Citrate Synthase Stabilizes the Enethiolate of Acetyldithio Coenzyme A†

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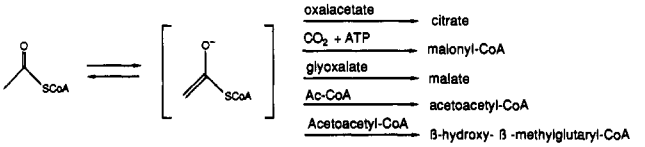
ABSTRACT: Citrate synthase catalyzes the slow condensation of acetyldithio-CoA [Ac(=S)CoA] with oxalacetate to form thiocitrate [Wlassics, I. D., Stille, C., & Anderson, V. E. (1988) *Biochim. Biophys. Acta 952*, 269]. During the transient approach to steady state an observable amount of the dithioester absorbance disappears. The amplitude of the decrease in absorbance corresponds to 0.32, 0.03, and 0.02 enzyme equiv at pH 8.3, 7.5, and 6.6, respectively. The difference spectra from before and after the transient exhibit the dithioester λ_{max} at 306 nm. Acid quenching of a stoichiometric reaction between Ac(=S)CoA and citrate synthase following the transient quantitatively regenerates Ac(=S)CoA, indicating carbon–carbon bond formation had not yet occurred. The apparent first-order rate constant of the transient is independent of Ac(=S)CoA concentration and increases with decreasing pH, being 0.007, 0.016, and 0.04 s^{-1} at pH 8.3, 7.5, and 6.6, respectively. 2-Fluoroacetyldithio-CoA is a better inhibitor of citrate synthase, K_i = 300 nM, and substrate, $V_{max} = 2 \times 10^{-3}$ s^{-1}, than Ac(=S)CoA. ^{1}H NMR experiments indicate that citrate synthase catalyzes the exchange of the α-hydrogens of Ac(=S)CoA with turnover numbers of 0.13 and 0.54 s^{-1} at pD 7.9 and 7.2, respectively. Analysis of the proton and deuterium decoupled ^{13}C NMR spectra of [2-^{13}C]Ac(=S)CoA that has exchanged 37% of the α-hydrogens in the presence of citrate synthase indicates that the relative proportions of CH_3, CH_2D, CHD_2, and CD_3 were 0.29, 0.39, 0.25, and 0.07, respectively. This statistical distribution indicates each exchange event is independent. The data indicate that citrate synthase stabilizes the ionized form of Ac(=S)CoA by 5 kcal/mol relative to the un-ionized form, that the ionized dithioester is on the reaction pathway, and that below pH 8.3 the slow carbon–carbon bond forming reaction is responsible for the 10^6 decrease in V_{max} caused by substituting sulfur for oxygen in the thioester carbonyl.

The thioester linkage in AcCoA[1] is believed to promote the acidity of the hydrogens adjacent to the carbonyl. This enhanced acidity is believed to be used enzymatically in a variety of enzyme reactions that formally condense the carbanion obtained from AcCoA with a variety of compounds containing an electrophilic carbonyl to form a new carbon–carbon bond (Scheme I). Citrate synthase provides a prototype for this reaction, condensing AcCoA with oxalacetate to yield citrate after hydrolysis of the CoA thioester. Eggerer (1965) demonstrated citrate synthase's ability to promote ionization of AcCoA by observing that tritium from solvent was incorporated into AcCoA in the presence of (*S*)-malate. The rate of this reaction is, however, very slow and does not provide unequivocal evidence for the intermediacy of a carbanion in the reaction sequence. Any favorable interactions developed in a concerted transition state could cause a significant stabilization of a carbanion that would lead to the very slow exchange with solvent observed in the absence of oxalacetate.

Acetyldithio-CoA [Ac(=S)CoA] is a very slow substrate for citrate synthase, $V_{max} = 4 \times 10^{-4}$ s^{-1}, in spite of the fact that the α-hydrogens have a pK_a of 12.5 (Wlassics et al., 1988), which is over 7 orders of magnitude more acidic than the α-hydrogens of thioesters (Lienhard & Wang, 1968). The ionization of the dithioester is spectrally observable since the absorbance of the thiocarbonyl at 306 nm disappears upon enethiolization. We have used these properties to demonstrate that citrate synthase binds to the protonated form of Ac(=S)CoA and promotes the acidity of the α-hydrogen by 3.8 pH units, and, once ionized at the enzyme active site, the enethiolized form of the dithioester reacts very slowly with oxalacetate.

Scheme I



Experimental Procedures

Materials. Citrate synthase, from porcine heart, and oxalacetic acid as the *cis*-enol were purchased from Sigma, and fresh solutions were prepared daily. The crystalline enzyme solution was pelleted by centrifuging the NH_4SO_4 suspension in an Eppendorf 5414 microcentrifuge. The pellet was resuspended in an aliquot of 0.1 M sodium phosphate buffer (pH 7.5) containing 10 mM EDTA. The concentration of the citrate synthase was determined by measuring the absorbance at 280 nm (ϵ_{280} = 140 000 cm^{-1} M^{-1}) using a λ-3B Perkin-Elmer spectrophotometer. 2-Fluoroacetyl chloride was from Alfa.

Ac(=S)CoA. CoA dithioesters were synthesized as described by Wlassics et al. (1988). Acetyl chloride (5 mmol) was added to a solution of thiophenol (5 mmol) in pyridine (10 mmol), quantitatively yielding acetylthiophenol. The

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[1] Abbreviations: Ac(=S)CoA, *S*-thionoacetyl coenzyme A; AcAc(=S)CoA, *S*-(3-oxo-1-thionobutyryl)-CoA; FAc(=S)CoA, *S*-(2-fluorothionoacetyl) coenzyme A; CoA, coenzyme A; OAA, oxalacetic acid; EDTA, ethylenediaminetetraacetic acid; DTNB, 3-carboxy-4-nitrophenyl disulfide; Tris, tris(hydroxymethyl)aminomethane; MES, 2-(*N*-morpholino)ethanesulfonic acid; HPLC, high-pressure liquid chromatography; FPLC, fast protein liquid chromatography.

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acetylthiophenol was reacted with Lawesson's reagent (4 mmol) in toluene at 75 °C for 8 h (Pedersen et al., 1978). The dithioester was separated from the unreacted thioester by chromatography on silica gel, and the final product was judged free of thioester by the absence of a ^{1}H NMR signal at 2.4 ppm. CoA (12 μmol) in 4 mL of EtOH:0.5 M sodium bicarbonate, pH 8.5 (1:1 v/v), was transesterified by adding acetyldithiophenol (60 μmol). A homogeneous solution was obtained by the dropwise addition of ca. 1 mL of ethyl acetate. After 20 min, the solution was acidified by the addition of 20 μL of concentrated phosphoric acid and extracted three times with equal volumes of ethyl acetate. Ac(=S)CoA with an A_{259}/A_{306} ratio of 1.3 was obtained directly. [2-^{13}C]Ac(=S)-CoA was synthesized from [2-^{13}C]acetyl chloride (Sigma) by the same procedure.

2-Fluoroacetyldithio-CoA [FAc(=S)CoA]. 2-Fluoroacetyl chloride (13 mmol) was added dropwise to a solution of thiophenol (13 mmol) and pyridine (39 mmol) in 2 mL of dry toluene. After 5 min, the pyridinium chloride precipitate was removed by centrifugation and the 2-fluoroacetylthiophenol was purified by chromatography on a 20 × 1.5 cm silica gel column eluted with petroleum ether:ethyl ether (90:10 v/v) with a 45% yield: ^{1}H NMR ($CDCl_3$) δ 7.5 (m, 5 H), 4.90 (d, J_{FCH} = 45.7 Hz, 2 H). The thioester was converted to the dithioester by adding 2 mmol of 2-fluoroacetylthiophenol to 1.6 mmol of Lawesson's reagent and 6 mL of dry toluene and heating to 95 °C for 34 h under nitrogen. The dithioester was purified from unreacted thioester on the same silica gel column in a purified yield of 25%: ^{1}H NMR δ 7.5 (m, 5 H), 5.28 (d, J_{FCH} = 49.5 Hz, 2 H); ultraviolet λ_{max} = 308 nm.

CoA was transesterified as above, only the reaction time was limited to 10 min. The FAc(=S)CoA was purified by HPLC on an Econosphere (Alltech) 4.6 mm × 25 cm octadecylsilyl column eluted isocratically at 1.0 mL/min with 10% methanol and 90% 0.01 M sodium phosphate, pH 4.9. The FAc(=S)CoA eluted 10 min after injection and was characterized by the A_{259}/A_{306} ratio of 1.4. The solution was lyophilized immediately and stored desiccated at −20 °C.

Ethyl-CoA. Ethyl iodide (358 μmol in 2 mL of ethanol) was added to 2 mL of CoA (8 μmol) in pH 9.2 sodium carbonate. A single-phase solution was obtained by the dropwise addition of ca. 1 mL of ethyl acetate. After 20 min, a negative DTNB test for unreacted thiols was obtained. The solution was acidified to pH 4.0 with phosphoric acid and extracted three times with 2 mL of ethyl acetate. The concentration of ethyl-CoA was determined spectrophotometrically by assuming ϵ_{259} = 15 000 cm^{-1} M^{-1}.

Kinetic Measurements. A Perkin-Elmer λ-3B spectrophotometer thermostated at 25 ± 0.1 °C and interfaced with an IBM computer was used for data acquisition. The steady-state production of CoA with varying AcCoA concentrations in the presence of 2.2 mM oxalacetate and 3.8 nM citrate synthase was determined in the presence of DTNB at 412 nm (ϵ_{412} = 14 150 cm^{-1} M^{-1}) without correction for the pH variation (Riddles et al., 1979). The concentrations of DTNB used varied from 1.29 mM at pH 8.3 and pH 7.5 to 2.58 mM at pH 6.6 to ensure that the measured initial steady-state velocity was not a function of the concentration of DTNB.

The steady-state reaction of Ac(=S)CoA was monitored by following the decrease of dithioester at 306 nm, ϵ_{306} = 11 000 cm^{-1} M^{-1} (Wlassics et al., 1988) for 1–4 h. Varying concentrations of Ac(=S)CoA were incubated with 1.2 mM oxalacetate, 6 μM citrate synthase, and 0.1 M Tris-HCl (pH 7.5 or 8.3) or 0.1 M sodium phosphate (pH 6.6). Steady-state kinetic constants and their standard errors were obtained from a nonlinear least-squares fit to eq 1, where v is the steady-state

$$v/E_t = \frac{V_{max}S}{K_m + S} \quad (1)$$

velocity, E_t is the enzyme concentration, and S is the substrate concentration.

Inhibition constants for FAc(=S)CoA and for ethyl-CoA were determined by using varying concentrations of the inhibitor at varying concentrations of AcCoA and the same assay system and buffers. The inhibition constants were obtained from a nonlinear least-squares fit to

$$v/E_t = \frac{V_{max}S}{K_m(1 + I/K_i) + S} \quad (2)$$

where I is the inhibitor concentration and K_i is the inhibition constant.

Transient Kinetic Measurements. In the reactions with Ac(=S)CoA a relatively rapid transient disappearance in dithioester absorbance was observed. To study the transient disappearance reaction, 1.0-mL solutions containing 10 mM EDTA, 2.2 mM oxalacetate, 26 μM citrate synthase, and 0.1 M sodium phosphate (pH 6.6 or 7.5) or 0.1 M Tris-HCl (pH 8.3) were centrifuged for 2 min in an Eppendorf 5414 microcentrifuge to remove any particulates. After transferring 900 μL of the solution to a quartz cuvette, varying amounts of Ac(=S)CoA were added, and the decrease in 306-nm absorbance was monitored over 7.5 min. The time courses obtained were fit to eq 3 by a nonlinear least-squares routine

$$A = A_0 - \mathrm{Amp}[1 - \exp(-k_1 t)] - k_2 t \quad (3)$$

where A_0 is the initial absorbance, Amp is the amplitude of the transient phase, and k_1 is the observed first-order rate constant of the transient. The slow steady-state disappearance was accounted for by the addition of a linear term with proportionality constant k_2. A limiting value for the amplitude of the burst, Amp_{max}, at saturating Ac(=S)CoA was obtained from a nonlinear least-squares fit to

$$\mathrm{Amp} = \frac{\mathrm{Amp}_{max}S}{K_m + S} \quad (4)$$

Difference Spectra. To obtain a difference spectrum of the transient phase, the reactions were followed by scanning repetitively from 313 to 295 nm for 4 min at pH 7.5 (16 scans at 15 s/scan) and from 313 to 285 nm for 8 min at pH 8.3 (16 scans at 30 s/scan). Before and after spectra of the reaction were also taken by scanning once from 415 to 230 nm. To verify that little change in absorbance occurred at 287 nm during the transient phase, this wavelength was monitored continuously for 8 min in a separate experiment. For FAc(=S)CoA a difference spectrum on binding was generated by placing equal volumes of a 20 μM solution of FAc(=S)CoA in 0.1 M sodium phosphate, pH 7.4, in one side of a double-chambered quartz curvette and 40 μM citrate synthase with 2 mM OAA in the same buffer in the second side. An initial spectrum was recorded, the contents of both chambers were mixed by repeated inversion, and a second spectrum was recorded 4 min after mixing. The difference spectra for the enethiolization of dithioacetylphenol in ethanol were generated by recording spectra before and after the addition of 5 equiv of sodium hydroxide. To establish that the loss of absorbance was not due to ethanolysis, the original dithioester spectrum was regenerated following the addition of excess acid.

Intermediate Analysis by Acid Quenching. A 900-μL reaction mixture containing 0.6 mM oxalacetate and 27 μM

citrate synthase in Tris-HCl, pH 8.3, was initiated by the addition of 32 μM Ac(=S)CoA. The reaction was monitored at 306 nm in the usual manner for 10 min until the burst was finished, and then the reaction was quenched to ca. pH 4.0 by adding 100 μL of 1 M acetic acid. The acidified mixture was centrifuged at 4500 rpm for 30 min in a centrifree micropartition system (Amicon) to remove the enzyme. The filtrate was titrated to pH 7.0 with Na_3PO_4 and loaded on a 5 mm × 6 cm Mono-Q anion-exchange column (Pharmacia) equilibrated with 20 mM MES, pH 7.0. The CoA derivatives were analyzed by eluting at 1 mL/min for 10 min with 20 mM MES followed by a 30-min linear gradient to 20 mM MES plus 0.4 M KCl (pH 7.0) that was maintained for 10 min. The column effluent was monitored at either 254 or 306 nm. The column was standardized by the elution of pure samples of Ac(=S)CoA and CoA. The single peak detected was tested for the presence of free thiol by assay with DTNB.

Enzyme-Catalyzed Exchange. The ability of citrate synthase to catalyze the exchange of the α-hydrogens of Ac(=S)-CoA with solvent was measured by ^1H NMR in sodium phosphate, pD 7.2 or 7.9. The pD was determined by adding 0.4 to the reading obtained from a pH meter with a Corning combination glass electrode (Glasoe & Long, 1960). For these experiments the oxalacetate and the citrate synthase pellet were dissolved directly in the D_2O buffer. At pD 7.9, Ac(=S)CoA (6.5 mM) and oxalacetate (25 mM) were added in a 5-mm NMR tube, and a ^1H NMR spectrum was acquired (75 scans on a Bruker 400-MHz NMR). Citrate synthase, 40 and 18 μM final concentrations at pD 7.9 and 7.2, respectively, was added to initiate the reaction. The decrease in the intensity of the α-hydrogen resonance at 2.81 ppm relative to the pantetheine methyl resonance at 0.754 ppm was monitored by accumulating 14 spectra in 10-min blocks. Omission of the citrate synthase or oxalacetate or replacing the oxalacetate with 2.8 mM (*S*)-malate resulted in a less than 10% decrease in the relative intensity after 80 min.

To determine if multiple exchanges of the α-hydrogens occur on each residence at the active site, the distribution of deuterons on the α-methyl group was determined by integrating the isotope-shifted ^{13}C NMR signals from a sample of [2-^{13}C]Ac(=S)CoA that had exchanged 37% of the α-hydrogens. A 0.7-mL solution containing 5 mM [2-^{13}C]Ac(=S)CoA was reacted with 14 mM oxalacetate and 11 μM citrate synhtase in 0.1 M sodium phosphate, pD 7.9. The reaction was monitored by ^1H NMR until the intensity of the α-hydrogen signal at 2.81 ppm had decreased by 37% relative to the pantetheine methyl signal at 0.754 ppm. The reaction was quenched to pH 4.0 with 1 M NaH_2PO_4/HCl. The ^1H and ^2H decoupled NOE suppressed spectra (Freeman et al., 1972) of the partially exchanged [2-^{13}C]Ac(=S)CoA was obtained. The spectra were acquired on a Bruker 250-MHz NMR equipped with an X-nucleus decoupler, and the lock signal channel was attached to a ^2H bandpass box (38.8 MHz) to decouple the ^2H. The pulse delay time was systematically increased until the relative intensities of the CH_3, CH_2D, CHD_2, and CD_3 peaks remained constant. Negligible exchange of the α-hydrogens of Ac-(=S)CoA with solvent was observed at pD 4.4 in the presence of 15.5 mM oxalacetate and 10 μM citrate synthase over a period of 8 h.

Results

pH Variation of Kinetic Parameters. The steady-state rate constants as a function of pH for Ac(=S)CoA and AcCoA in the presence of saturating oxalacetate are shown in Table I. Earlier studies (Wlassics et al., 1988) indicated the product of the reaction with Ac(=S)CoA was largely thiocitrate. Over the pH range from 6.6 to 8.3 the K_m for Ac(=S)CoA decreased a factor of 4 while the K_i for ethyl-CoA increased 2-fold. Also of note are the 330-fold lower K_i and 10-fold greater V_{max} for FAc(=S)CoA than observed for the K_m and V_{max} of Ac(=S)CoA at pH 6.6.

Table I: Steady-State Kinetic Parameters

compound	pH	K_m (μM)	V_{max} (s^{-1})	K_i (μM)
Ac-CoA	6.6	25 ± 6	209 ± 20	
	7.5	14 ± 3	201 ± 14	
	8.3	11 ± 2	222 ± 16	
Ac(=S)CoA	6.6	103 ± 9	(2.2 ± 0.03) × 10^{-4}	
	7.5	53 ± 8	(4.0 ± 0.001) × 10^{-4}	
	8.3	24 ± 1	(3.0 ± 0.06) × 10^{14}	
FAc(=S)CoA	6.6		(2 ± 0.1) × 10^{-3}	0.3 ± 0.08
	7.5			
	8.3			
ethyl-CoA	6.6			143 ± 40
	7.5			197 ± 50
	8.3			270 ± 50

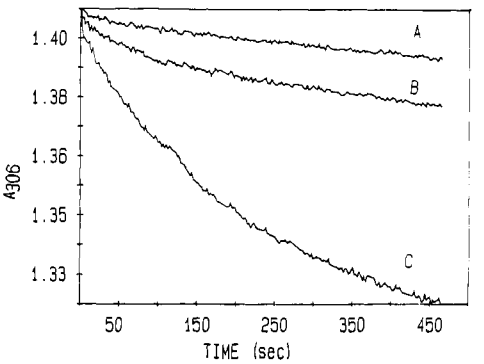


FIGURE 1: Traces showing the rapid decrease and the slower steady-state decrease of the dithioester absorbance at 306 nm as a function of time at pH 6.6 (A), pH 7.5 (B), and pH 8.3 (C). Ac-(=S)CoA, 121 μM, is nearly saturating, and the concentrations of citrate synthase (26 μM) and oxalacetic acid (2.2 mM) were fixed.

The transient decrease in dithioester absorbance at 306 nm that occurs when Ac(=S)CoA is added to a reaction mixture containing 26 μM citrate synthase and 2.2 mM oxalacetic acid shown in Figure 1 demonstrates that there is a rapid disappearance of the chromophore followed by a much slower steady-state decrease. The amplitude of the burst was a function of the concentration of Ac(=S)CoA, citrate synthase, and pH. The amplitude of the burst as a function of the Ac(=S)CoA concentration could be adequately described by a rectangular hyperbola (data not shown), so the maximum change could be obtained from a fit to eq 3. Assuming the decrease in absorbance is due to the total loss of absorbance of the dithioester, ϵ_{306} = 11 000 cm^{-1} M^{-1}, the fraction of enzyme having a nonabsorbing intermediate present at saturation can be determined. This fraction decreases from 0.31 ± 0.05 at pH 8.3 to 0.035 ± 0.002 at pH 7.5 and 0.018 ± 0.002 at pH 6.6. The rate of the burst was not detectably a function of the Ac(=S)CoA concentration, but as the pH decreased, the rate increased dramatically from (7 ± 1) × 10^{-3} s^{-1} at pH 8.3 to (1.6 ± 0.2) × 10^{-2} s^{-1} at pH 7.5 and (4 ± 0.5) × 10^{-2} s^{-1} at pH 6.6.

Difference Spectra. A difference spectrum generated by subtracting the ultraviolet spectrum obtained after the transient phase at pH 8.3 from the initial spectrum is shown in Figure 2A. The difference is positive at all wavelengths, has a λ_{max} at 306 nm, consistent with the spectra of dithioesters, and compares favorably with the difference spectra for the enethiolization of dithioacetylphenol in ethanol promoted by the addition of NaOH (Figure 2C). This suggests that the transient phase is due to the complete disappearance of the

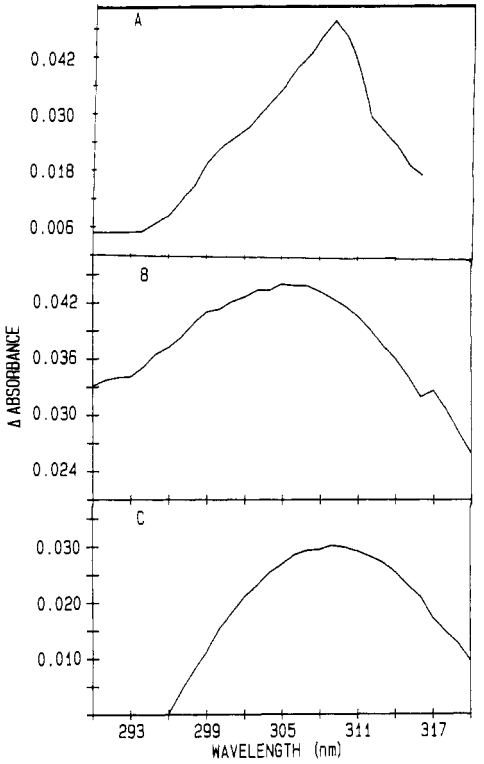


FIGURE 2: Difference spectra of dithioesters upon enethiolization. (A) Reaction between Ac(=S)CoA (121 μM), oxalacetic acid (2.2 mM), and citrate synthase (26 μM) at pH 8.3 between t = 0 s and t = 240 s. (B) Reaction between 20 μM citrate synthase, 10 μM FAc(=S)CoA, and 1.0 mM oxalacetic acid at pH 7.4. (C) Reaction between dithioacetylphenol and 5 equiv of NaOH in ethanol.

dithioester absorbance and is not just a result of the change in environment of the dithioester. The initial increase in absorbance at 306 nm corresponded to the amount of Ac(=S)-CoA added. Consequently, no detectable rapid reaction occurred during the mixing time. Figure 2B shows the difference spectra for the rapid reaction of FAC(=S)CoA with citrate synthase. At pH 7.4 over 40% of the dithioester absorbance disappeared in the presence of excess citrate synthase, indicating that FAc(=S)CoA can enethiolize when bound at the active site and that this process is significantly enhanced by the fluoro substitution.

Intermediate Analysis. By quenching the reaction between 32 μM Ac(=S)CoA and 27 μM citrate synthase in the presence of 0.6 mM oxalacetate with acetic acid, we hoped to recover the citrate synthase bound intermediate presumed to be present because of the rapid decrease in absorbance at 306 nm. During the burst 11% of the absorbance at 306 nm disappeared. This is consistent with the K_m of 24 μM for Ac(=S)CoA and the fraction of bound substrate that ionizes determined from the maximum burst amplitude. If the enzyme-bound intermediate is a stabilized carbanion, the acid quench would regenerate starting material. A single peak observed on chromatography of the quenched reaction was identified as Ac(=S)CoA by coelution with authentic material and the A_{259}/A_{306} ratio of 1.3. Over 95% of the initial dithioester was recovered as Ac(=S)CoA in all of the quenched reactions. No significant amounts of free thiol were detected in the Ac(=S)CoA fractions, and no peak absorbing at 259 nm was observed to elute with a retention time of authentic CoA or citryl-CoA.

Enzyme-Catalyzed Exchange. The ^{1}H NMR experiments shown in Figure 3 show that citrate synthase catalyzes the rapid exchange of the α-hydrogens of Ac(=S)CoA in the presence of oxalacetate. This exchange is faster at pD 7.2 than

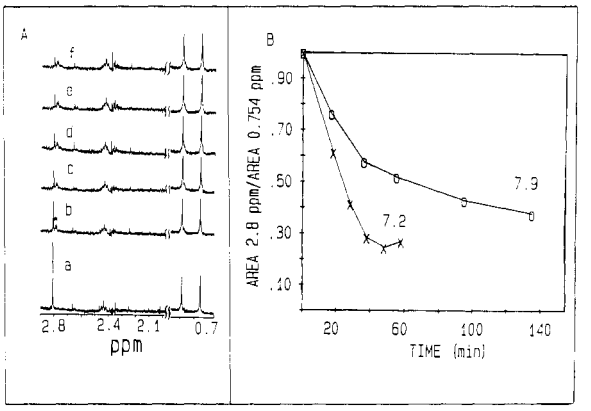


FIGURE 3: Citrate synthase (24 μM) catalyzed exchange of the α-hydrogens of Ac(=S)CoA (5.7 mM) with D_2O in the presence of oxalacetic acid (25 mM) at pD 7.2. (A) ^{1}H NMR spectra acquired at 0 min (a), 18 min (b), 28 min (c), 38 min (d), 48 min (e), and 58 min (f). The peak labeled with an asterisk was present in a control ^{1}H NMR spectra of just citrate synthase. (B) Plot of relative intensity of the α-hydrogens to the high-filed pantetheine methyl as a function of time at pD 7.2 (×) and 7.9 (O). The connecting lines have no theoretical significance.

at pD 7.9. The decrease in intensity is not described by a first-order decay, presumably because the Ac(=S)CoA concentration is greater than K_m and at higher fractional conversions the fully exchanged material will inhibit the exchange of the remaining protons. The initial rate of exchange at both pD values is estimated by using the first time point. This procedure will systematically underestimate the exchange rate, but the accuracy of the data does not warrant a more sophisticated analysis. Additional systematic underestimation arises from collecting the data over 10 min and using the midpoint as a time estimate. The initial exchange rates estimated from eq 5 are 0.54 and 0.13 s^{-1} at pD 7.2 and 7.9,

$$k_{ex} = \frac{F3[\text{Ac(=S)CoA}]}{t[\text{citrate synthase}]} \quad (5)$$

respectively. F is the fractional decrease in intensity of the α-hydrogen signal at time t. The factor of 3 arises from the three protons of the exchanging methyl group. Control experiments demonstrated that both citrate synthase and oxalacetate were required for the exchange to occur. The oxalacetate could not be replaced by 2.8 mM (*S*)-malate over the same time frame (results not shown).

To determine if multiple proton exchanges occur each time the Ac(=S)CoA binds to citrate synthase, the distribution of deuterium in the acetyl methyl groups was determined by analysis of the ^{1}H and ^{2}H decoupled NOE suppressed ^{13}C NMR spectra of [2-^{13}C]Ac(=S)CoA after 37% of the α-hydrogens had exchanged with solvent. As can be seen in Figure 4, the NOE suppressed spectrum showed a statistical distribution with areas of 0.29, 0.39, 0.25, and 0.07, for CH_3, CDH_2, CD_2H, and CD_3 methyl groups at δ 39.099, 38.848, 38.595, and 38.333, respectively, relative to the methanol internal standard at 49.000 ppm. When the same experiment was performed without the NOE suppression, the same peaks were observed but the distribution of the relative areas was 0.60, 0.20, 0.20, and <0.1, respectively, corroborating the assignment of the peak farthest downfield as being the CH_3 peak. Less than 10% of the α-hydrogens exchanged in control experiments omitting citrate synthase.

Discussion

Enzyme Stabilization of the Enethiol(ate) of Ac(=S)CoA. The data presented in this paper are consistent with the

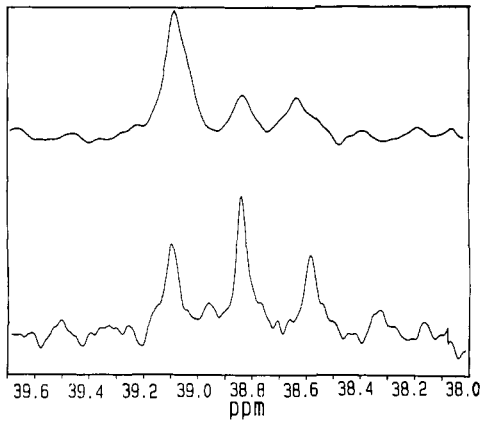


FIGURE 4: ^{13}C NMR of [2-^{13}C]Ac(=S)CoA where 37% of the α-hydrogens have been exchanged in the presence of citrate synthase and oxalacetate. The NOE suppressed (bottom) and unsuppressed (top) spectra show the distribution of the CH_3, CH_2D, CHD_2, and CD_3 methyl groups for the multiple proton exchange experiment.

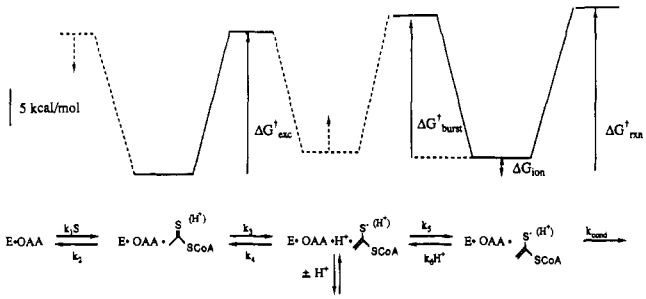


FIGURE 5: Reaction coordinate diagram for the partitioning of the citrate synthase–oxalacetate–Ac(=S)CoA complex at pH 7.5 as determined in this paper. The solid lines indicate values that have been defined absolutely. The dashed lines indicate values that only have limits. The direction of the dashed arrow indicates if the limiting value is a maximum or minimum. The relative energy of the initial complex and the enethiol(ate) intermediate is determined from the burst amplitude. The heights of the four transition states are established by the observed distribution of deuterium in unreacted but exchanged Ac(=S)CoA, the rate of proton exchange with solvent, k_{obs} for the approach to steady state, and V_{max} as discussed in the text.

minimal reaction sequence shown in Figure 5. Two key conclusions of this study are included in this scheme: citrate synthase stabilizes the enethiol(ate) form of the dithioesters, and the enzyme enethiol(ate) complex is on the reaction pathway and not an unproductive complex.

The biphasic time courses shown in Figure 1 indicate that an intermediate with an altered dithioester absorbance is being formed in increasing amounts as the pH is increased. Three lines of evidence suggest the intermediate accumulating is the enethiolized form of the dithioester. The difference spectra between the beginning and end of the rapid reaction has a λ_{max} at 306 nm and approximates the spectra of dithioesters, indicating the entire chromophore has been lost. Second, the intermediates present on the enzyme can be investigated by designing a reaction mixture so that a significant fraction of the Ac(=S)CoA will be bound to the enzyme and subsequently quenched with acid. Under the conditions chosen, 11% of the dithioester absorbance disappeared, and still essentially all of the CoA-containing material was recovered as Ac(=S)CoA. This is consistent with an enol(ate) intermediate since analogous experiments with acetyldithiophenol enolized in basic ethanol regenerated the dithioester on acidification. If the slow step in the reaction were subsequent to the carbon–carbon bond forming reaction, it would be expected that either CoA or citryl-CoA analogues would be recovered in the quenched solution. Finally, the rate constant of 0.54 s^{-1} for exchange of the α-hydrogens with solvent at pD 7.2 is significantly faster than the observed first-order rate constant for the transient phase of the reaction of $(1.6 \pm 0.2) \times 10^{-2}$ s^{-1} at pH 7.5, establishing that enolization occurs fast enough to be the cause of the observed transient time courses.

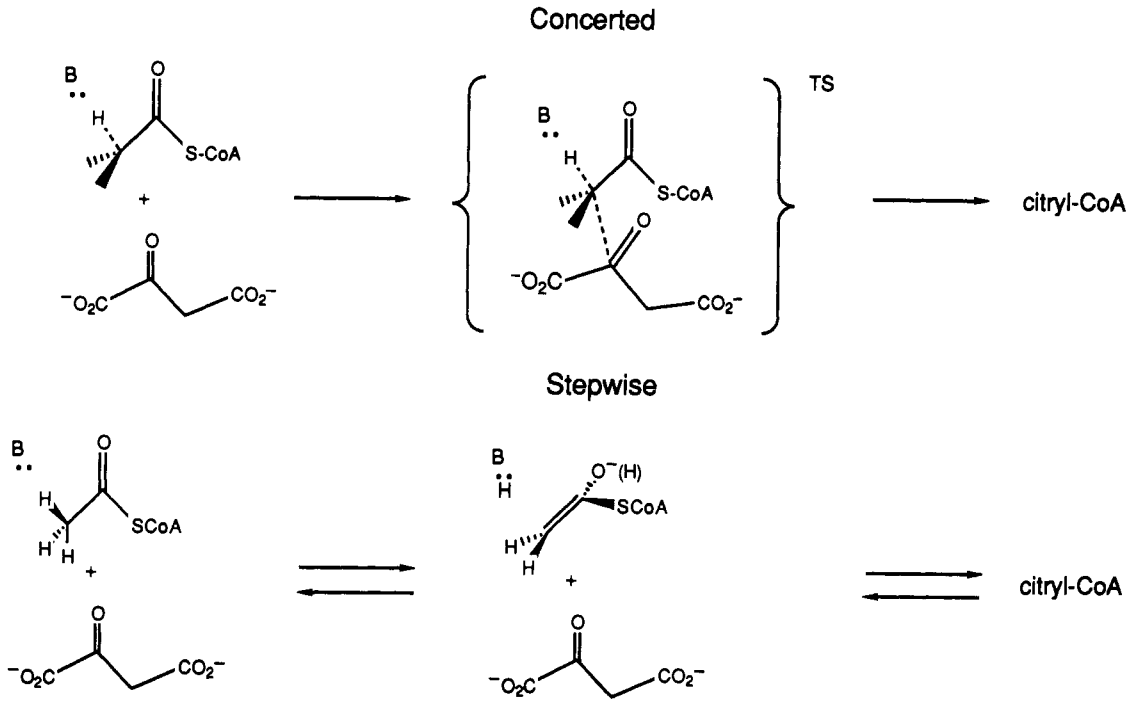
The results with FAc(=S)CoA further support the preference of the active site for the enethiolized form of the dithioester. The spectrally determined pK_a of FAc(=S)CoA (data not shown) of 9.3 is 3 pK_a units lower than the pK_a of Ac(=S)CoA and it binds 330-fold more tightly at pH 6.6. The difference spectra (Figure 2B) indicate that at pH 7.4 over 40% of the FAc(=S)CoA is bound in the enethiolized form. From the observed K_i of 300 nM at pH 6.6 we can calculate an effective K_i of the enethiolate of FAc(=S)CoA as being 620 pM, or 3.9×10^4 times less than the K_m for AcCoA. This value compares with the K_i values of 70 nM and 9 μM for the negatively charged carboxymethyl-CoA (Bayer et al., 1981) and neutral acetonyl-CoA (Rubenstein & Dryer, 1980), respectively.

Relative Energies of Bound Intermediates. Three distinct enzyme–Ac(=S)CoA intermediate complexes must exist prior to the carbon–carbon bond forming step. The observation that citrate synthase catalyzes the exchange of the α-protons of Ac(=S)CoA implies the initial Michaelis complex contains the fully protonated substrate. The existence of two enol(ate) complexes is required since the exchange rate observed in the ^1H NMR is over an order of magnitude faster than k_{obs} obtained from the observed transient. If exchange were to occur only from the spectrally detected intermediate, it could occur no faster than the observed first-order rate constant for the transient. An additional requirement for the exchange rate to be faster than the k_{obs} for the transient is that the high-energy intermediate must return to the Michaelis complex faster than it is converted to the more stable intermediate, i.e., $k_4 > k_5$.

The pK_a of the bound Ac(=S)CoA, i.e., the equilibrium constant between the first and third intermediates in Figure 5, can be estimated at pH 7.5 and 8.3 from the maximum burst stoichiometries of 0.035 and 0.31 to be 8.9 and 8.7, respectively. This is a maximum value for the pK_a on the enzyme because it is possible that not all of the remaining enzyme is present as the ternary complex with protonated Ac(=S)CoA. In experiments at higher pH values, the steady-state rate had increased and the rate of the transient decreased to such an extent that it became difficult to detect the burst. The decrease of at least 3.7 pH units in the effective pK_a of Ac(=S)CoA indicates citrate synthase must promote its ionization by providing 5 kcal/mol more favorable interaction with the enethiolized form of the dithioester. The energy levels depicted represent the relative energies at pH 7.5. At higher pH the energy level of the third intermediate will decrease. The energy level of the second intermediate complex is dashed because its level is uncertain. The lowest possible value shown is derived from the assumption that a 1% burst would have been reproducibly detected and consequently this intermediate must be over 2.3 kcal/mol higher in energy than the Michaelis complex.

Relative Transition-State Energies. The transition-state energies are defined by four experiments. The partitioning of the initially bound Ac(=S)CoA can be determined by examining the distribution of deuterium in the α-methyl group. If each exchange event is independent, the fraction of CH_3, CH_2D, CHD_2, and CD_3 groups can be predicted if the fractional deuterium incorporation, x, is known as $(1 - x)^3$, $3(1 - x)^2x$, $3(1 - x)x^2$, and x^3, respectively. For our experiment with an experimental x of 0.37, the observed integrations

Scheme II



of 0.29, 0.39, 0.25, and 0.07 compare with the calculated values of 0.25, 0.44, 0.26, and 0.05, respectively. This agreement suggests each exchange event was independent. This independence is achieved only if the reprotonated Ac(=S)CoA preferentially dissociates from the enzyme, i.e., $k_2 > k_3$. Because of the potential for intramolecular primary and secondary isotope effects, a rigorous analysis was not undertaken, but a simple model with no isotope effects and assuming equal rates of exchange and dissociation indicated over 10% of the methyl group would be CD_3. A large primary intramolecular deuterium isotope effect would increase this value.

The kinetic barrier between the Michaelis complex and the first unstable complex is calculated from the rate constant for the exchange of the α-hydrogens with solvent detected in the ^1H NMR by assuming that exchange with solvent ($\pm H^+$) is rapid. If exchange of the removed proton with solvent is partially or completely rate limiting, this transition-state energy would be overestimated.

The height of the barrier between the two enethiol(ate) complexes is determined by the k_{obs} for the pre-steady-state transient. The observed first-order rate is given by eq 6, where

$$k_{obs} = \frac{\left\{\frac{k_3k_5}{(k_3 + k_4 + k_5)}\right\}S}{\frac{k_2k_4 + k_2k_5 + k_3k_5}{k_1(k_3 + k_4 + k_5)} + S} + \frac{\left\{\frac{k_2k_4}{(k_2 + k_3 + k_4)}\right\}H^+}{\frac{k_2k_4 + k_2k_5 + k_3k_5}{k_6(k_2 + k_3 + k_4)} + H^+} \quad (6)$$

the rate constants are defined in Figure 5. When the pH < pK_a, the protonation rate constant must be faster than the deprotonation rate constant; i.e., the second term is greater than the first. If $k_2 > k_3$ and $k_4 > k_5$ (vide supra), then the observed rate constant for the burst is given by k_6H^+; i.e., k_{obs} may be used to estimate the kinetic barrier between the two enethiol(ate) intermediates. As indicated by eq 6, the observed variation of k_{obs} with pH is accounted for by this model.

The k_{cat} for the overall reaction defines the free energy difference between the most populated intermediate and the highest subsequent transition-state energy. At pH 7.5 the small burst amplitude and existence of rapid exchanges implicate the initial Michaelis complex as the most populated intermediate and a transition state following formation of the spectrally detected complex as having the highest energy.

Concerted vs Stepwise Mechanism. The mechanistic role of the thioester linkage in CoA esters has long been presumed to be the promotion of the acidity of the α-hydrogens. This resulted in the hypothesis that many of the condensation reactions utilizing AcCoA proceeded through a discrete carbanion intermediate. Dewar and Dieter (1988), using theoretical calculations on gas-phase model systems, have come to a similar conclusion. Double isotope effect studies have experimentally demonstrated that the analogous condensation of AcCoA and glyoxalate to form (*S*)-malate catalyzed by malate synthase proceeds via an enol(ate) intermediate (Clark et al., 1988). The stepwise and the alternative concerted reaction pathways are shown in Scheme II. Eggerer (1965) and Srere (1967) demonstrated that citrate synthase was capable of forming the enol(ate) intermediate by measuring the exchange rate of the α-hydrogens of AcCoA with solvent in the presence of (*S*)-malate. The rate of the reaction at saturating AcCoA concentrations, however, is only 2×10^{-3} s^{-1}, which is 1.4×10^5 times slower than the V_{max} of 283 s^{-1} (Singh et al., 1970) with oxalacetate replacing (*S*)-malate. Remington et al. (1982) have suggested that His274 is the active-site base and that the proton removed from AcCoA is shielded from exchange with solvent. However, with Ac(=S)CoA the exchange of the removed proton with solvent must be faster than 0.54 s^{-1}.

The alternative possibility is that the bond-forming and bond-breaking reactions are concerted and the exchange reaction is kinetically much slower than condensation and is consequently unobservable in the presence of oxalacetate. The primary arguments in favor of a concerted reaction are the inversion of stereochemistry at the methyl group of AcCoA (Lenz et al., 1971) and the lack of exchange on a turnover time scale (Eggerer, 1965).

Our results with the FAc(=S)CoA strongly implicate a stepwise mechanism for the dithioesters. If carbon–carbon

bond formation were not possible from the enethiol(ate), the spectrally detected intermediate would be a dead-end complex and increasing its concentration would decrease the rate in the absence of other effects. The 2-fluoro substitution, which enhances the stability of the enethiolate, results in a significant increase in rate, which suggests that the enethiol(ate) is catalytically competent intermediate.

The stepwise reaction apparent with the dithioesters cannot be extrapolated to the condensation of AcCoA. The thiocarbonyl substitution has enhanced the acidity of the α-hydrogens by an estimated factor of 10^7 (Wlassics et al., 1988). It would not be surprising that an active site designed to accommodate a concerted proton transfer will promote ionization of an analogous molecule that is favored by this large amount. The other difficulty with the extrapolation is the 10^6 difference in rates between the thiol and dithioesters. A thiocarbonyl double bond is 0.4 Å longer than a carbon–oxygen double bond (Storer & Carey, 1985). This steric difference may move the nucleophilic α-carbon beyond an essential reacting distance with the electrophilic carbon of oxalacetate. For this interpretation to be correct, not only must the lowest energy conformation be beyond a reactive distance but the conformation must be rigid enough to prevent easy access to a reactive conformation.

A second potential explanation for the extremely slow turnover is that the electron density in the enethiol(ate) is localized on the sulfur and in AcCoA it is localized to a much greater extent on the nucleophilic α-carbon. Gas-phase calculations indicate nearly half of the negative charge remains on the α-carbon after a thioester (Dewar & Dieter, 1988) is deprotonated. The rationale for the enhanced acidity of the α-hydrogens of the dithioesters is the comparative weakness of the carbon–sulfur double bond, so a much greater fraction of the negative charge would be localized on the thiocarbonyl sulfur. The 10-fold increase in activity observed with FAc(=S)CoA could be accounted for by this hypothesis. The inductive effect of the fluorine would return some of the electron density to the α-carbon. This hypothesis is not supported by model reactions. The metalated enethiolates of methyl dithioacetate condense readily with carbonyls at −20 to −78 °C to yield 3-hydroxydithioesters (Lawson et al., 1984; Meyers et al., 1978). Additionally, Ac(=S)coA serves as the nucleophile with AcCoA as the electrophile to produce 3-oxobutyryldithio-CoA in the presence of thiolase from *Zooglea ramigera* faster than the physiological dimerization reaction shown in eq 7 and 8 (V. Anderson, unpublished observation).

$$\text{AcCoA} + \text{AcCoA} \rightarrow \text{AcAcCoA} \quad (7)$$

$$\text{AcCoA} + \text{Ac(=S)CoA} \rightarrow \text{AcAc(=S)CoA} \quad (8)$$

Finally, the condensation reaction between Ac(=S)CoA and oxalacetate may be thermodynamically unfavorable at the active site of the enzyme. This explanation is again doubtful because the equilibrium constant for the condensation of Ac(=S)CoA with AcCoA is approximately 30-fold greater than that for the dimerization (V. Anderson, unpublished observation). The substitution of sulfur for the carbonyl oxygen consequently weakens the α-carbon–hydrogen bond by roughly 2 kcal/mol more than the α-carbon–carbon bond.

Summary. The utility of dithioesters as spectroscopic probes of enzyme-catalyzed reactions has been demonstrated by showing that citrate synthase stabilizes an enethiol(ate) form of Ac(=S)CoA. On the basis of the relative rates of the condensation reaction and enethiolization of Ac(=S)CoA and the enhanced V_{max} observed with FAc(=S)CoA, we believe the condensation reaction of oxalacetate with CoA dithioesters catalyzed by citrate synthase is a stepwise process.

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Registry No. Ac(=S)CoA, 113947-51-6; [2-^{13}C]Ac(=S)CoA, 118355-23-0; CoA, 85-61-0; ethyl-CoA, 70019-68-0; citrate synthase, 9027-96-7; oxalacetate, 328-42-7; 2-fluoroacetyldithio-CoA, 118355-22-9; thiophenol, 108-98-5; [2-^{13}C]acetylthiophenol, 118355-24-1; Lawesson's reagent, 19172-47-5; [2-^{13}C]acetylthiophenol dithioester, 118355-25-2; [2-^{13}C]acetyl chloride, 14770-40-2; 2-fluoroacetyl chloride, 359-06-8; 2-fluoroacetylthiophenol, 370-04-7; 2-fluoroacetylthiophenol dithioester, 118355-26-3; ethyl iodide, 75-03-6; acetyl-CoA, 72-89-9.

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