Two Functional Domains of Coenzyme A Activate Catalysis by Coenzyme A Transferase

PANTETHEINE AND ADENOSINE 3'-PHOSPHATE 5'-DIPHOSPHATE*

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Carol A. Fierke and William P. Jencks
From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254

Studies of the reactivity of succinyl-CoA:3-keto acid CoA transferase with a small coenzyme A analog, methylmercaptocaptopionate, have shown that noncovalent interactions between the enzyme and the side chain of CoA are responsible for a rate acceleration of ~10^15, which is close to the total rate acceleration brought about by the enzyme (Moore, S. A., and Jencks, W. P. (1982) J. Biol. Chem. 257, 10893-10907). We report here that interaction between the enzyme and the pantetheine moiety of CoA provides the majority of the rate acceleration and destabilization of the enzyme-thiol ester intermediate that is observed with CoA substrates. The role of the adenosine 3'-phosphate 5'-diphosphate moiety of CoA is to provide 6.9 kcal/mol of binding energy in order to pull the pantetheine moiety into the active site. The enzyme-thiol ester intermediate, E-pantetheine, was generated by reaction of pantetheine with the thiol ester of enzyme and methylmercaptocaptopionate. E-Pantetheine undergoes hydrolysis with k_{hyd} = 2 min^{-1}, 140-fold faster than E-CoA, and reacts with acetoacetate with k_{EAc}= 3 x 10^6 M^{-1} min^{-1}, only 10-fold slower than E-CoA. However, in the reverse direction acetoacetyl-pantetheine reacts with CoA transferase (k_{EAc=SP} = 220 M^{-1} min^{-1}) 1.6 x 10^6 times slower than acetoacetyl-CoA. The equilibrium constant for the reaction of pantetheine with E-CoA is ~8 x 10^{-6}.

Enzyme catalysis involves the use of intrinsic binding energy to stabilize the transition state for reactions of specific substrates. It is equally important for catalysis that the intrinsic binding energy should not be expressed in the enzyme-substrate complex; instead, it is utilized to overcome destabilization and loss of entropy so that the transition state can be reached easily (1-5). Enzymes differ from most chemical catalysts in their ability to utilize the intrinsic binding energy of nonreacting groups on specific substrates to bring about large rate increases. For example, noncovalent interactions between the enzyme succinyl-CoA:3-ketoacid CoA transferase (3-oxoacid CoA transferase, EC 2.8.3.5) and nonreacting portions of the coenzyme A molecule provide a rate increase of 3 x 10^12 that is close to the total rate increase of 6 x 10^13 for catalysis by this enzyme (6). We report here that destabilization and the expression of observed binding energy are brought about by different portions of the coenzyme A molecule. Interaction between the enzyme and the pantetheine moiety provides the majority of substrate destabilization and rate acceleration, while the interaction with the 3'-phospho-ADP moiety provides binding energy that overcomes this destabilization and permits significant binding of acyl-CoA substrates to the enzyme.

EXPERIMENTAL PROCEDURES

Materials—Succinic acid, DTNB, IAA, and disodium EDTA were recrystallized, and MMP and diketen were distilled before use. Commercial products include the dilithium salt of coenzyme A (95% pure, P-L Biochemicals), disodium adenosine 3',5'-diphosphate (98% pure, Sigma), and pantetheine (U. S. Biochemicals). Potassium acetate was prepared by the method of Seeley (7) and the concentration was determined by the method of Walker (8). Pantetheine was synthesized by reduction of pantethines with a 10-fold molar excess of sodium borohydride in water at 4 °C, with the pH maintained below 8.5, purified by chromatography on a Sephadex G-10 column, and concentrated by lyophilization. The free thiol concentration was measured with DTNB (10). Acetoacetyl-CoA and acetoacetyl-pantetheine were prepared from diketen and CoA or pantetheine, as described previously (6). Traces of remaining free thiol were removed by reaction with IAA (6). The concentration of acetoacetyl-SR was determined at 310 nm in 67 mM Tris sulfate, 5 mM magnesium sulfate, pH 8.1, 25 °C (9).

CoA transferase was purified from pig hearts to a specific activity of 285 pmol/min/mg (6). For some experiments the protein was concentrated to 10 mg/ml using a Centricon microconcentrator (Amicon Corp.).

Preparation of E-MMP—E-CoA was formed by adding 5 µl of 1.2 mM AcAc-CoA to 405 µl of 6.4 mM CoA transferase, 0.92 mM EDTA, and 0.18 M Tris sulfate, pH 8.1, at 25 °C. To this was added 75 µl of 250 mM MMP, 65 µl of 0.88 mM IAA, and 15 µl of 0.15 M sodium borohydride solution to achieve >60% E-MMP. This was incubated for 2 min, which is >10 times for the reaction of IAA and thiol under these conditions (6). To shift the equilibrium towards E-MMP, 75 µl of 260 mM MMP and 15 µl of 2.5 M Tris were added, followed by incubation for 4 min and addition of 50 µl of 250 mM MMP, 10 µl of 2.5 M Tris, and 5 µl of 0.1 M sodium borohydride solution. Finally, after incubation for 4.5 min, 25 µl of 250 mM MMP and 5 µl of 2.5 M Tris were added and incubated for another 30 min. The E-MMP was purified on a gel filtration centrifuge column as described by Peneffsky (11). A solution of E-MMP (250 µl) was applied to a 2.5-ml Sephadex G-50 (fine) column that had been equilibrated with 0.0 mM EDTA in 0.1 M Tris sulfate, pH 8.1, before centrifugation. This procedure produced E-MMP at final concentrations of 3.4 μM CoA transferase (90% E-MMP) in 0.5 mM EDTA, 0.1 M Tris sulfate, pH 8.1, with <0.6 mM potassium iodide and <44 μM free thiol. Higher concentrations of E-MMP were obtained with shorter periods of centrifugation.

Kinetics—Enzyme activity was measured by following the initial linear decrease in the absorbance of AcAc-CoA at 310 nm under standard conditions: 50 mM IAA-treated AcAc-CoA, 10 mM potassium succinate, 0.1 M Tris sulfate, pH 8.1, 0.5 mM EDTA, 0.2 mM IAA, and 5 mM magnesium sulfate at 25 °C (12, 13). For measurements of the concentration of E + E-MMP the assay buffer also contained 20-50 μM CoA, which reacts rapidly with inactive E-MMP to form active E-MMP.

* The abbreviations used are: 3'-phospho-ADP, adenosine 3'-phosphate, 5'-diphosphate; AcAc, acetoacetate; AcAc-CoA, acetoacetyl-CoA; MMP, methylmercaptocaptopionate; IAA, iodacetamide; SP, pantetheine; 3',5'-ADP, adenosine 3',5'-diphosphate; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); E, enzyme (in this paper, CoA transferase); Suc, succinate.

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Pantetheine catalyzes the formation of active enzyme from the inactive thiol ester derivative, E-MMP, with a second-order rate constant of $k_{sp} = 5.9 \pm 0.3 \text{ M}^{-1} \text{min}^{-1}$. This reaction can be completely inhibited by low concentrations of MMP, as shown in Fig. 1. Since MMP does not bind to E-MMP under these conditions (6), this inhibition requires the formation of an E-pantetheine thiol ester intermediate, E-SP. This intermediate can either hydrolyze to form active enzyme or react with MMP to regenerate inactive E-MMP (Scheme 1) with the rate constants $k_{h}^{yd}$ and $k_{MMMP}$, respectively. The ratio of these rate constants, $k_{MMMP}/k_{h}^{yd} = (1.3 \pm 0.1) \times 10^6 \text{ M}^{-1}$, was obtained from the concentration of MMP necessary to give 50% inhibition of the reactivation rate after correcting for the hydrolysis of E-MMP (dashed line, Fig. 1). The solid line in Fig. 1 was calculated from this ratio and $k_{sp}$.

$$E\cdot\text{MMP} + SP \xrightarrow{k_{sp}} E\cdot\text{SP} \xrightarrow{k_{h}^{yd}} E\cdot\text{CoA} \xrightarrow{k_{MMMP}} E\cdot\text{MMP}$$

**Scheme 1**

Addition of very low concentrations of acetoacetate to the reaction mixture of E-MMP and pantetheine in the presence of MMP causes the observed rate constant for reactivation to increase, as shown in Fig. 2. The rate constant levels off as it approaches the rate constant for the formation of E-SP, $k_{SP}^{yd} [SP]$, which is shown by the dashed lines. Thus, the E-SP intermediate can react with acetoacetate as well as with water. This reaction provides a new pathway for the formation of free enzyme that competes with the reaction of E-SP and MMP to form inactive enzyme (Scheme 1). At high concentrations of acetoacetate all of the E-SP reacts to give free enzyme and the rate-limiting step becomes the formation of E-SP. Rate constant ratios of $k_{AcAc}/k_{MMMP} = (1.1 \pm 0.2) \times 10^3$ and $k_{AcAc}/k_{hyd} = (1.5 \pm 0.2) \times 10^6 \text{ M}^{-1}$ were obtained from the concentration of acetoacetate needed to relieve half of the MMP inhibition. Similar experiments with succinate gave rate constant ratios of $k_{succ}/k_{MMMP} = (3.1 \pm 0.5) \times 10^3$ and $k_{AcAc}/k_{hyd} = (4 \pm 1) \times 10^6 \text{ M}^{-1}$. The $k_{MMMP}/k_{hyd}$ ratio is different from the corresponding ratio for E-CoA (Table I); this shows that the reactivation does not occur through any E-CoA that might somehow have been formed from traces of CoA that were not removed by reaction with IAA.

**Table 1**

| Rate constant | E-SP$^a$ | E-CoA$^b$ | E-MMP$^a$ |
|---------------|---------|-----------|-----------|
| $k_{AcAc}/k_{MMMP}$ | $1.1 \times 10^3$ | $1.9 \times 10^3$ | |
| $k_{AcAc}/k_{h}^{yd}$ | $3.1 \times 10^4$ | $3.1 \times 10^4$ | |
| $k_{AcAc}/k_{MMMP}$ | $1.3 \times 10^4$ | $1.1 \times 10^4$ | |
| $k_{hyd}$, min$^{-1}$ | $2 \pm 1$ | $0.014$ | $0.002$ |
| $k_{succ}$, M$^{-1}$ min$^{-1}$ | $8 \times 10^5$ | $5 \times 10^5$ | $1.6 \times 10^2$ |
| $k_{AcAc}$, M$^{-1}$ min$^{-1}$ | $3 \times 10^6$ | $3 \times 10^6$ | $2.4 \times 10^3$ |

$^a$Values measured at standard conditions, as described in the text.
$^b$Values measured by Pandy (N. Pandey, C. A. Fierke, and W. P. Jencks, unpublished experiments) in the presence of 67 mM Tris sulfate, pH 8.1, at 25 °C, ionic strength maintained at 0.9-1.0 with sodium sulfate.

*Ref. 6.*

$^c$Calculated from $k_{t} = k_{MMMP}$ at pH 8.1, 25 °C (unpublished experiments).

**Fig. 1. Inhibition of the reaction of E-MMP with pantetheine by MMP, which reacts with E-SP to regenerate E-MMP.**

E-MMP was prepared exactly as described under "Experimental Procedures." Enzyme reactivation was initiated by a 5-fold dilution of E-MMP into reaction mixtures at 25 °C to a final concentration of 0.69 μM E-MMP, 0.1 mM Tris sulfate, pH 8.1, 0-2 mM potassium acetoacetate and either: 29 mM pantetheine and 18.9 mM MMP (●); 6.2 mM pantetheine and 9.8 mM MMP (△); 6.2 mM pantetheine and 19.8 mM MMP (▲). The dashed lines refer to 0.2 or 29 mM pantetheine with no added MMP. Aliquots (5 μl) were diluted into 2.5 ml of assay buffers. The enzyme activity, total enzyme activity and first-order rate constants were calculated from a plot of log(100% - % activity) versus time. The solid line is theoretical and the dashed line is the rate constant for hydrolysis of E-MMP.

**Fig. 2. Reaction of E-pantetheine with acetoacetate.**

E-MMP was prepared exactly as described under "Experimental Procedures." Enzyme reactivation was initiated by a 5-fold dilution of E-MMP into reaction mixtures at 25 °C to a final concentration of 0.69 μM E-MMP, 0.1 mM Tris sulfate, pH 8.1, 0-2 mM potassium acetoacetate and either: 29 mM pantetheine and 18.9 mM MMP (●); 6.2 mM pantetheine and 9.8 mM MMP (△); 6.2 mM pantetheine and 19.8 mM MMP (▲). The dashed lines refer to 0.2 or 29 mM pantetheine with no added MMP. Aliquots (5 μl) were diluted into 2.5 ml of assay buffers. The enzyme activity, total enzyme activity and first-order rate constants for reactivation were measured as described in Fig. 1. The lines are theoretical.

**Activation of Catalysis by CoA Transferase**
MMP. The remaining E-SP was assayed by addition of a high concentration of MMP that converts E-SP back to inactive E-MMP (Scheme 1). The control reaction mixture contained 33-66 μM potassium succinate, which increases the rate of disappearance of E-SP >10-fold. The solid triangles in Fig. 3A show that in the presence of 35 mM 3',5'-ADP there is a lag in the formation of free enzyme that can be ascribed to the accumulation of up to 50% E-SP before hydrolysis. The sigmoid curve can be fit by the rate constant of 0.66 min⁻¹ for the formation of E-SP under these conditions and hₙₐₜ = 0.35 ± 0.1 min⁻¹ for the hydrolysis of E-SP, as shown by the solid line in the figure. The solid circles in Fig. 3B show that there is no less buildup of an intermediate in the absence of 3',5'-ADP, consistent with faster hydrolysis of E-SP in the absence of this inhibitor. The data are consistent with the solid line, which was calculated from the rate constant of 0.5 min⁻¹ for the formation of E-SP under these conditions and hₙₐₜ = 2 ± 1 min⁻¹. This rate constant is 140 times larger than the value of hₙₐₜ = 0.014 min⁻¹ for E-CoA; it shows that E-SP is even more activated toward nonspecific reactions than E-CoA, which undergoes hydrolysis ~50 times faster than acetyl-CoA (6) (Table I).

Rate constants for other reactions of E-SP were calculated from the observed rate constant ratios and the value of hₙₐₜ = 2 min⁻¹. These rate constants are compared with those for E-CoA in Table I.

These results suggested that CoA transferase should catalyze the reaction of AcAc-SP and succinate to form succinyl-pantetheine, although this reaction was not detected previously (9). As predicted, CoA transferase (69 μM, 138 μM active sites) was found to catalyze the disappearance of AcAc-SP, (measured spectrophotometrically at 310 nm with 0.47 mM AcAc-SP, 6 mM potassium succinate, 0.5 mM EDTA, and 0.1 M Tris sulfate, pH 8.1, at 25 °C). The reaction followed first-order kinetics for >9 half-lives with a rate constant of 0.03 min⁻¹. This gives values of kₙₐₜ/Kₘ = 220 ± 40 M⁻¹ min⁻¹ and Kₘ > 1 mM, which may be compared with values of kₙₐₜ/Kₘ = 4 × 10⁻⁵ M⁻¹ min⁻¹ and Kₘ = 0.2 mM for the reaction of CoA transferase with AcAc-CoA under identical conditions (6).

**DISCUSSION**

As shown in Table I, the reactivity of E-SP is similar to that of E-CoA. It reacts with specific substrates, succinate and acetoacetate, 5-10-fold slower and reacts with nonspecific reagents as fast (MMP) or up to 140-fold faster (water) compared with E-CoA. In the reverse direction, AcAc-SP reacts with CoA transferase 2 × 10⁶-fold slower than AcAc-CoA. Thus, the equilibrium constant for the formation of E-SR from E and AcAc-SR is much less favorable for E-SP (Kₑᵤ = kₙₐₜ/kₙₐₜ = 220/(3 × 10⁸) = 7 × 10⁻⁷) than for E-CoA (Kₑᵤ = 9) (15); the equilibrium constant for the reaction of SP with E-CoA is ~8 × 10⁻⁸. When E-SP is compared to the nonspecific thiol ester E-MMP (6), the reactivity is increased by a factor of >5 × 10⁶ towards specific substrates and 10⁷ towards nonspecific reagents. However, E-SP is 1.9 kcal/mol less stable than E-MMP. These facts are summarized in the reaction coordinate diagram shown in Fig. 4A.

The noncovalent interactions between CoA transferase and CoA can therefore be split into two functional domains. The pantetheine moiety provides activation and destabilization, which are used to increase the reactivity of E-pantetheine to a level very close to that of E-CoA. The interactions between pantetheine and CoA transferase cause a net destabilization of the E-SP intermediate compared with E-MMP, E-CoA,

![FIG. 3. Determination of the rate constant for hydrolysis of E-SP in the presence (A) or absence (B) of 3',5'-ADP. The reactions were initiated by a 3-fold dilution of E-MMP (10 μM, 60% E-MMP) into reaction mixtures at 25 °C to give a final concentration of 1.4 μM CoA transferase, 0.28 μM pantetheine, 0.5 mM EDTA, 0.1 mM Tris sulfate, pH 8.1, and either: nothing (O); 33 μM potassium succinate (C); 34.7 mM adenosine 3',5'-diphosphate (D); or 34.7 mM 3',5'-ADP and 66 μM potassium succinate (D). Aliquots (5 μl) of the reaction mixture were quenched by dilution into 30 μl of 24 mM MMP in 0.5 mM EDTA, 0.1 mM Tris sulfate, pH 8.1, 25 °C and incubated for 2 min. This quench decreases the rate of formation of E by the reaction of E-MMP with SP by >98%. An aliquot (5 μl) of this solution was then diluted into 2.5 ml of assay buffer and the enzyme activity and total enzyme activity were assayed as described in Fig. 1. The enzyme activity was corrected for inhibition by MMP in the assay (<5%). The lines are theoretical assuming either a single first-order reaction or two consecutive first-order reactions (14).](image1)

![FIG. 4. Reaction coordinate diagrams for thiol transfer reactions involving E-CoA, E-MMP, and E-SP with acetoacetate at pH 8.1, 25 °C. The values of ΔG° and ΔG‡ were calculated from the constants in Table I and Ref. 6 for a standard state of 1 M.](image2)
and nonenzymic thiol esters, as shown by the less favorable equilibrium constant for its formation (Fig. 4A). The remainder of the CoA molecule, 3′-phospho-ADP, provides 6.9 kcal/mol of observed binding energy. This binding energy causes the large increase in $k_{cat}/K_M$ for AcAc-CoA compared with AcAc-SP and the large stabilization of E-CoA relative to E-SP at equilibrium. Addition of 3′,5′-ADP to the reaction of acetoacetyl pantetheine with CoA transferase does not increase the chemical reactivity of E-SR, as measured by the slower hydrolysis rate of E-CoA compared with E-SP and the decrease in the hydrolysis rate of E-SP in the presence of 3′,5′-ADP (Fig. 3). Thus, in order for the enzyme to utilize the binding energy of the 3′-phospho-ADP moiety it must be covalently attached to pantetheine.

Fig. 4B shows the energy profiles of these reactions normalized to the energy of the transition state, which may have a similar structure for the three substrates. The left side shows that $E\text{-CoA}$ and $E\text{-SP}$ are activated, or destabilized, relative to $E\text{-MMP}$ by 11.1–12.5 kcal mol$^{-1}$, so that they can reach the transition state easily. However, on the right side it is apparent that AcAc-SP cannot reach the transition state easily because it lacks the 3′-phospho-ADP group that provides binding energy. The pantetheine group is highly reactive only after it has been forced into the active site; it was attached to the enzyme to prepare $E\text{-SP}$ by a chemical reaction. However, the pantetheine group does also provide some binding energy to stabilize the transition state, in addition to destabilization in the ground state, because AcAc-SP is much more reactive than the short chain AcAc-MMP substrate (Fig. 4).

The nature of the substrate-induced rate acceleration in this system differs from that in phosphoglucomutase, which was shown by Ray and coworkers to transfer phosphate from the enzyme to the hydroxyl group of glucose 1-phosphate $\sim 10^{20}$ faster than to water. Most of this rate increase can be brought about by binding of two separate molecules, xylose and phosphite, which results in phosphorylation that is $2 \times 10^6$ faster than with water (16, 17). In contrast, the different parts of CoA must be covalently linked so that the 3′-phospho-ADP moiety can provide the binding energy to drive the activation by the pantetheine group.

REFERENCES
1. Pauling, L. (1946) Chem. Eng. News 24, 1375–1380
2. Wolfenden, R. (1972) Acc. Chem. Res. 5, 10–18
3. Lienhard, G. E. (1975) Science 189, 149–154
4. Fersht, A. R. (1974) Proc. R. Soc. Lond. B Biol. Sci. 187, 397–407
5. Jencks, W. P. (1975) Adv. Enzymol. Relat. Areas Mol. Biol. 43, 219–410
6. Moore, S. A., and Jencks, W. P. (1982) J. Biol. Chem. 257, 10985–10997
7. Seely, H. W. (1955) Methods Enzymol. 1, 624–627
8. Walker, P. G. (1954) Biochem. J. 58, 699–704
9. White, H., and Jencks, W. P. (1976) J. Biol. Chem. 251, 1688–1699
10. Ellman, G. L. (1959) Arch. Biochem. Biophys. 82, 70–77
11. Penefsky, H. S. (1979) Methods Enzymol. 56, 527–530
12. Hersh, L. B., and Jencks, W. P. (1967) J. Biol. Chem. 242, 3486–3488
13. Hersh, L. B., and Jencks, W. P. (1967) J. Biol. Chem. 242, 3481–3486
14. Atkins, P. W. (1982) Physical Chemistry, 2nd Ed. p. 940, Freeman and Co., San Francisco
15. White, H., Solomon, F., and Jencks, W. P. (1976) J. Biol. Chem. 251, 1700–1707
16. Ray, W. J., Jr., and Long, J. W. (1976) Biochemistry 15, 3993–4006
17. Ray, W. J., Jr., Long, J. W., and Owens, J. D. (1976) Biochemistry 15, 4006–4017