Devillez, E. J., and Buschlen, K. (1967), *Comp. Biochem. Physiol. 21*, 541.
Folk, J. E., Piez, K. A., Carroll, W. R., and Gladner, J. (1960), *J. Biol. Chem. 235*, 2272.
Folk, J. E., and Schirmer, E. W. (1963), *J. Biol. Chem. 238*, 3884.
Gates, B. J. (1972), Ph.D. Thesis, University of Georgia.
Gates, B. J., and Travis, J. (1969), *Biochemistry 8*, 4483.
Kleine, R. (1969), *Z. Vergl. Physiol. 56*, 142.
Lacko, A. G., and Neurath, H. (1970), *Biochemistry 9*, 4680.
Laskowski, M. (1955), *Methods Enzymol. 2*, 33.
Linke, V. R., Zwilling, R., Herbold, D., and Pfleiderer, G. (1969), *Hoppe Zeller's Z. Physiol. Chem. 350*, 877.
Matsubara, H., and Sasaki, R. M. (1969), *Biochem. Biophys. Res. Commun. 35*, 175.
McMeekin, T. L., and Marshall, K. (1952), *Science 116*, 142.
Nelson, C. A. (1971), *J. Biol. Chem. 246*, 3895.
Prahl, J. W., and Neurath, H. (1966), *Biochemistry 5*, 4137.
Reeck, G. R., and Neurath, H. (1972), *Biochemistry 11*, 3947.
Schachman, H. K. (1967), Ultracentrifugation in Biochemistry, New York, N. Y., Academic Press, p 82.
Warburg, O., and Christian, W. (1942), *Biochem. Z. 310*, 384.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.
Zwilling, R., Pfleiderer, G., Sonneborn, H. H., Kraft, V., and Stucky, I. (1969), *Comp. Biochem. Physiol. 28*, 1275.

Interaction of a Spin-Labeled Analog of Acetyl Coenzyme A with Citrate Synthase. Paramagnetic Resonance and Proton Relaxation Rate Studies of Binary and Ternary Complexes†

S. W. Weidman, G. R. Drysdale,* and A. S. Mildvan

ABSTRACT: A spin-labeled analog of acetyl coenzyme A (CoA), R·CoA (3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl-CoA thio ester), has been prepared and shown, by magnetic resonance techniques, to bind to pig heart citrate synthase. Paramagnetic resonance studies indicated that the bound R·CoA was "partially immobilized," with a dissociation constant of 10^{-4} M and 2.2 mol bound per enzyme dimer. The bound radical enhanced the longitudinal relaxation rate of water protons by a factor of 3.4. Acetyl-CoA and CoASH displaced R·CoA from the enzyme. The dissociation constants for both, as shown by competition studies, were approximately equal to that of R·CoA. Paramagnetic resonance and proton relaxation rate studies showed that oxaloacetate formed a ternary complex, R·CoA–enzyme–oxaloacetate. The affinity of the enzyme for R·CoA was not affected by oxaloacetate binding, but the paramagnetic resonance spectrum of the bound radical changed to one of a spin-labeled substrate covalently bound to chymotrypsin by electron paramagnetic resonance. Weiner (1969) and Mildvan and Weiner (1969a,b) studied the noncovalent interaction of a spin-labeled analog of NAD^+ with alcohol dehydrogenase by paramagnetic resonance and by measurements of the relaxation rates of water and substrate protons. The information that may be obtained by such studies includes: (1) determination of the number of binding sites and the binding constant of the analog; (2) detection of ternary complexes of the analog with enzyme substrates or substrate analogs, and their thermodynamic properties; and (3) kinetic and structural information concerning binary and ternary complexes containing the analog.

This paper concerns the application of these techniques to characteristic of a "fully immobilized" nitroxide. The enhancement of the relaxation rate of water protons by the bound radical in the ternary R·CoA–enzyme–oxaloacetate complex increased by about 40% over that in the R·CoA–enzyme complex. From a large series of mono-, di-, and tricarboxylic analogs of oxaloacetate and citrate, ternary R·CoA–enzyme–ligand complexes were observed only with citrate, (*R*)-malate, and (*R*,*S*)-tartrate by the paramagnetic resonance method. The paramagnetic resonance spectra of the bound R·CoA in these ternary complexes were similar and slightly more "fully immobilized" than that in the oxaloacetate–enzyme–R·CoA system. The dissociation constants of citrate, (*R*)-malate, and (*R*,*S*)-tartrate from their corresponding ternary complexes were 0.5, 0.4, and ~2 mM, respectively. Stereochemical arguments are presented to account for the selectivity of R·CoA–enzyme–ligand complex formation.

Probing active sites of enzymes by magnetic resonance techniques using paramagnetic ions or spin-labeled substrate analogs has been exploited by several groups (Mildvan and Cohn, 1970). McConnell (1967) first investigated the motion of a spin-labeled substrate covalently bound to chymotrypsin by electron paramagnetic resonance. Weiner (1969) and Mildvan and Weiner (1969a,b) studied the noncovalent interaction of a spin-labeled analog of NAD^+ with alcohol dehydrogenase by paramagnetic resonance and by measurements of the relaxation rates of water and substrate protons. The information that may be obtained by such studies includes: (1) determination of the number of binding sites and the binding constant of the analog; (2) detection of ternary complexes of the analog with enzyme substrates or substrate analogs, and their thermodynamic properties; and (3) kinetic and structural information concerning binary and ternary complexes containing the analog.

This paper concerns the application of these techniques to

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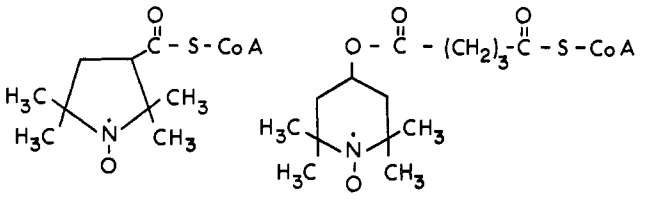
pig heart citrate synthase (EC 4.1.3.7). This enzyme catalyzes the condensation of acetyl-CoA with oxaloacetate to form citrate and CoASH. Two spin-labeled analogs of acetyl-CoA were prepared and used in these studies.



R · Co A

I

G · Co A

II

Experimental Section

Synthesis of R·CoA. 2,2,5,5-Tetramethylpyrrolidine-3-carboxamide-1-oxyl (Frinton Laboratories) was converted to the acid by the method of Rozantsev and Krinitskaya (1965). The acid chloride was prepared as follows: 18.6 mg (100 μmol) of the dry acid was placed in a small round-bottomed flask equipped with a drying tube and dissolved in 0.3 ml of anhydrous pyridine (Rozantsev, 1970). To the resulting dark yellow solution was added 9 μl (124 μmol) of thionyl chloride. A white precipitate of pyridine hydrochloride immediately formed. The solution was stirred for 15 min. Approximately 60 μl (20 μmol) of the acid chloride solution was added to 1 ml of an aqueous solution containing 10 μmol of CoASH (P-L Biochemicals), 20 mg of $NaHCO_3$, and 1.2 mg of EDTA at pH 7.0 and 0°. The EDTA was necessary to complex trace metal ions which catalyze reaction of the nitroxide with free sulfhydryls (McConnell and McFarland, 1970). The solution was stirred vigorously with a glass stirring rod for about 20 sec, and nitrogen was bubbled through the solution for 10 min while the pH was maintained near 7.0 by the addition of solid $NaHCO_3$. If the nitroprusside test for free SH was not negative at this point, more acid chloride solution was added (Stadtman, 1957). The reaction mixture was then passed through a 1 × 10 cm column of Sephadex G-10 in water at 4°. The early fractions containing the bulk of the material having an absorbance maximum at 260 nm were pooled and placed on a 1 × 10 cm column of DEAE-cellulose (DE-52, Whatman) equilibrated with 0.003 M HCl at 4°. A linear LiCl gradient with 50 ml of 0.003 M HCl in the mixing flask and 50 ml of 0.15 M LiCl in 0.003 M HCl in the reservoir elutes the R·CoA at aproximately 0.1 M LiCl (Moffatt and Khorana, 1961). The fractions containing the radical CoA ester were pooled, sodium acetate was added to neutralize HCl, and the material was lyophilized to near dryness. Water was added to the residue to bring the total volume to 1–2 ml. The solution was then desalted as above on a Sephadex G-10 column. The overall yield of R·CoA is approximately 50%. The extinction coefficient of R·CoA at 260 nm was estimated to be 16×10^3 M^{-1} cm^{-1}. The concentration of paramagnetic R·CoA molecules could also be estimated from its paramagnetic resonance spectrum by comparison of the amplitude of the center line in an R·CoA solution with the corresponding line in a solution of 2,2,5,5-tetramethylpyrrolindine-3-carboxyl-1-oxyl of known concentration, since the line widths are equal for the mid-field lines. The radical concentration as determined by paramagnetic resonance was 75–80% of the concentration determined spectrophotometrically. The concentration of paramagnetic molecules was used in all calculations of dissociation constants. The 20–25% discrepancy may be caused by inaccuracy in the estimate of the extinction coefficient, or more probably by the presence of diamagnetic CoA ester arising from the acid-catalyzed disproportionation of the nitroxide moiety (Rozantsev, 1970). The pH of solutions of R·CoA should not be allowed to fall below 2.0, to avoid this disproportionation. Failure to neutralize the 0.003 M HCl prior to lyophilization led to a product which had intense absorbance at 350 nm and which was diamagnetic. The R·CoA preparations contain no R–COOH or oxidized CoA. The nitroxide thio ester gives a negative test for free sulfhydryl with Ellman's reagent (Ellman, 1959), but yields the expected amount of free sulfhydryl upon reaction with hydroxylamine at neutral pH.

Synthesis of G·CoA. The hydrogen glutarate of 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl was synthesized by the method given in Rozantsev (1970) for the hydrogen phthalate: yield, 40%; mp 80.0–83.0°. The infrared spectra of the acid and its sodium salt, as well as the nmr spectrum of the sodium salt reduced with sodium dithionite, were consistent with the above structure. The CoA ester was prepared and purified as described for R·CoA.

Materials. Tris-chloride buffer (0.04 M) at pH 7.5 was used in all magnetic resonance studies. Acetyl-CoA was prepared by the modified method of Simon and Shemin (1953) and was purified by chromatography on DEAE-cellulose as described for the radical CoA ester. The following acids were purchased from Sigma Chemical Co.: oxaloacetic, citric, D-(*R*)- and L-(*S*)-malic,[1] α-ketoglutaric, succinic, *cis*- and *trans*-aconitic, maleic, fumaric, tricarballylic, D-(*R*)- and L-(*S*)-lactic and *threo*-D_8-(2*R*,3*S*)-isocitric (monopotassium salt). *meso*-(*R*,*S*)-Tartaric acid was obtained from Aldrich Chemical Co. The above acids were used without further purification and were neutralized to pH 7.5 with Tris base before use.

Pig heart citrate synthase, obtained as an ammonium sulfate suspension from Boehringer-Mannheim, was desalted on Sephadex G-25. Several batches of the enzyme had specific activities of 140–200 units (U)/mg with the standard DTNB assay system of Srere *et al.* (1963). The specific activity of the pure enzyme was reported by Singh *et al.* (1970) to be 180 U/mg. The concentration of enzyme solutions was determined spectrophotometrically at 280 nm, using an absorbance index ($E^{1\%}_{1\,cm}$) of 17.8. For some experiments the enzyme was concentrated in a Schleicher and Schuell collodion bag apparatus (A. H. Thomas).

Magnetic Resonance Studies. Electron paramagnetic resonance spectra of 0.025–0.05-ml samples of solutions containing the analog were obtained in quartz capillaries using Varian E-3 and E-4 spectrometers. The temperature was controlled at 24.5 ± 1.0°. Binding of the analog was measured by comparison of the peak-to-peak amplitude of the high-field line in the paramagnetic resonance spectrum of the sample containing the analog plus enzyme to that of a solution of equal concentration of analog in buffer alone.

The longitudinal relaxation rate, $1/T_1$, of water protons in solutions containing analog and/or citrate synthase was measured with a precision of ±1% by the pulsed method at 24.3 MHz as previously described by Mildvan and Cohn (1963), using the NMR Specialties PS 60W pulsed nmr spectrometer. The theory of relaxation rate enhancement has recently been reviewed by Mildvan and Cohn (1970).

[1] No oxaloacetate could be detected in the (*R*)-malic acid with malic dehydrogenase and NADH.

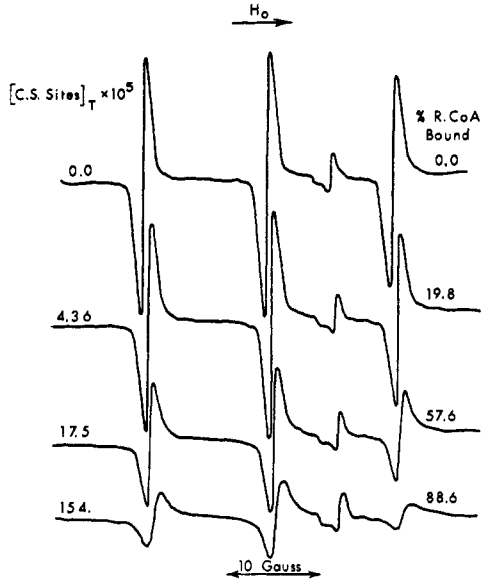


FIGURE 1: Paramagnetic resonance spectra of R·CoA in the presence and absence of citrate synthase. The total R·CoA concentration was 1.19×10^{-5} M in 0.04 M Tris-chloride at pH 7.5 and 24.5° for all spectra. Concentrations of citrate synthase are expressed as active site concentrations assuming a mol wt of 48,000 per active site. The gain and modulation amplitudes (1.0 G) were the same for all spectra. The signal between the mid- and high-field signals is a quartz impurity resonance.

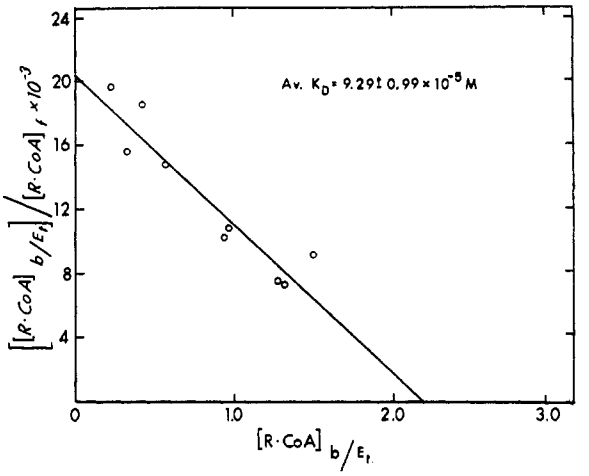


FIGURE 2: Scatchard plot of the paramagnetic resonance data for R·CoA binding to citrate synthase. The line intersecting the abscissa at $[R\cdot CoA]_b/E_t = 2.2$ was determined by a least-squares fit of the data.

Results

Binding of R·CoA to Citrate Synthase. R·CoA has a typical nitroxide paramagnetic resonance spectrum (Figure 1; Hamilton and McConnell, 1968). The addition of citrate synthase to a solution of the analog causes the amplitudes of all three lines of the paramagnetic resonance spectrum to decrease (Figure 1). Comparison of the amplitude of each line with that of the corresponding line in the standard yields unequal estimates for the per cent radical bound to the enzyme. The largest change in amplitude relative to the standard was observed for the high-field line with intermediate values for the low-field line and lowest values for the middle-field line. If the bound radical had been "fully immobilized," equal estimates would have been expected for the per cent bound using the low- and high-field lines with a slightly lower value resulting from the middle-field line (Taylor *et al.*, 1969). Thus, the bound radical must contribute to the observed spectrum (which is a summation of narrow and broad spectra) and is therefore only "partially immobilized." This conclusion was supported by the failure to observe distinct shoulders or new peaks in the low- and high-field regions of the paramagnetic resonance spectrum when the instrumental gain and modulation amplitude were increased (Figure 3). By comparison with published spectra of partially immobilized nitroxides in aqueous glycerol solutions, a rotational correlation time, τ_c, of the order of 5×10^{-9} sec can be estimated (Hsia and Piette, 1969; Hoff *et al.*, 1971). The corresponding τ_c for R·CoA in buffer would be of the order of 10^{-10} sec.

The amplitude of the high-field line was used to calculate the per cent R·CoA bound to the enzyme. The results of titrations described below indicate that the bound radical makes, at most, a 2 or 3% contribution to the amplitude of this line; but an accurate estimation of this contribution was not possible.

The enzyme was titrated with R·CoA to determine the dissociation constant of the radical–enzyme complex and the number of moles of R·CoA bound per mole of enzyme. A molecular weight of 96,000 for the enzyme was used (Singh *et al.*, 1970; Wu and Yang, 1970a). The saturation fraction covered was 10–80% (Deranleau, 1969). A Scatchard plot of the data is shown in Figure 2 (Scatchard, 1949). Extrapolation of the line to $[R\cdot CoA]b/E_t = 2.2$ fits the data with the dissociation constant, K_D, for the radical–enzyme complex of 9.3×10^{-5} M. Previous work has indicated that citrate synthase is a dimer containing two similar subunits (Singh *et al.*, 1970; Wu and Yang, 1970a). There were no indications of secondary, weaker, nonspecific binding.

Some variation was noted in K_D from one set of experiments to another employing different desalted enzyme and R·CoA preparations. This variation was probably caused by incomplete desalting; since, as will be shown, the dissociation constant for R·CoA increases with increasing ionic strength.

The molar relaxivity of R·CoA ($1/T_{1P}[R\cdot CoA]$) was found to be 230 ± 24 M^{-1} (Table I). This value is comparable to that reported for other nitroxides at 24.3 MHz, *e.g.*, 2,2,6,6-tetramethyl-4-hydroxypiperidine-1-oxyl (282 M^{-1} sec^{-1}, Mildvan and Weiner, 1969a) and *N*-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide (245 M^{-1} sec^{-1}, Taylor *et al.*, 1969).

Binding of R·CoA to citrate synthase causes an enhancement of the effect of the radical on the relaxation rate of water protons (Table I). The observed enhancement, ϵ^*, is the weighted average of the enhancements of the free, ϵ_f, and bound, ϵ_b, R·CoA

$$\epsilon^* = \frac{[R\cdot CoA]_f}{[R\cdot CoA]_t}\epsilon_f + \frac{[R\cdot CoA]_b}{[R\cdot CoA]_t}\epsilon_b \qquad (1)$$

where the subscripts f, b, and t refer to the concentrations of free, bound, and total radical CoA, respectively (Mildvan and Weiner, 1969a,b). By definition, $\epsilon_f = 1$. ϵ_b may be calculated from eq 1 using the value for the fraction R·CoA bound obtained by paramagnetic resonance measurements. The data presented in Table I show that $\epsilon_b = 3.4$, that is, the bound radical is 3.4 times more effective in relaxing water protons than the unbound radical, and that ϵ_b is not a function of the saturation fraction. The magnitude of this bound enhancement is much lower than that found for a spin-labeled

TABLE I: Effect of R·CoA and Its Complex with Citrate Synthase on the Proton Relaxation Rate of Water.

Addn to 0.04 M Tris-Chloride Buffer (Concn (mM))	$1/T_1$ or Proton Relaxation Rate (sec^{-1})	$1/T_{1P}$ (sec^{-1})	ϵ^* [a]	$[R\cdot CoA]_f/[R\cdot CoA]_t$ [b]	ϵ_b [c]
None	0.417				
R·CoA (1.34)	0.720	0.303			
R·CoA (2.68)	1.030	0.613	1.00	1.00	
R·CoA (0.608)	0.580	0.163			
C.S.[d] (0.201)	0.468				
R·CoA (0.608) + C.S. (0.201)	0.779	0.311	1.91	0.671	3.8
R·CoA (0.10)	0.446	0.029			
C.S. (0.221) + R·CoA (0.10)	0.554	0.086	2.97	0.380	4.2
					3.4 ± 0.4[e]

[a] Calculated from the definition of ϵ^*. [b] Determined by epr. [c] Calculated from *a* and *b* using eq 1. [d] Citrate synthase site concentration assuming 2.0 sites per enzyme molecule. [e] Average of 14 determinations.

analog of NAD^+ with alcohol dehydrogenase for which ϵ_b varied from 80 to 15 depending on the site occupancy (Mildvan and Weiner, 1969a). The observed ϵ_b is about one-half of that found for a strongly immobilized covalently bound nitroxide on creatine kinase (Taylor *et al.*, 1969). Although the enhancement is a function of the access of water to the nitroxide, as well as the extent of immobilization of the radical and its water of hydration, this relatively low ϵ_b value is consistent with the partially immobilized state of the bound R·CoA, indicated by the paramagnetic resonance spectrum.

Displacement of R·CoA from Citrate Synthase by Acetyl-CoA and CoASH. When increasing concentrations of acetyl-CoA or CoASH were added to a solution of R·CoA and citrate synthase, the bound R·CoA was progressively displaced from the enzyme. These results are illustrated in Table II for acetyl-CoA. Although the data of Table II show a progressive decrease to a limiting value of K_D as the acetyl-CoA concentration is raised, we are not prepared to attribute this change to a variation in affinity with site occupancy. The analog, acetyl-CoA, and CoASH appear to be competing for the same site on the enzyme, and the dissociation constants for acetyl-CoA and CoASH (14.5 and 11.1×10^{-5} M, respectively) were approximately the same as that determined for R·CoA.

Formation of the R·CoA–Citrate Synthase–Oxaloacetate Ternary Complex. Previous work of Srere (1966) has shown that oxaloacetate forms a tight binary complex with citrate synthase with a dissociation constant of 6×10^{-7} M in 4 M urea. When oxaloacetate is added to a solution of R·CoA and the enzyme, the concentration of the unbound radical and hence the affinity of the enzyme for the radical do not change detectably (Figure 3). However, oxaloacetate does promote a profound decrease in the rotational freedom of the bound radical, as indicated by the disappearance from the low-gain spectrum of the contribution of the bound radical (Figure 3). The middle- and low-field lines are decreased in amplitude and narrowed. Therefore, the low-gain paramagnetic resonance spectrum consists almost entirely of that of the unbound radical.

When the gain and modulation amplitudes of the paramagnetic resonance spectrometer were increased, the broadened and shifted peaks of the bound and immobilized radicals in the oxaloacetate ternary complex were observed (Figure 3). The peak-to-peak distance between the low- and high-field lines (56.3 G) is comparable to that found for a covalently bound and highly immobilized spin label on creatine kinase (62 G, Taylor *et al.*, 1969) and to the value which can be estimated from the data of Mildvan and Weiner (1969a) for the highly immobilized NAD^+ analog bound to alcohol dehydrogenase (61 G).

TABLE II: Displacement of R·CoA from Citrate Synthase by Acetyl-CoA and Calculation of the Acetyl-CoA–Citrate Synthase Dissociation Constant.

[Acetyl-CoA] (mM)[a]	Free R·CoA (%)[b]	Calcd K_D, Acetyl-CoA (10^5 M)[c]
0	35.3	
0.202	44.2	19.7
0.404	53.0	17.4
0.607	61.8	14.4
0.889	72.1	12.4
1.07	76.2	11.7
1.58	83.3	11.6
		14.5 ± 2.7[d]

[a] Citrate synthase site concentration and $[R\cdot CoA]_t$ were 0.352 and 0.230 mM, respectively, in 0.04 M Tris-chloride, pH 7.5. [b] Determined by epr. [c] Calculated, assuming K_D-$(R\cdot CoA) = 11.1 \times 10^{-5}$ M (determined from a simultaneous experiment with no addition of acetyl-CoA). [d] Average of all determinations.

A paramagnetic resonance titration based upon the decrease to a limiting value of the amplitude of the middle- and low-field lines in the low-gain spectra with increasing oxaloacetate concentration indicated the binding of oxaloacetate was stoichiometric with enzyme sites with a dissociation constant of $\leq 10^{-5}$ M. Figure 4 shows the change in enhancement of the relaxation rate of water protons as oxaloacetate is added to the R·CoA–enzyme system. Although the overall changes in the relaxation rates were small, the results were reproducible. The enhancement of the relaxation rate of water protons caused by the bound radical in the ternary complex, ϵ_t, was about $1.4\epsilon_b$. An upper limit of 4.4×10^{-5} M on the dissociation constant for oxaloacetate from the ternary complex was obtained. An increase in the enhancement would be

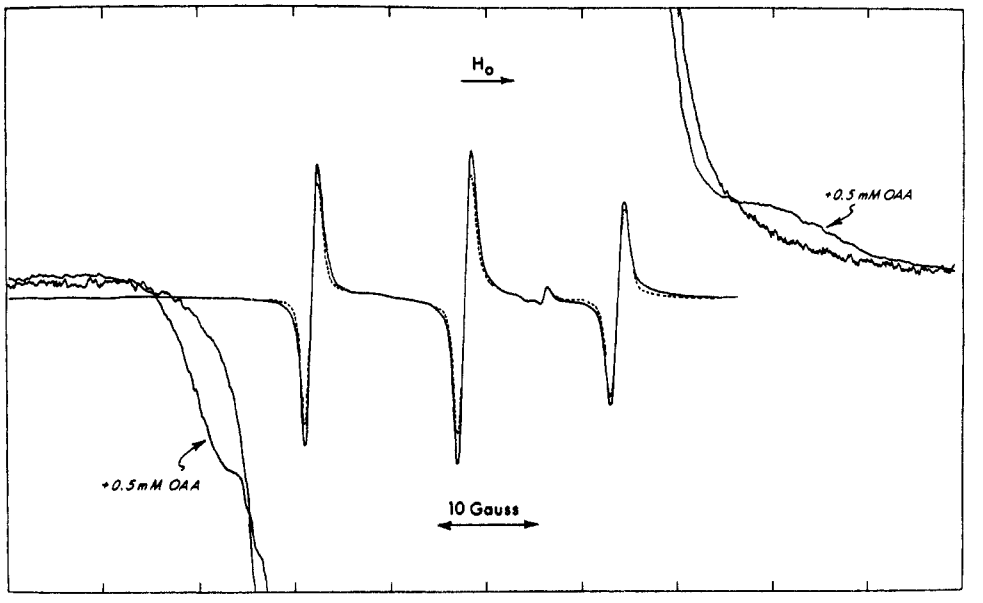


FIGURE 3: R·CoA–citrate synthase system in the presence (– – – –) and absence (————) of 0.50 mM Tris-oxaloacetate. The spectra which are off-scale are higher gain and higher modulation amplitude (6.3 G) recordings of the on-scale spectra. In both cases the concentration of citrate synthase active sites and total R·CoA concentration were 0.340 and 0.235 mM, respectively. The apparent noise level is lower in the high gain spectra in the presence of oxaloacetate because it was retraced by hand.

expected upon immobilization of R·CoA, if water access to the nitroxide were unchanged. However, larger enhancements have been observed for almost fully immobilized nitroxides on creatine kinase and alcohol dehydrogenase. The relatively small increase in the enhancement by the bound radical may be caused by a decrease in the accessibility of water protons to the paramagnetic center in the ternary complex.

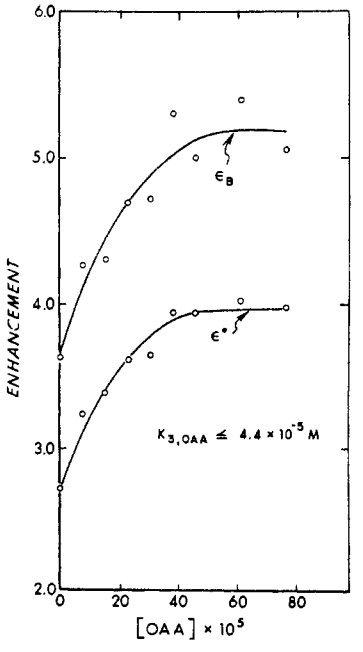


FIGURE 4: Change in the proton relaxation rate of the H_2O protons as a function of oxaloacetate concentration in the citrate synthase –R·CoA system. Titration of 0.297 mM citrate synthase (active sites) and 0.262 mM R·CoA with a concentrated solution of Tris-oxaloacetate in the same system. The bound enhancement, ϵ_b, was calculated from the observed enhancement, ϵ^*, and the per cent unbound R·CoA (determined by paramagnetic resonance), using eq 1.

Effects of Various Carboxylic Acids on the Citrate Synthase–R·CoA Binary Complex. The following carboxylic acids (Tris salts) have been examined for their effect on the citrate synthase–R·CoA system: citric, *threo*-D_S-(2*R*,3*S*)-isocitric, *cis*- and *trans*-aconitic, tricarballylic, α-ketoglutaric, succinic, maleic, fumaric, (*R*)- and (*S*)-malic, (*S*,*S*)- and (*R*,*R*)-tartaric, (*R*,*S*)-tartaric, and (*R*)- and (*S*)-lactic. Of these substances, only (*R*)-malate, citrate, and *meso*-(*R*,*S*)-tartrate have been found to form a ternary complex (enzyme–R·CoA–acid), as evidenced by a change in the rotational mobility of R·CoA over that in the binary enzyme–R·CoA system. The formation of binary enzyme complexes, which excluded the radical, was noted with the remainder of the carboxylic acids. These data will be presented and discussed in another paper.

The effect of adding (*R*)-malate, citrate, and (*R*,*S*)-tartrate on the affinity of the enzyme for the radical is shown in Figure 5. It is seen that the addition of (*R*)-malate or citrate in concentrations less than 2–4 mM causes a significant decrease and increase, respectively, in the dissociation constant of R·CoA. These additions cause the paramagnetic resonance spectrum of the bound R·CoA to change to that characteristic of a more fully immobilized nitroxide. The low- and high-field regions of the paramagnetic resonance spectra of the bound R·CoA (under conditions of high gain and modulation) in the ternary complexes involving (*R*)-malate and citrate are similar but both are somewhat different from that of the oxaloacetate–enzyme–R·CoA complex (Figure 6). The peak-to-peak distances are 57.8 and 57.3 G, respectively, for the (*R*)-malate and citrate ternary complexes. The dissociation constants of (*R*)-malate and citrate from their ternary complexes were estimated to be 0.4 and 0.5 mM, respectively, from the change in R·CoA dissociation constant with increasing ligand concentration. Further additions of (*R*)-malate or citrate to the enzyme–R·CoA system in concentrations greater than 2–4 mM caused slight decreases in the affinity of the enzyme for R·CoA which appeared to be linear with added ligand concentration. It is not known whether this decreased affinity for R·CoA is caused by weak binding of the added ligand at

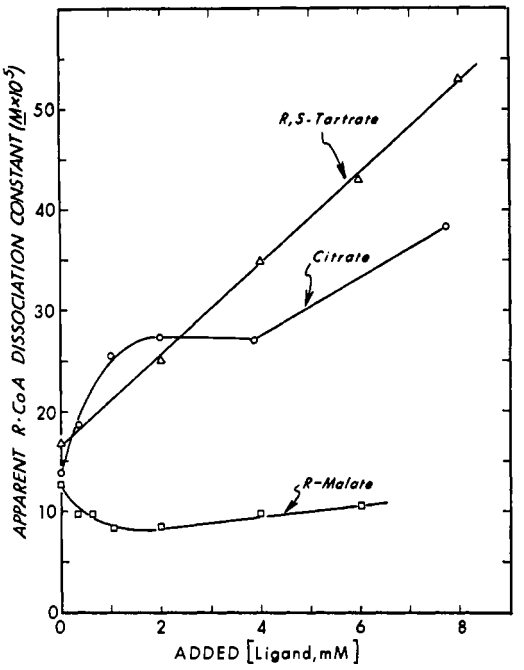


FIGURE 5: The effect of added citric, (*R*)-malic, and (*R,S*)-tartaric acids (all as Tris salts) on the apparent dissociation constant of R·CoA from the enzyme.

another site which excludes R·CoA binding or an ionic strength effect. The sensitivity of citrate synthase to inorganic salts has been well documented by other workers (Kosicki and Lee, 1966; Wu and Yang, 1970b; Poulsen and Sarkissian, 1971).

The addition of increasing concentrations of (*R,S*)-tartrate to the enzyme–R·CoA system causes a progressive decrease in the affinity of the enzyme for the radical. However, the rotational mobility of the remaining bound radical is decreased compared to the binary system. The bound R·CoA appears to be as fully immobilized as that in the other ternary complexes discussed above ((*R*)-malate and citrate), the peak-to-peak distance being ~58.7 G (Figure 6). The detection of the (*R,S*)-tartrate–enzyme–R·CoA complex as well as estimation of the dissociation constant of R·CoA from the ternary complex is complicated by the displacement of R·CoA from the enzyme by the added ligand. An estimation of the dissociation constant of (*R,S*)-tartrate from its ternary complex (~2 mM) was made from the change in the relative amplitudes of the middle- and high-field lines in the low-gain paramagnetic resonance spectra as increasing concentrations of the ligand were added. Since addition of still higher tartrate concentrations displaces increasing amounts of bound R·CoA, there appears to be another mode of binding to the enzyme available to (*R,S*)-tartrate which excludes R·CoA. The properties of the ternary complexes involving oxaloacetate, (*R*)-malate, citrate, and (*R,S*)-tartrate are summarized in Table III.

The binding of (*R*)-malate and citrate to the enzyme–R·CoA system was also studied by the proton relaxation method. The total changes in relaxation rates were small (4–6%). The facts that these changes are small and are contributed to by a multiplicity of effects (immobilization changes, changes in extent of R·CoA binding), along with the moderate enhancement upon formation of the binary complex, make any interpretation of the changes in enhancement tenuous. Paramagnetic resonance was found to be a more sensitive technique in this system for the detection and determination of dissociation

TABLE III: Properties of R·CoA–Citrate Synthase–X Ternary Complexes.

X[a]	K_X^3 (mM)[b]	$K^3_{R\cdot CoA}$[c] / $K^2_{R\cdot CoA}$
Oxaloacetate	≤0.01	1.0
D-Malate	0.4	0.7
Citrate	0.5	2.0
(*R,S*)-Tartrate	~2	*d*

[a] Added as Tris salt. In all ternary complexes the bound R·CoA is strongly immobilized. [b] Dissociation constant of X from its ternary complex. [c] Ratio of the dissociation constant of R·CoA from ternary complex (inflection points in Figure 5) to that of the R·CoA–citrate synthase binary complex. [d] Could not be determined.

constants of ternary complexes with citrate, (*R*)-malate, and (*R,S*)-tartrate under the conditions employed than was the proton relaxation rate method.

Studies with G·CoA. Paramagnetic resonance studies indicated that G·CoA binds to citrate synthase and that the bound G·CoA was "partially immobilized" with a dissociation constant approximately the same as that of the R·CoA–enzyme complex. The bound G·CoA was not as immobilized as was R·CoA in the R·CoA–enzyme system. The addition of oxaloacetate or (*R*)-malate to the G·CoA–enzyme system had a similar effect to the corresponding additions to the R·CoA–enzyme system, in that they both changed the relative amplitudes of the three lines in the low-gain paramagnetic resonance spectra indicating that the rotational motion of bound G·CoA was slowed. However, no peaks or shoulders could be resolved in low- and high-field regions of the paramagnetic resonance spectra, comparable to those seen in the R·CoA system. Thus, the rotational freedom of G·CoA is greater than that of R·CoA in the ternary radical–enzyme–ligand complexes.

Discussion

Binding of R·CoA to citrate synthase appears to be specific, since there is only one class of binding sites and the radical is displaced by acetyl-CoA and CoASH. Furthermore, the substrates oxaloacetate and citrate form ternary complexes with R·CoA and the enzyme. The formation of ternary R·CoA–enzyme–ligand complexes is quite stereospecific with respect to the ligand, since only oxaloacetate, citrate, (*R*)-malate, and (*R,S*)-tartrate formed such complexes out of many substances examined. On the other hand, the formation of binary or ternary complexes is not apparently stereospecific with respect to R·CoA. The pyrrolidine carbon to which the carboxyl is attached is a chiral center, so that the analog used is racemic at this position. There was no experimental evidence, however, that one diastereomer of R·CoA bound better or differently than the other. Apparently, the position taken by the pyrrolidine system in binding is not sensitive to chirality at the carboxyl-substituted position. Although it appears rather surprising that the two diastereomers bind with equal facility, several reasonable explanations can be offered. In the first place, examination of space filling models indicates that if the two enantiomeric acids (R·COOH) are oriented in

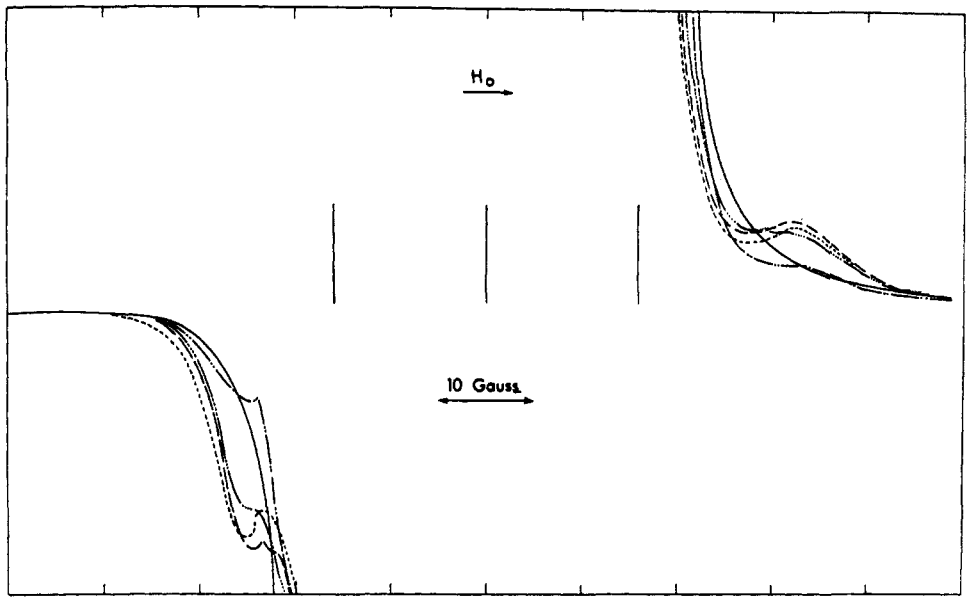


FIGURE 6: Low- and high-field portions of the paramagnetic resonance spectra of bound R·CoA in ternary R·CoA–enzyme–ligand complexes involving oxaloacetate (· · · · —), citrate (– –), (*R*)-malate (- - - -), and (*R*,*S*)-tartrate (· · —). Also shown is the R·CoA spectrum in the binary R·CoA–enzyme system (——). The instrumental gain and modulation amplitudes were the same for all spectra. All spectra were retraced by hand after alignment using the lower gain–lower modulation portion of the spectrum (line positions indicated by vertical lines in center portion of figure). The enzyme and total R·CoA concentrations were the same for all spectra. The ligand and bound R·CoA concentrations were as follows: [oxaloacetate], 1 mM; $[R \cdot CoA]_b$, 0.13 mM; [citrate], 5 mM; $[R \cdot CoA]_b$, 0.14 mM; [(*R*)-malate], 10 mM; $[R \cdot CoA]_b$, 0.15 mM; [(*R*,*S*)-tartrate], 8 mM; $[R \cdot CoA]_b$, 0.08 mM. The bound R·CoA concentration in the binary system was 0.13 mM.

space so that the configurations are equivalent at the chiral centers, the positions taken by the pyrrolidine rings are similar. The only difference between the enantiomers oriented in this fashion is that the ring atoms are displaced out of coincidence by one ring atom. Alternatively, and more probably, this lack of stereospecificity may indicate that the pyrrolidine system is not completely accommodated in the active site region in these complexes. If this is indeed so, the usefulness of the probe is not negated, since it is still a sensitive reporter of structure changes in the system.

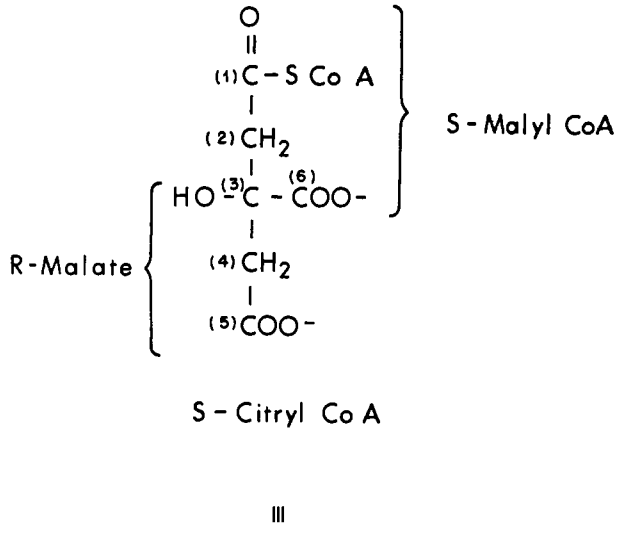
The behavior of the spin-labeled acetyl-CoA analogs allows some deductions to be made about their mode of binding to the protein. First, the analogs clearly bind at a portion of the CoA ester binding site since they bind at one site per monomer and are displaced by CoASH and acetyl-CoA. Since the bound analogs are "partially immobilized" in the enzyme–analog complexes, this must mean that the CoA portion of the analog makes contacts with the surface of the enzyme which partially restrict the rotational freedom available to the nitroxide through the pantetheine moiety. It seems unlikely that the binding of the acyl portion of the radicals would be important in the interaction with the protein because of the limited immobilization seen. This suggests that the other CoA esters also form binary complexes in which the acyl moiety is not directly involved since the binary dissociation constants for R·CoA, G·CoA, acetyl-CoA, and CoASH are all about the same.

Upon formation of ternary R·CoA–enzyme–ligand complexes, the motion of the nitroxide is strongly restricted (Figures 4 and 6). This further restriction of motion is probably caused by steric interactions near the acyl end caused either by a conformational change in the protein or by direct steric interactions with the second ligand. Qualitatively, the properties of the G·CoA–enzyme–ligand complexes are comparable to the corresponding R·CoA complexes, with the exception that there is more freedom of bound nitroxide motion in all of the G·CoA complexes. The less immobilized nature of bound G·CoA is consistent with the additional degrees of rotational freedom present in this molecule provided by the three additional methylenes and the additional ester groups. The observation that the motion of bound G·CoA is slowed upon formation of ternary G·CoA–enzyme–ligand complexes is interpreted to be a result of added hindrance of motion in the acyl portion of this acyl-CoA derivative. This is the region of the molecule which is analogous to the acetyl group of acetyl-CoA.

Since the formation of ternary complexes involving R·CoA is stereospecific with respect to the second ligand, it is useful to briefly review the stereochemistry of the condensation of oxaloacetate with acetyl-CoA as catalyzed by citrate synthase. (1) The acetyl group of acetyl-CoA approaches the *si* face of the C-2 carbonyl carbon of oxaloacetate (Hanson and Rose, 1963; Martius and Schorre, 1950a–c). (2) It has been proposed that the initial product of the condensation of acetyl-CoA with oxaloacetate is enzyme-bound (*S*)-citryl-CoA, since Eggerer and Remberger (1963) found that citryl-CoA is a substrate for the enzyme. (3) The reaction proceeds with inversion of configuration at the methyl group of acetyl-CoA (Klinman and Rose, 1971; Eggerer *et al.*, 1970; Rétey *et al.*, 1970). (4) The product of the reaction, citrate, has (*pro-R*)- and (*pro-S*)-carboxymethyl ends with the *pro-R* group originating from oxaloacetate and the *pro-S* group from acetyl-CoA. (5) The monofluoro derivatives of either oxaloacetate or acetyl-CoA are both substrates of the enzyme with the erythro diastereomer of monofluorocitrate being the product in either case (Fanshier *et al.*, 1962, 1964). However, the isomer which inhibits citrate metabolism arises from monofluoroacetyl-CoA and has the configuration 1*R*,2*R* (1-fluoro-2-hydroxy-1,2,3-propanetricarboxylic acid) (Carrell *et al.*, 1970). The other enantiomer (1*S*,2*S*) arises from monofluorooxaloacetate.

A partial insight into the observed stereoselectivity of ternary R·CoA–enzyme–ligand complex formation can be obtained by using (*S*)-citryl-CoA as a model for a real intermediate or transition state in the normal catalytic reaction. In the discussion which follows the changes in configuration about the carbonyl carbon atom of oxaloacetate during the reaction are examined in order to provide a stereochemical basis for our observations regarding the observed ligand specificity of ternary ligand–enzyme–R·CoA complex formation. These arguments do not require that (*S*)-citryl-CoA itself is a real or transition state intermediate during the reaction.

The carbon skeleton of (*S*)-citryl-CoA is numbered as shown in structure III. The binding sites for the presumptive



III

intermediate, (*S*)-citryl-CoA, on the enzyme may be considered to consist of two partially overlapping subsites: (a) an (*R*)-malate subsite which recognizes and interacts with the portion of (*S*)-citryl-CoA derived from oxaloacetate (carbon atoms 3, 4, 5, and 6); (b) an (*S*)-malyl-CoA subsite which recognizes and interacts with the half-acetyl–half-oxaloacetate portion of (*S*)-citryl-CoA (carbon atoms 1, 2, 3, and 6).

The configurational relationships involved are illustrated with Newman projections shown in Figure 7. Because the analogs and substrates we have investigated are all flexible molecules, no conformational analysis is possible. To minimize unwarranted conformational inferences, we have used Newman projections. Figure 7 shows two Newman projections of (*S*)-citryl-CoA. The projection in the top half of Figure 7 looks down the C-3–C-4 bond axis toward the end of the molecule contributed by oxaloacetate. The top half of Figure 7, also shows similar projections of (*R*)- and (*S*)-malates, respectively, which look down the C-2–C-3 axes of these compounds. The (*R*)-malate projection and *not* the (*S*)-malate projection is superimposable with the projection of (*S*)-citryl-CoA shown in Figure 7. In other words, (*R*)-malate looks like carbons 3, 4, 5 and 6 of (*S*)-citryl-CoA to the enzyme.

The bottom half of Figure 7 shows a projection of (*S*)-citryl-CoA looking along the C-3–C-2 bond axis from the end of the molecule contributed by oxaloacetate toward the end contributed by acetyl-CoA. Also shown in the bottom half of Figure 7 are projections of (*S*)- and (*R*)-malyl-CoA, viewed along the C-2–C-3 bond axes of these molecules. It is clear that the projection of (*S*)-malyl-CoA and *not* that of (*R*)-malyl-

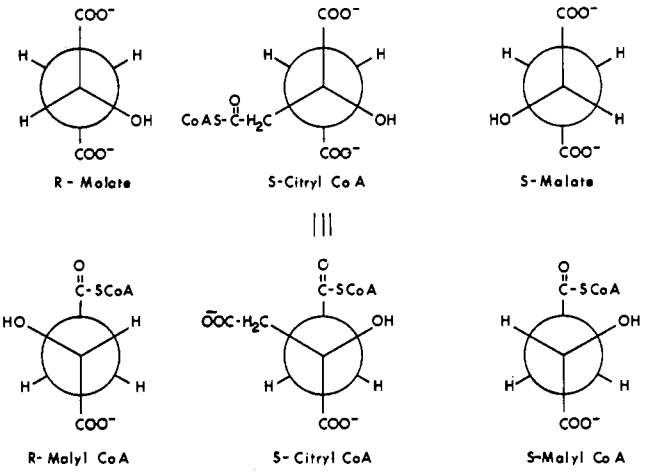


FIGURE 7: Newman projections of (*R*)- and (*S*)-malate, (*R*)- and (*S*)-malyl-CoA, and (*S*)-citryl-CoA.

CoA is superimposable with the projection of (*S*)-citryl-CoA shown in the bottom half of Figure 7. Therefore, (*S*)-malyl-CoA would look like carbons 1, 2, 3, and 6 of (*S*)-citryl-CoA. This is the end which would be formed one-half from carbons 1 and 2 of oxaloacetate and one-half from acetyl-CoA. This argument is compatible with the experimental observation that only the *S* enantiomer of malyl-CoA is hydrolyzed by citrate synthase, presumably at the (*S*)-malyl-CoA subsite of the enzyme (Eggerer *et al.*, 1964).

The above arguments provide rationale for the stereospecificity of ligand binding in the ternary complex. The (*R*,*S*)-tartrate–enzyme–R·CoA ternary complex can be explained on this same basis with an –OH substituted for a hydrogen on C-4 of (*S*)-citryl-CoA. (*R*,*S*)-Tartrate might be expected to have binding characteristics similar to both (*R*)- and (*S*)-malate, depending on the mode of binding. Experimentally, (*R*,*S*)-tartrate was observed to cause both a restriction of motion of R·CoA (like (*R*)-malate) and to be competitive with R·CoA binding (like (*S*)-malate). Neither (*R*,*R*)- nor (*S*,*S*)-tartrates were found to have any effect on the rotational motion of bound R·CoA, and increasing concentrations of either displaced a progressively larger concentration of bound R·CoA. Thus, both (*R*,*R*)- and (*S*,*S*)-tartrates behaved similarly to (*S*)-malate.

The effect of the tartrates on the R·CoA–enzyme system correlates with the stereospecificity shown by the enzyme in monofluorocitrate synthesis. As mentioned earlier, the product of the enzyme-catalyzed condensation of (*R*,*S*)-monofluorooxaloacetate with acetyl-CoA is 1(*S*)-fluoro-2(*S*)hydroxypropanetricarboxylic acid. Figure 8 shows Newman projections of (*S*,*S*)- and (*R*,*R*)-monofluorocitryl-CoA's along with the three tartrates. In the top half of the figure, it can be seen that the configurations are equivalent at both chiral centers of (*R*,*S*)-tartrate and (*S*,*S*)-monofluorocitryl-CoA. Thus, it is apparent that the three-dimensional structure of enzyme at the active site will allow interaction with reactant or product analogs carrying a bulky group in the configuration shown in the projections of (*R*,*S*)-tartrate and the monofluorocitryl-CoA's, but *not* if the configuration is as illustrated in the projection of (*R*,*R*)- and (*S*,*S*)-tartrate.

The observation of a citrate–enzyme–R·CoA ternary complex is also consistent with the stereochemical model, (*S*)-citryl-CoA, with citrate bound to the enzyme by its (*R*)-malate-like end. That this may be so is indicated by the sim-

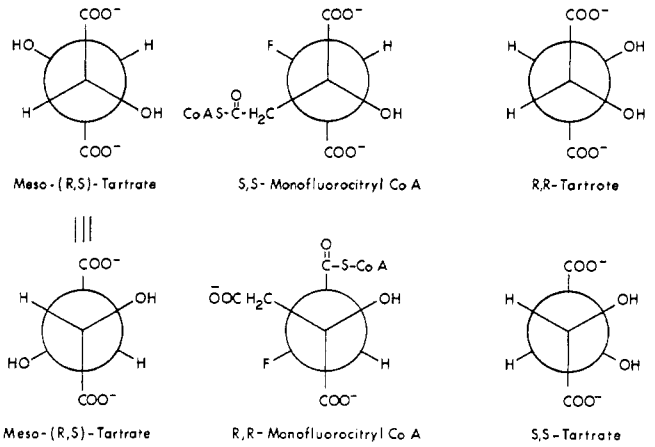


FIGURE 8: Newman projections of (*R*,*R*)- (*R*,*S*)- and (*S*,*S*)-tartrate and (*R*,*R*)- and (*S*,*S*)-monofluorocitryl-CoA.

ilarity of dissociation constants of citrate and (*R*)-malate from their respective ternary ligand–enzyme–R·CoA complexes (Table III).

The above arguments have provided a qualitative correlation for ligand specificity in terms of a single kind of ligand–enzyme–R·CoA complex with product-like stereochemistry of the small ligand.

The reaction must involve a series of at least three complexes along the reaction path, each with a unique active site conformation: (1) oxaloacetate–enzyme–acetyl-CoA; (2) (*S*)-citryl-CoA–enzyme (or its equivalent); and (3) citrate–enzyme–CoASH. In addition, several abortive complexes are possible.

One could naively propose that oxaloacetate–enzyme–R·CoA with its relatively mobile radical (compared to the other observed ternary complexes, Figure 6) is an analog of (1) and that citrate–enzyme–R·CoA is an analog of (3). Although we feel that the observed ternary ligand–enzyme–R·CoA complexes may well be analogs of one or another of the ternary complexes along the catalytic path, the assignment of an observed complex as an analog of a particular intermediate is not presently possible for several reasons. The central portions of the electron paramagnetic resonance (epr) spectra are partially obscured by the spectrum of free R·CoA, so that we cannot be certain that the strongly immobilized spectrum represents the only species present in the ternary complexes. The observed difference in rotational mobility of bound R·CoA in the different ternary complexes (oxaloacetate *vs.* the others, Figure 6) could then result from the mixing of weakly and strongly immobilized signals. If the observed signals arise from a single species we still cannot distinguish whether differences in rotational mobility result from differing active site conformations or differing ligand–R·CoA interactions within the same active site conformation. Finally, if there were order of magnitude differences in dissociation constants for R·CoA and the ligand from these ternary complexes (Table III), one might have some basis for structurally distinguishing one complex from another and for assigning them as analogs of particular intermediates. However, with the exception of the dissociation constant of oxaloacetate from its ternary complex, the differences in dissociation constants for the various ternary complexes are relatively small. Experiments which we hope will shed light on this subject are in progress.

It was surprising that a ternary (*S*)-malate–enzyme–R·CoA complex was not observed in light of Eggerer's (1965) finding that it, and not (*R*)-malate, induces the exchange of the acyl methyl protons of acetyl-CoA with those of the medium in the presence of the enzyme. Using the above stereochemical considerations, it might have been expected that (*R*)-malate, and not (*S*)-malate, would have induced this exchange. However, Srere (1967) confirmed Eggerer's finding and we (Weidman, S. W., and Drysdale, G. R., unpublished observations) have also demonstrated exchanges under experimental conditions which are similar to those prevailing during our paramagnetic resonance measurements. However, the addition of (*S*)-malate to the enzyme–R·CoA system has no apparent effect on the rotational mobility of bound R·CoA. The only experimentally observed effect of (*S*)-malate is the progressive displacement of bound R·CoA with increasing addition of ligand with no evidence for any change in rotational mobility. Therefore, if an (*S*)-malate–enzyme–R·CoA complex is formed, either the mobility of the bound R·CoA is not changed detectably compared to that in the R·CoA–enzyme system or the competing displacement of bound R·CoA caused by (S)-malate binding at another site is masking the change in mobility. The site of (*S*)-malate binding which is competitive with R·CoA binding could conceivably be the region of the CoA binding site that binds the pyrophosphate moiety. Whatever the reason for the failure to detect a ternary (*S*)-malate–enzyme–R·CoA complex, it is apparent that (*S*)-malate behaves differently from oxaloacetate, citrate, (*R*)-malate, and (*R*,*S*)-tartrate when added to the enzyme–R·CoA system. We conclude that (*S*)-malate forms a ternary complex with the enzyme and acetyl-CoA which does not correspond to any of the ones detected with R·CoA and small ligands.

In conclusion, these experiments using a spin-labeled analog of acetyl-CoA have resulted in the detection and determination of thermodynamic properties of paramagnetic binary and ternary complexes involving the probe and of diamagnetic binary complexes involving the CoA substrates with citrate synthase. They have also provided an insight into the stereochemical constraints of the catalytic reaction. In future publications we plan to report results of studies which employ competition with R·CoA to detect other diamagnetic binary and ternary complexes and, in addition, studies on the interaction of a spin-labeled analog of oxaloacetate with citrate synthase (Weidman and Drysdale, 1972). We expect that these studies will provide more information about the catalytic mechanism.

The paramagnetic analogs used in these studies on citrate synthase should prove useful for the study of certain other enzymes which bind acyl-CoA esters. In preliminary studies, R·CoA has been found to interact with ATP citrate lyase (Weidman, S. N., Srere, P., Cottam, G. L., Das, N., and Drysdale, G. R., unpublished observation) and with carnitine acetyltransferase (Weidman, S. W., and Drysdale, G. R., unpublished observation) but not with acyl-CoA dehydrogenase (Drysdale, G. R., and Weidman, S. W., unpublished observations) or pyruvate carboxylase (Mildvan, A. S., Scrutton, M. C., and Weidman, S. W., unpublished observations).

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References

Carrell, H. L., Glusker, J. P., Villafranca, J. J., Mildvan, A. S., Dummel, R. J., and Kun, E. (1970), *Science 170*, 1412.

Deranleau, D. A. (1969), *J. Amer. Chem. Soc. 91*, 4044, 4050.

Eggerer, H. (1965), *Biochem. Z. 343*, 111.

Eggerer, H., Buckel, W., Lenz, H., Wunderwald, P., Gottschalk, G., Cornforth, J. W., Donninger, C., Mallaby, R., and Redmond, J. W. (1970), *Nature (London) 226*, 517.

Eggerer, H., and Remberger, U. (1963), *Biochem. Z. 337*, 202.

Eggerer, H., Remberger, U., and Grünwälder, C. (1964), *Biochem. Z. 339*, 436.

Ellman, G. (1959), *Arch. Biochem. Biophys. 82*, 70.

Fanshier, D. W., Gottwald, L. K., and Kun, E. (1962), *J. Biol. Chem. 237*, 3588.

Fanshier, D. W., Gottwald, L. K., and Kun, E. (1964), *J. Biol. Chem. 239*, 425.

Hamilton, C. L., and McConnell, H. M. (1968), *in* Structural Chemistry and Molecular Biology, Rich, A., and Davidson, N., Ed., San Francisco, Calif., W. H. Freeman, p 115.

Hanson, K. R., and Rose, I. A. (1963), *Proc. Nat Acad. Sci. U. S. 50*, 981.

Hoff, A. J., Oosterbaan, R. A., and Deen, R. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 14*, 17.

Hsia, J. C., and Piette, L. H. (1969), *Arch. Biochem. Biophys. 129*, 296.

Hummel, J. P., and Dreyer, W. J. (1962), *Biochim. Biophys. Acta 63*, 530.

Klinman, J. P., and Rose, I. A. (1971), *Biochemistry 10*, 2267.

Kosicki, G. W., and Lee, L. P. K. (1966), *J. Biol. Chem. 241*, 3571.

Kosicki, G. W., and Srere, P. A. (1961), *J. Biol. Chem. 236*, 2560.

Martius, C., and Schorre, G. (1950a), *Justus Liebigs Ann. Chem. 570*, 140.

Martius, C., and Schorre, G. (1950b), *Justus Liebigs Ann. Chem. 570*, 143.

Martius, C., and Schorre, G. (1950c), *Z. Naturforsch. B 5*, 170.

McConnell, H. M. (1967), *in* Magnetic Resonance in Biological Systems, Ehrenberg, A., Malmström, B. G., and Vanngard, J., Ed., New York, N. Y., Pergamon, p 313.

McConnell, H. M., and McFarland, B. G. (1970), *Quart. Rev. Biophys. 3*, 91.

Mildvan, A. S., and Cohn, M. (1963), *Biochemistry 2*, 910.

Mildvan, A. S., and Cohn, M. (1970), *Advan. Enzymol. 33*, 1.

Mildvan, A. S., and Weiner, H. (1969a), *Biochemistry 8*, 552.

Mildvan, A. S., and Weiner, H. (1969b), *J. Biol. Chem. 9*, 2465.

Moffatt, J. G., and Khorana, H. G. (1961), *J, Amer. Chem. Soc. 83*, 663.

Poulsen, L. L., and Sarkissian, I. V. (1971), *Life Sci. 10*, 91.

Rétey, J., Lüthy, J., and Arigoni, D. (1970), *Nature (London) 226*, 519.

Rozantsev, E. G. (1970), Free Nitroxyl Radicals, New York, N. Y., Plenum Press.

Rozantsev, E. G., and Krinitskaya, L. A. (1965), *Tetrahedron 21*, 491.

Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51*, 660.

Simon, E. J., and Shemin, D. (1953), *J. Amer. Chem. Soc. 75*, 2520.

Singh, M., Brooks, G. C., and Srere, P. A. (1970), *J. Biol. Chem. 245*, 4636.

Srere, P. A. (1966). *J. Biol. Chem. 241*, 2157.

Srere, P. A. (1967), *Biochem. Biophys. Res. Commun. 26*, 609.

Srere, P. A., Brazil, H., and Gonen, L. (1963), *Acta Chem. Scand. 17*, S 129.

Stadtman, E. R. (1957), *Methods Enzymol. 3*, 931.

Taylor, J. S., Leigh, J. S., and Cohn, M. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 219.

Weidman, S. W., and Drysdale, G. R. (1972), 164th National Meeting of the American Chemical Society, New York, N. Y., Sept, Abstracts.

Weiner, H. (1969), *Biochemistry 8*, 526.

Wu, Jang-Yen, and Yang, J. T. (1970a), *J. Biol. Chem. 245*, 212.

Wu, Jang-Yen, and Yang, J. T. (1970b), *J. Biol. Chem. 245*, 3561.