (Scheme 2), as Figure 4A shows for the reaction of E-NAA. Figure 4A shows that the appearance of enzyme activity follows pseudo-first-order kinetics for >90% of the reaction under conditions in which the reactivation is essentially irreversible. The observed first-order rate constants are linearly dependent on the concentration of CoA (Figure 4B); no evidence of saturation behavior

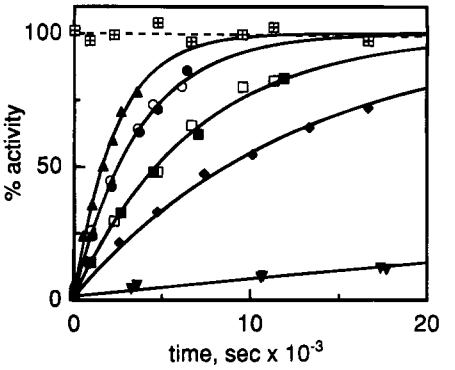


FIGURE 5: Reaction of E-NAC with succinate. E-NAC (3.4 μM) was prepared as described in Materials and Methods. The reaction was initiated by a 5-fold dilution of this solution into 0.5 mM EDTA/0.1 M Tris−sulfate (pH 8.1, 25 °C) containing sufficient potassium succinate to give the following final concentrations: 0 (▼), 0.0625 (◆), 0.125 (■, □), 0.25 (●, ○), or 0.375 M (▲); the open symbols indicate that the ionic strength of the reaction was maintained at 1.5 with sodium sulfate. At various times an aliquot was diluted into assay buffer and assayed for CoA transferase activity, as described in Materials and Methods. The total enzyme activity (crossed boxes) was assayed after incubation in assay buffer containing 50 μM CoA for 4 min prior to the addition of AcAc-CoA. First-order rate constants were calculated from plots of log-(100%−%activity) against time.

is observed. The second-order rate constants for reactivation of E-NAA and E-NAC by CoA, k_{CoA}, were determined from the slopes defined by the data in Figure 4B and are consistent with those determined in the previous experiment; both sets of numbers are given in Table 1.

We also measured a first-order rate constant of 0.23 ± 0.02 s^{-1} for the reactivation of 0.034 μM E-MMP by 0.17 μM CoA in 0.1 M Tris−sulfate buffer/0.5 mM EDTA at pH 8.1 and 25 °C. Under these conditions, the spontaneous hydrolytic reactivation of E-MMP is very slow, and noncovalent binding of CoA is not significant (Moore & Jencks, 1982a). The second-order rate constant for the reaction of 0.17 μM CoA with E-MMP is therefore $k_{CoA} = k_{obs}/[CoA] = (1.3 \pm 0.1) \times 10^6$ M^{-1} s^{-1}. This value of k_{CoA} is ≈10-fold larger than that measured by Moore and Jencks (1982a) for the reactivation of E-MMP in an assay mixture containing CoA and substrates. This difference probably arises from inhibition of the reactivation by CoA in these earlier experiments caused by the binding of AcAc-CoA to E-MMP. Similar inhibition of the CoA-dependent reactivation of E-MMP by acetyl-CoA was observed previously (Moore & Jencks, 1982a).

Reactivity of E-SR with Carboxylate Ions. The inability of the enzyme to catalyze the turnover of E-NAC and E-NAA in a standard activity assay shows that these thiol esters do not react rapidly with succinate. However, Figure 5 shows that E-NAC does reactivate very slowly in the presence of succinate. The appearance of enzyme activity follows pseudo-first-order kinetics for >80% of the reaction. This reactivation is unaffected by the addition of 2 mM iodoacetamide, which reacts with thiols, by the addition of 2 mM borohydride, which specifically inactivates E-CoA (Fierke, 1984), or by a 5-fold dilution of the reaction mixture. This indicates that the reactivation is not caused by a small concentration of E-CoA that was formed from trace amounts of CoA. Incubation of E-NAC with acetoacetate under similar conditions resulted in an analogous slow reactivation of the enzyme. Figure 6 shows that the observed first-order

Table 2: Reactivity of Thiol Esters of CoA Transferase with Carboxylate Substrates

E-SR	succinate		acetoacetate	
	k_{cat}/K_M (M^{-1} s^{-1})	K_M (M)	k_{cat}/K_M (M^{-1} s^{-1})	K_M (M)
E-MMP[a]	$(2.7 \pm 0.5) \times 10^{-4}$	≥1	$(4.0 \pm 0.5) \times 10^{-4}$	0.25 ± 0.03
E-NAC[a]	$(1.2 \pm 0.1) \times 10^{-3}$	≥1	$(3.2 \pm 0.3) \times 10^{-3}$	0.14 ± 0.02
E-NAA[a]	$(1.1 \pm 0.1) \times 10^{-3}$	≥1	$(2.8 \pm 0.2) \times 10^{-3}$	0.12 ± 0.01
E-Pant[b]	1.3×10^4	$\geq 2 \times 10^{-4}$	5×10^4	$\geq 1 \times 10^{-4}$
E-CoA[c]	8.3×10^4	0.02	5×10^5	9×10^{-5}
E-deamino-CoA[d]	2×10^3	≥0.05	nd	nd
E-dephospho-CoA[d]	5×10^4	0.007	nd	nd

[a] 0.1 M Tris−sulfate/0.5 mM EDTA, pH 8.1, 25 °C, measured as described in the legend to Figure 6. [b] Fierke & Jencks, 1986. [c] 67 mM Tris−sulfate, pH 8.1, 25 °C; ionic strength maintained at 1.0 with sodium sulfate (N. Pandy, C. A. Fierke, and W. P. Jencks, unpublished data). [d] Determined from steady state kinetics with 10.4 nM E, 0.1 M Tris−sulfate buffer, 0.08 M sodium sulfate and 2 mM EDTA, pH 8.1, 25 °C.

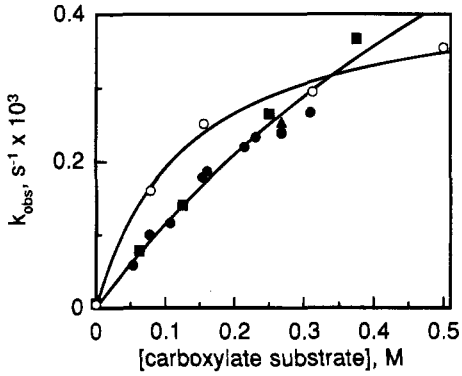


FIGURE 6: Reaction of E-NAC with succinate and acetoacetate. E-NAC (3.4 μM) was diluted 5-fold into 0.5 mM EDTA/0.1 M Tris−sulfate (pH 8.1, 25 °C) containing varying concentrations of either potassium succinate (■), potassium succinate with the ionic strength maintained at 1.5 with sodium sulfate (●), potassium succinate with 30 mM 3′,5′-ADP (+xtus), or potassium acetoacetate (○). The first-order rate constants were determined as described in the legend to Figure 5. The solid lines were calculated from the Michaelis-Menten equation and the rate constants shown in Table 2.

rate constants for reactivation increase with increasing concentrations of succinate or acetoacetate. Saturation behavior is observed at high concentrations of acetoacetate and, to a lesser extent, succinate. Kinetic constants for the reactions of E-NAC with succinate and acetoacetate were obtained by fitting these data to the Michaelis−Menten equation and are shown in Table 2.

Figure 5 shows that the succinate-catalyzed reactivation of E-NAC is insensitive to ionic strength over the range μ = 0.5−1.5 M. However, addition of sodium sulfate to maintain the ionic strength at 1 M was found to increase k_{cat} and K_M for reactivation by acetoacetate by ≈50% to k_{cat} = $(7.6 \pm 1.3) \times 10^{-4}$ s^{-1} and $K_M = 0.20 \pm 0.07$ M (data not shown); k_{cat}/K_M was not affected.

Rate constants for the reactivation of E-NAA and E-MMP by carboxylate ions were also measured, as described earlier for E-NAC (Table 2). The rate constant for the reactivation of E-MMP by succinate is somewhat larger than that previously reported (Moore & Jencks, 1982a) because 18.5 mM potassium iodide was present in the earlier experiments; iodide inhibits reaction of E-MMP with water, succinate, thiols, and sodium borohydride with $K_I = 1.3 \pm 0.2$ mM (Fierke, 1984). The addition of either 30 mM 3′,5′-ADP to E-NAC or 50 mM pantoamide (Figure 1) to E-MMP did not accelerate the reactivation of these enzyme thiol esters by succinate. This shows that these fragments of CoA do not bring about a significant increase in the reactivity of the enzyme thiol ester by binding to the E-SR intermediate at these concentrations.

Table 3: Inhibition Constants for CoA and Derivatives under Steady State Conditions

inhibitor or substrate	K_I (mM)[a]	K_s (mM)
coenzyme A	1.7 ± 0.5 (0−5.2 mM)	
desulfo-CoA	2.7 ± 0.7 (0−2.24 mM)	
acetoacetyl-3′-dephospho-CoA		0.2−1.0[b]
acetyl-CoA		1.7[c]
acetoacetyl-CoA		0.2−1.0[c]
succinyl-CoA		2−7[c]
5′-ADP	45 ± 10 (0−89 mM)	
desulfopantetheine	110 ± 15 (0−100 mM)	
pantothenol[d]	120 ± 15 (0−113 mM)	

[a] Measured as described in Materials and Methods and in the legend to Figure 7, with 2.4 nM CoA transferase, 8.3 mM succinate, 45−190 μM acetoacetyl-CoA, 0.1 M Tris−sulfate buffer, and 2 mM EDTA, μ = 1.0 M with Na_2SO_4 (pH 8.1, 25 °C). Figures in parentheses indicate the range of inhibitor concentrations included in the measurements. In all cases, inhibition was strictly competitive with respect to acetoacetyl-CoA. [b] Calculated according to the method of White and Jencks (1976a), from K_M measured with 5 nM CoA transferase, 2−5 mM succinate, 10−230 μM acetoacetyldephospho-CoA, 0.1 M Tris−sulfate, and 2 mM EDTA, μ = 0.3 M with Na_2SO_4 (pH 8.1, 25 °C). [c] Calculated from the data of White and Jencks (1976a); conditions were 2.1 nM CoA transferase, 0.08 M Tris−sulfate, 5 mM $MgSO_4$, and 0.32 M Na_2SO_4, pH 8.1, 25 °C. [d] See Figure 1.

Deamino-CoA and 3′-Dephospho-CoA. To further investigate the structural features of CoA that determine the high reactivity of the E-CoA thiol ester, we determined the steady state kinetic parameters for catalysis by CoA transferase of reactions of succinate (2−5 mM) with AcAc-deamino-CoA (0.01−1.0 mM) and AcAc-3′-dephospho-CoA (0.01−0.23 mM). The reactions were followed by monitoring the disappearance of thiol ester at 310 nm. The results, given in Table 2, show that removal of the 3′-phosphate group has little effect on k_{cat}/K_M for the reaction of the enzyme thiol ester intermediate, E-SR, with succinate and that removal of the adenosine amino group of CoA reduces k_{cat}/K_M for this reaction by a factor of ≈40.

Inhibition of Steady State Turnover by Fragments and Derivatives of CoA. Equilibrium constants for the dissociation of acetoacetyl-CoA and succinyl-CoA from the enzyme, calculated from the data of White and Jencks (1976a), are shown in Table 3. The very low reactivity of acetoacetyl and succinyl thioesters of NAA, NAC, and MMP precluded direct kinetic determination of the dissociation constants of these truncated substrates, and although acetoacetylpantetheine is reactive enough to allow direct observation of its turnover in the steady state, only a lower limit of $K_M \geq 1$

Table 4: Rate and Equilibrium Constants for the Reaction of Free Enzyme with Thiol Esters of Acetoacetate and Succinate To Form Enzyme Thiol Esters, E-SR

	E + acetoacetyl-SR		E + succinyl-SR	
SR[a]	k_{cat}/K_M (M^{-1} s^{-1})	$K_{Eq(E+AcAcSR)}$	k_{cat}/K_M (M^{-1} s^{-1})	$K_{Eq(E+SuccSR)}$
CoA	1.3×10^7 [b]	9[c]	3.3×10^4 [b]	0.24[c]
Pant	3.7[d]	7×10^{-5} [d]	2×10^{-2} [d]	1×10^{-6} [d]
NAA	5×10^{-5} [e]	2×10^{-2} [f]	5×10^{-7} [g]	5×10^{-4} [h]
NAC	5×10^{-4} [e]	0.14[f]	5×10^{-6} [g]	4×10^{-3} [h]
MMP	9×10^{-7} [e]	2×10^{-3} [f]	2×10^{-8} [g]	6×10^{-5} [h]

[a] Thiols used were coenzyme A (CoA), pantetheine (Pant), *N*-acetylaletheine (NAA), *N*-acetylcysteamine (NAC), and methyl mercaptopropionate (MMP). [b] 67 mM Tris−sulfate, pH 8.1, 25 °C; ionic strength maintained at 1.0 with sodium sulfate (N. Pandy, C. A. Fierke, and W. P. Jencks, unpublished data). [c] White et al., 1976. [d] Fierke and Jencks (1986), or calculated from the rate constants therein. [e] Calculated from $k_{cat}/K_{M(E+AcAcSR)} = K_{Eq(E+AcacSR)}k_{cat}/K_{M(E-SR+acetoacetate)}$, using values for $k_{cat}/K_{M(E-SR+acetoacetate)}$ from Table 2. [f] Calculated from $K_{Eq(E+AcAcSR)} = K_{Eq}^{E-SR}K_{Eq(E+AcAcCoA)}$. K_{Eq}^{E-SR}, the equilibrium constant for the formation of E-SR from the reaction of E-CoA and RSH, is from Table 1; $K_{Eq(E+AcAcCoA)} = 9$ (White et al., 1976). [g] Calculated from $k_{cat}/K_{M(E+SuccSR)} = K_{Eq(E+SuccSR)}k_{cat}/K_{M(E-SR+Succ)}$ and values of $k_{cat}/K_{M(E-SR+Succ)}$ from Table 2. [h] Calculated from $K_{Eq(E+SuccSR)} = K_{Eq}^{E-SR}K_{Eq(E+SuccCoA)}$; K_{Eq}^{E-SR} is from Table 1 and $K_{Eq(E+SuccCoA)} = 0.24$ (White et al., 1976).

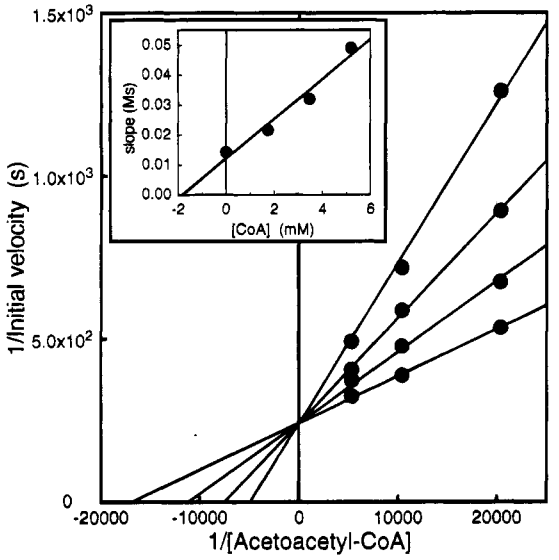


FIGURE 7: Lineweaver−Burk plots showing inhibition by coenzyme A with respect to acetoacetyl-CoA under steady state conditions with 2.4 nM CoA transferase, 8.3 mM succinate, 49−190 μM AcAc-CoA, 0.1 M Tris−SO_4, and 2 mM EDTA, μ = 1.0 M with Na_2SO_4 (pH 8.1, 25 °C). An inhibition constant for CoA of K_I = 1.7 ± 0.5 mM was determined from a plot of the slopes against the concentration of CoA (inset).

mM could be determined for this substrate (Fierke & Jencks, 1986). Information about ground state binding interactions in the enzyme−substrate complexes therefore was obtained by measuring the binding of fragments and derivatives of CoA to the enzyme in steady state inhibition experiments. Figure 7 shows that CoA itself inhibits the initial rate of the CoA transferase-catalyzed reaction of AcAc-CoA with succinate under steady state conditions. The competitive inhibition by CoA with respect to AcAc-CoA indicates that the CoA is binding to the free enzyme and not to E-CoA. An inhibition constant for CoA of K_I = 1.7 ± 0.5 mM was calculated from these data. Inhibition constants that were determined for a number of fragments and derivatives of CoA are reported in Table 3.

DISCUSSION

Expression of Binding Energy with Coenzyme A in the Michaelis Complexes. Coenzyme A and all of the fragments and derivatives of the CoA molecule that were examined as inhibitors of turnover in the steady state inhibit the enzyme competitively with respect to AcAc-CoA. This indicates that these molecules bind to the free enzyme, not to E-CoA, and that binding of the inhibitors to the enzyme prevents the productive binding of AcAc-CoA. These observations are consistent with binding of the molecules listed in Table 3 to the CoA-binding site on the enzyme, as might be expected from their structural similarity to CoA. Further support for this conclusion, at least for inhibition by CoA and desulfo-CoA, is provided by the fact that the dissociation constants for these two inhibitors are similar to the dissociation constants of ≈1 mM found for AcAc-CoA, Succ-CoA, and other CoA-derived substrates listed in Table 3.

The data in Table 3 show that the free enzyme binds desulfo-CoA ≈40-fold more strongly than it binds desulfopantetheine, indicating that the terminal nucleotide domain of CoA provides ca. −2.2 kcal/mol of binding energy [$\Delta\Delta G = -RT \ln(K_1/K_2)$] to stabilize noncovalent complexes of CoA and related compounds with the enzyme. The dissociation constant for the Michaelis complex with AcAc-Pant therefore would be expected to be larger than that for AcAc-CoA by ≈40-fold, i.e., $K_s \approx 8-40$ mM. This is consistent with a lower limit of $K_M \geq 1$ mM that was determined experimentally for AcAc-Pant (Fierke & Jencks, 1986). The similarity of the dissociation constants for AcAc-CoA and AcAc-dephospho-CoA, which lacks the 3′-phosphate group of CoA, shows that this phosphate group does not contribute significantly to the observed binding energy in the Michaelis complex. This result indicates that 5′-ADP is an appropriate analogue of the nucleotide domain of CoA (Figure 1), despite its lack of a 3′-phosphate group. Comparison of the dissociation constant for 5′-ADP with those for CoA and desulfo-CoA shows that deletion of the pantetheine moiety from CoA causes a decrease in affinity for the enzyme of 17−26-fold, which corresponds to a loss of ca. 1.8 kcal/mol of binding energy. Pantothenol, in which the thiol terminal cysteamide portion of pantetheine is replaced by a hydroxyl group (Figure 1), binds with the same affinity as desulfopantetheine, showing that the cysteamide moiety does not contribute to the stability of noncovalent complexes with the enzyme and, therefore, that all of the ca. −1.8 kcal/mol of binding energy that is provided by the pantetheine moiety when CoA is bound noncovalently at the active site arises from binding of the pantoic acid portion of the molecule. The binding energies that are contributed by different portions of the CoA molecule when CoA is binding noncovalently at the active site are summarized in Table 5.

Free Energy Changes for the Formation of E-NAC and E-NAA from E-CoA. The equilibrium constants for the formation of E-NAC and E-NAA from the reaction of E-CoA with NAC or NAA (Table 1) show that the stability of these three thiol esters of the enzyme decreases in the order E-CoA > E-NAC > E-NAA. E-NAC is less stable than E-CoA by ≈2.4 kcal/mol, and E-NAA is less stable than E-CoA by ≈3.7 kcal/mol. These results can be compared with those for E-Pant and E-MMP, which are less stable than E-CoA by ≈7.2 and ≈4.9 kcal/mol, respectively (Fierke & Jencks, 1986; Moore & Jencks, 1982a).

Table 5: Expression of Noncovalent Binding Energy Derived from the Binding of Different Portions of the Coenzyme A Molecule to the Enzyme at Key Points along the Reaction Coordinate

	expressed binding energy (kcal/mol)[a]			
	Michaelis complexes[b]	E-CoA[c]	TS(1)[d]	TS(2)[e]
nucleotide domain[f]	−2.2	−7.2	−8.9	−8.5
pantoic acid domain[f]	−1.8	+4.8	−5.2	−4.9
amide group[g]	N/A[i]	+1.3	+1.4	+1.4
other groups[h]	N/A[i]	+3.5	−6.6	−6.3
total[j]	−4.0[k]	−2.4[j]	−14.1[j]	−13.4[j]

[a] Negative numbers represent stabilizing interactions and positive numbers indicate destabilizing interactions with the enzyme. [b] Binding energy with CoA that is expressed in the Michaelis complexes, E·AcAc-CoA and E·Succ-CoA; calculated from the data in Table 3 as described in the text. [c] Binding energy with CoA expressed in the E-CoA intermediate, calculated from the data in Table 1 as described in the text. [d] Binding energy with CoA expressed in the transition state for the reaction of the enzyme with AcAc-CoA to form E-CoA, calculated from the data in Table 4 as described in the text. [e] Binding energy with CoA expressed in the transition state for the reaction of the enzyme with Succ-CoA to form E-CoA, calculated from the data in Table 4 as described in the text. [f] See Figure 1. [g] The amide group within the pantoic acid domain (Figure 1). [h] Portions of the pantoic acid domain other than the amide group (Figure 1). [i] The data in Table 3 do not allow the effects of binding to smaller regions within the pantoic acid domain of CoA to be separated. [j] The total binding energy derived from nonreacting portions of CoA (i.e., the nucleotide and pantoic acid moieties), calculated from a direct comparison of the experimental data from Tables 1, 2, and 4 for reactions involving CoA and NAC. [k] Obtained from the sum of the binding energies for binding to the nucleotide domain (−2.2 kcal/mol) and the pantoic acid domain (−1.8 kcal/mol).

The large difference in free energy between the enzyme thiol esters E-CoA and E-Pant, which differ in structure only by the presence or absence of the terminal nucleotide domain of CoA (Figure 1), indicates that binding of the nucleotide domain of CoA to the enzyme stabilizes E-CoA by ca. −7.2 kcal/mol (Fierke & Jencks, 1986). However, the data in Table 1 show that E-NAC, which lacks the pantoic acid domain of E-Pant, is more stable than E-Pant by ca. −4.8 kcal/mol. Thus, interaction of the pantoic acid domain with the active site in E-Pant *destabilizes* the enzyme thiol ester by ≈4.8 kcal/mol. Comparison of the equilibrium constants for the formation of E-NAA and E-NAC shows that interaction of the enzyme with the terminal acetamide group of NAA destabilizes E-CoA by ≈1.3 kcal/mol. Thus, the destabilizing effect of the pantoic acid domain in E-CoA comprises an unfavorable interaction of ca. +1.3 kcal/mol with the amide group of the pantoic acid domain (Figure 1) and destabilizing interactions with the remainder of the pantoic acid domain that destabilize E-CoA by an additional amount of 4.8 − 1.3 = +3.5 kcal/mol. The binding energies that result from interactions with different portions of the CoA molecule in the E-CoA intermediate are summarized in Table 5.

This analysis shows that binding of the nucleotide and pantoic acid domains of CoA have opposing effects on the stability of E-CoA. This is illustrated in Figure 8A, which shows overlaid free energy profiles that are normalized to the energy of the free enzyme and free substrate in the ground state, for the reactions of AcAc-CoA, AcAc-Pant, and AcAc-NAC with succinate to give Succ-CoA, Succ-Pant, and Succ-NAC, respectively. Figure 8A shows that E-Pant, which lacks the nucleotide domain of CoA, is ≈7.2 kcal/mol less

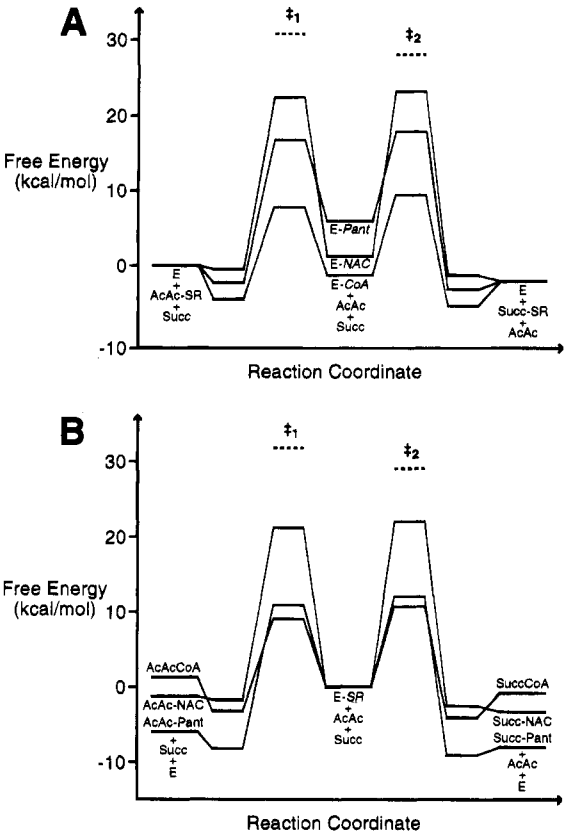


FIGURE 8: (A) Overlaid free energy profiles for catalysis by CoA transferase of the reactions of Acac-CoA, AcAc-Pant, and AcAc-NAC with succinate (Succ) to form acetoacetate (AcAc) and, respectively, Succ-CoA, Succ-Pant, and Succ-NAC. Free energies were calculated from the data in Tables 1−4, as described in the text, and are normalized to the free energy of the ground state containing the enzyme and the free substrates, AcAc-SR and succinate. Energy levels are shown for the following ground and transition states (left to right): enzyme plus free substrates, AcAc-SR and succinate; the Michaelis complex of the enzyme with AcAc-SR; the transition state ($\ddagger_1$) for the formation of the thiol ester intermediate, E-SR, from the reaction of the enzyme with AcAc-SR in the first half-reaction; the enzyme thiol ester intermediate, E-SR; the transition state ($\ddagger_2$) for the reaction of E-SR with succinate in the second half-reaction; the Michaelis complex of the enzyme with Succ-SR; and the ground state containing the enzyme together with the free products, Succ-SR and acetoacetate. The dashed lines that appear above the transition states show the relative free energies of the transition states for the uncatalyzed reactions of AcAc-SR with acetate ($\ddagger_1$) and of acetyl-SR with succinate ($\ddagger_2$), normalized to the free energy of the ground state containing AcAc-SR and free acetate (Moore & Jencks, 1982b). (B) As in part A, but with the free energy profiles normalized to the free energy of the enzyme thiol ester intermediate, E-SR, illustrating the different effects of the pantoic acid and nucleotide domains on the reactivity of E-CoA toward acetoacetate and succinate.

stable than E-CoA when both are compared to the ground state of free enzyme plus free substrate. This shows that binding to the nucleotide domain of CoA is strongly stabilizing. In contrast, E-NAC, which lacks the pantoic acid portion of E-Pant, is *more* stable than E-Pant by ca. −4.8 kcal/mol. This shows that interactions between the enzyme and the pantoic acid domain of coenzyme A are strongly *destabilizing*. Thus, in E-CoA, unfavorable interactions with the pantoic acid domain are offset by strong binding to the nucleotide moiety, resulting in a modest net stabilization of E-CoA compared to E-NAC (Figure 8A). This stabilization favors the formation of E-CoA and acetoacetate from AcAc-CoA and the free enzyme and results in an equilibrium

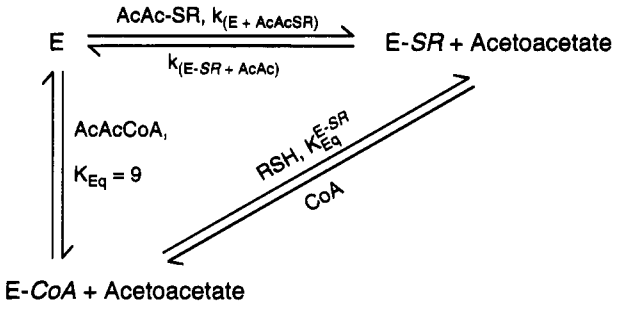
constant of $K_{eq} = 9$ for this reaction (White et al., 1976). This equilibrium constant is approximately 10^2 times larger than the equilibrium constant for the formation of E-NAC from the analogous reaction of the free enzyme with AcAc-NAC (Table 4).

Reactivities of Enzyme Thiol Esters toward Carboxylate Substrates. The values of k_{cat}/K_M for the reactions of succinate and acetoacetate with E-Pant, which lacks only the nucleotide domain of CoA, are within an order of magnitude of those found with E-CoA (Fierke & Jencks, 1986). In contrast, the short-chain enzyme thiol ester E-MMP is 10^8–10^9-fold less reactive than E-CoA toward succinate (Moore & Jencks, 1982a). Table 2 shows that the reactivity of E-MMP with acetoacetate is reduced by a similar factor of $\approx 10^9$ compared with E-CoA. The data in Table 2 also show that the values of k_{cat}/K_M for the reactions of E-NAA and E-NAC with acetoacetate are smaller than those for the corresponding reactions of E-CoA by identical factors of 2×10^8 and that their reactions with succinate are both 8×10^7-fold slower than the reaction of E-CoA with succinate.

These effects on the reactivity of E-CoA are illustrated in Figure 8B, which shows the free energy profiles from Figure 8A normalized to the free energy of the enzyme thiol ester intermediate. Figure 8B also shows that, although removal of the terminal nucleotide domain of CoA has little effect on the reactivity of the thiol ester intermediate toward acetoacetate and succinate (Fierke & Jencks, 1986), removal of the pantoic acid domain from E-Pant, to give E-NAC, causes a reduction in k_{cat}/K_M for the reactions of the enzyme thiol ester with acetoacetate and succinate of $\approx 10^7$-fold. This corresponds to an increase of ≈9.9 kcal/mol in the activation barrier for reaching the transition state. We conclude that binding to the pantoic acid moiety stabilizes the transition state by approximately 10 kcal/mol, relative to the enzyme thiol ester intermediate. This represents a very large increase in the strength with which the enzyme binds to this region of CoA upon reaching the transition state and is responsible for a large fraction of the total rate acceleration of 10^{16}-fold that is brought about by the enzyme (Moore & Jencks, 1982b; Fierke & Jencks, 1986). The essentially identical reactivities of E-NAC and E-NAA toward acetoacetate and also toward succinate (Table 2) show that interaction of the enzyme with the terminal acetamide group of NAA does not contribute significantly to the activation of E-CoA for reaction with carboxylate substrates.

Formation of E-SR from the Reaction of Free Enzyme with Acetoacetyl-SR and Succinyl-SR. AcAc-MMP, AcAc-NAC, and AcAc-NAA, truncated analogues of the substrate AcAc-CoA, show no detectable reaction when incubated with the free enzyme under standard assay conditions. However, the rate constants for the formation of the corresponding enzyme thiol ester intermediates from these compounds can be calculated from the rate constants in Table 2 for the reaction in the reverse direction, i.e., the reaction of the enzyme thiol ester with acetoacetate, and the equilibrium constants for formation of the thiol ester intermediate from AcAc-SR and the free enzyme, $K_{Eq(E+AcAcSR)}$ (eq 1). As shown in Scheme 3 and eq 1, these equilibrium constants, $K_{Eq(E+AcAcSR)}$, can be calculated from the equilibrium constants given in Table 1 for the formation of E-SR from the reaction of E-CoA with RSH ($K_{Eq}^{E\text{-}SR}$), and $K_{Eq} = 9$ for the formation of E-CoA and acetoacetate from the free enzyme and AcAc-CoA

Scheme 3



(White et al., 1976).

$$k_{(E+AcAcSR)} = k_{(E-SR+AcAc)}K_{(E+AcAcSR)} = k_{(E-SR+AcAc)}(9K_{Eq}^{E\text{-}SR}) \quad (1)$$

Rate and equilibrium constants for the formation of E-SR and acetoacetate from the reactions of AcAc-SR with the free enzyme, together with corresponding numbers that were calculated in the same way for the reactions of the free enzyme with Succ-SR thiol ester substrates, are reported in Table 4. Data for the reactions of the free enzyme with AcAcCoA and AcAc-Pant calculated by this method agree well with the direct experimental values that are available for these two substrates (Fierke & Jencks, 1986) shown in Table 4.

The rate constants in Table 4 cover a range of 10^{13}. They show that deletion of the terminal nucleotide domain of AcAc-CoA, to give AcAc-Pant, results in a $\approx 3 \times 10^6$-fold decrease in k_{cat}/K_M; this corresponds to a destabilization of the transition state for the first half-reaction by ≈8.9 kcal/mol. Thus, the nucleotide domain of CoA binds strongly to the enzyme to stabilize the transition state, but only slightly more strongly than the ca. −7.2 kcal/mol that binding to this domain contributes to stabilization of the E-CoA intermediate. Removal of the pantoic acid domain in AcAc-Pant to give AcAc-NAC, decreases k_{cat}/K_M by an additional factor of $\approx 7 \times 10^3$. This difference in reactivity corresponds to a free energy difference of ≈5.2 kcal/mol, i.e, binding of the pantoic acid domain of CoA to the active site stabilizes the transition state for the formation of E-CoA by ca. −5.2 kcal/mol compared to the ground state of free enzyme plus free substrate. Thus, binding of the enzyme to the pantoic acid domain of CoA changes from being strongly favorable in the transition state to being highly unfavorable in E-CoA, which the pantoic acid domain *destabilizes* by ≈4.8 kcal/mol. This change of ≈10 kcal/mol in the affinity of the enzyme for the pantoic acid moiety is almost entirely responsible for the much higher reactivity of E-CoA and E-Pant toward carboxylate substrates, compared with the reactivity of the short-chain enzyme thiol esters E-NAA, E-NAC, and E-MMP. The effects of these changes in substrate structure on the rate of the formation of E-SR from the reaction of the free enzyme with thiol ester substrates are illustrated by the free energy profiles shown in Figure 8A; their corresponding effects on the reactivity of the E-SR intermediate are illustrated in Figure 8B, which shows the free energy profiles shown in Figure 8A normalized to the free energy of the E-SR thiol ester intermediate.

Table 4 also shows that removal of the terminal acetamide group of NAA, to give AcAc-NAC, results in a ≈10-fold

increase, rather than the expected decrease, in k_{cat}/K_M for the formation of the thiol ester intermediate. This result indicates that the binding of this region of the CoA molecule destabilizes the transition state by ≈1.4 kcal/mol, which is similar in magnitude to the destabilizing effect of this interaction in the E-CoA intermediate. Binding of this region of the substrate in the active site therefore appears to slow the formation of E-CoA slightly, but has no effect on the rate of reaction of E-CoA with carboxylate substrates. The observation of a noncovalent interaction with a specific substrate that *destabilizes* the transition state for an enzymatic reaction, as these results imply is the case for the interaction between the enzyme and the amide group in the pantoic acid domain of CoA, is unexpected, although the effect seen here is relatively small. It is possible that slightly unfavorable binding of this region of CoA in the transition state is required in order to allow the pantoic acid and nucleotide domains of CoA, which are covalently linked to the enzyme through this amide group, to interact productively with the active site and give rise to the very much larger changes in the expression of binding energy that their interactions bring about. Alternatively, it is conceivable that the relatively small difference of ≈10-fold between the rate constants for reactions of the NAC and NAA thiol ester substrates results from accumulated experimental uncertainties in the rate and equilibrium constants in Tables 1 and 2, from which these numbers were calculated. Thus, although we cannot state with certainty that interaction with the amide group of the pantoic acid domain causes significant destabilization of the transition state, binding to this region of CoA certainly does not contribute to transition state stabilization.

The data in Table 4 also show that the relative reactivities of Succ-CoA, Succ-Pant, Succ-NAA, and Succ-NAC are virtually identical to the relative reactivities of the corresponding acetoacetyl thiol ester substrates. Thus, the amount of binding energy that is derived from interactions with different portions of the CoA molecule in the transition state is essentially independent of whether the carboxylate substrate involved in the transition state is acetoacetate or succinate, as shown in Table 5.

Utilization of Binding Energy Derived from Binding Interactions with Coenzyme A for Catalysis. Figure 8A and the data in Table 5 show that binding to the terminal nucleotide domain of CoA stabilizes the E-CoA intermediate by ca. −7.2 kcal/mol compared to E-Pant. Binding to the nucleotide domain also stabilizes the transition state for the formation of E-CoA from AcAc-CoA or Succ-CoA by −8.5 to −9 kcal/mol, which corresponds to an increase in k_{cat}/K_M for the formation of E-CoA in the first half-reaction by a factor of $\approx 10^6$. The data in Table 5 show that binding to the nucleotide domain is relatively weak in the Michaelis complex, resulting in a reduction in K_M of only ≈40-fold. Thus, the great majority of the binding energy that is derived from binding of the nucleotide moiety is expressed preferentially in the transition state, where it brings about a reduction of 8.9 − 2.2 = 6.7 kcal/mol in the activation barrier required for reaching the transition state from the Michaelis complex. This difference in Gibbs energy corresponds to an increase in k_{cat} of 8×10^4-fold, which accounts for most of the increase in k_{cat}/K_M that is caused by binding to the nucleotide domain.

Binding to the pantoic acid domain increases k_{cat}/K_M for the formation of E-CoA by an additional factor of ≈7 × 10^3. From the weak binding of this region in the Michaelis complexes (Table 5), we can see that the energy derived from binding to the pantoic acid domain is also expressed preferentially in the transition state and results in an additional reduction of 3.4 kcal/mol in the activation barrier required for reaching the transition state from the Michaelis complex. Thus, most of the increase in k_{cat}/K_M that is brought about by binding to this domain once again arises from k_{cat}, which is increased by a factor of about 350; binding to this domain has a smaller effect on K_M for the thiol ester substrates, which are decreased by only ≈20-fold.

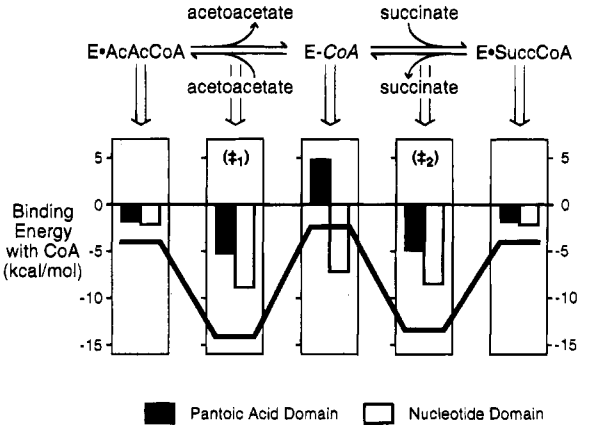


FIGURE 9: Diagram illustrating the expression of noncovalent binding energy derived from interaction of the enzyme with the CoA molecule at key points along the reaction coordinate (from Table 5). Below the reaction scheme are histograms showing the binding energies derived from binding to the pantoic acid domain (black bar) and to the terminal nucleotide domain of CoA (white bar) at key ground and transition states. The bold black line illustrates the total noncovalent binding energy derived from binding to the entire nonreacting portion of CoA, comprising the pantoic acid and nucleotide domains. The figure was constructed using the binding energies from Table 5.

Binding to the nucleotide domain in E-CoA is almost as strong as in the transition state. Interaction of this portion of the CoA molecule with the enzyme thus contributes binding energy, but increases the reactivity of E-CoA toward carboxylate substrates in the second half-reaction by only ≈10-fold (Figure 8B). The pantoic acid domain of CoA, on the other hand, *destabilizes* the thiol ester intermediate by ≈4.8 kcal mol. However, this destabilizing interaction in E-CoA, and its transformation into a strongly stabilizing interaction in the transition state, lowers the activation barrier for the reactions of E-CoA with acetoacetate and succinate by ≈10 kcal/mol and thereby accelerates the second half-reaction to bring about an increase in k_{cat} of $\approx 10^7$-fold, as shown in Figure 8. Binding to the amide group of the pantoic acid domain does not change significantly between the E-CoA intermediate and the transition state (Table 5), and so this large and critical change in the binding of the enzyme to CoA appears to involve only the small region of the CoA molecule containing the α, β, and γ carbon atoms of the pantoic acid domain and their substituents (Figure 1).

Figure 9 summarizes how the individual contributions from binding to the nucleotide and pantoic acid domains combine to give the total binding energy that is gained from binding to the nonreacting portions of CoA in the enzyme−substrate complexes, the E-CoA intermediate, and the transition states. The vertical boxes shown below each element of the reaction scheme in Figure 9 contain bars that show the amount of

binding energy that is obtained at each stage of the reaction from interaction of the enzyme with the pantoic acid domain and with the nucleotide domain of CoA. The bold line in Figure 9 shows the total binding energy that is obtained from noncovalent interactions between the enzyme and nonreacting regions of the CoA molecule; it represents the sum of the binding energies obtained from binding to the individual domains of CoA.

The pattern shown by the bold line in Figure 9 illustrates Pauling's description of catalysis by enzymes in terms of strong binding to the reacting complex in the transition states and weak binding to substrates and products in the ground states (Pauling, 1946). In the case of CoA transferase, however, the apparent simplicity of this pattern is deceptive. This is illustrated by the bars in Figure 9, which represent the individual contributions that are provided by binding to the pantoic acid and nucleotide domains toward the total binding energy that is derived from interactions with the CoA molecule at successive points along the reaction coordinate. It is apparent from Figure 9 that the nucleotide and pantoic acid domains of CoA play distinct roles in the CoA transferase reaction, which are defined by the different points along the reaction coordinate at which their binding energy is expressed. Thus, the changes in the total expressed binding energy that is gained from interactions with CoA are the result of a complex interplay of favorable and unfavorable noncovalent interactions between the enzyme and the different domains of the CoA molecule.

NAC and NAA are truncated analogues of CoA that contain only functionalities that are present in CoA itself; MMP, on the other hand, contains a methyl ester group that is not present in CoA (Figure 1). The fact that E-MMP is ≈2.5 kcal/mol less stable than E-NAC (Table 1) suggests either that E-MMP lacks a favorable binding interaction between the enzyme and the terminal acetamide group of NAC or that the methyl ester group of MMP is involved in unfavorable interactions with the protein that are not present in E-NAC and are not relevant to E-CoA. Calculation of interaction energies based on the truncated enzyme thiol esters E-NAA and E-NAC, rather than E-MMP (Moore & Jencks, 1982a; Fierke & Jencks, 1986), therefore represents a significant improvement in the analysis of the utilization of binding energy by this enzyme and allows the effects of binding of different portions of the pantetheine structure at the active site to be characterized.

Table 5 shows that the strengths of the interactions with the CoA substrate are essentially identical for the formation of E-CoA from either direction, i.e., from the reaction of E with AcAc-CoA or Succ-CoA. This shows that the binding of the enzyme to different portions of the CoA molecule in the ground and transition states of the reaction is independent of the identity of the specific carboxylate substrate.

The very large values of K_M, up to 1 M or more, for the reactions of carboxylate substrates with the nonspecific enzyme thiol ester derivatives E-NAA, E-NAC, and E-MMP (Table 2) emphasize the importance of specific binding interactions of the enzyme with coenzyme A in the catalytic process. These interactions presumably cause a change in the conformation of the enzyme (White et al., 1976; Fierke, 1984; Aldwin, 1980) that converts it to a catalytically active form that binds the carboxylate substrates strongly, with the catalytic groups held precisely in the optimal position for reaction. Our attempts to increase the reactivity of the truncated enzyme thiol esters E-NAC and E-MMP by binding 3′,5′-ADP or pantoamide (Figure 1) to the enzyme were unsuccessful. This suggests that a covalent linkage is required for transmission of the activating effect of the specific, noncovalent interactions with the nucleotide and pantoic acid domains of CoA to the site of the catalyzed reaction and is consistent with the results of previous experiments on CoA transferase (Fierke & Jencks, 1986). However, it contrasts with the behavior seen with phosphoglucomutase by Ray and coauthors (Ray & Long, 1976; Ray et al., 1976), who found that complementing xylose, a poor substrate for phosphoglucomutase, with phosphite results in a reactivity almost equal to that of the specific substrate, glucose 6-phosphate. Thus, although CoA transferase and phosphoglucomutase both exemplify the critical role played by noncovalent binding energy in enzyme catalysis, they appear to utilize two distinct mechanisms for coupling the differential expression of binding energy to the rate of the catalyzed reaction. The detailed mechanisms by which CoA transferase brings about these variations in the expression of binding energy are unknown; however, they surely include changes in the conformation of the enzyme between the free enzyme state and the E-CoA intermediate (White et al., 1976; Fierke, 1984; Aldwin, 1980), as well as changes in the interactions between the enzyme and the substrate that arise from changes in the structure of the reacting complex as it proceeds along the reaction coordinate.

In summary, CoA transferase derives a large fraction of its catalytic effect from the differential expression of binding energy gained from noncovalent interactions between the enzyme and nonreacting portions of the CoA molecule. We have characterized the distinct roles that the nucleotide and pantoic acid domains of CoA play in the catalytic process. We have shown that the nucleotide domain stabilizes the E-CoA intermediate by ca. −7.2 kcal/mol and accelerates its formation by a factor of 10^6 by stabilizing the transition state for the reaction of specific thiol ester substrates with the enzyme in the first half-reaction. Interactions with the pantoic acid domain destabilize E-CoA by ≈4.8 kcal/mol, but stabilize the transition state for its reaction with carboxylate substrates by ca. −5.2 kcal/mol, thereby increasing the rate of the second half-reaction by a factor of 10^8. Binding to each of these domains of CoA is weak in the ground states of the Michaelis complexes, and thus the effects of these changes in the expression of binding energy on k_{cat}/K_M appear almost entirely as increases in k_{cat}. The overall pattern for the expression of binding energy from interactions with nonreacting portions of the CoA molecule involves strong binding in the transition states and weak binding in the E-CoA and Michaelis complex ground states. This differential expression of binding energy is achieved by a combination of mechanisms, including stabilizing interactions that are strong in the transition states but weak in the Michaelis complexes, strongly destabilizing interactions in the E-CoA intermediate that are relieved in the transition states, and strongly stabilizing interactions in the E-CoA intermediate that are offset by other destabilizing interactions to give the relatively weak binding that is observed.

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