Magnetic Resonance Studies of the Proximity and Spatial Arrangement of Propionyl Coenzyme A and Pyruvate on a Biotin–Metalloenzyme, Transcarboxylase†

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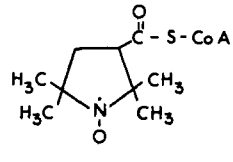
ABSTRACT: A spin-labeled ester of CoA, R·CoA (3-carboxy-2,2,5,5-tetramethyl-1-pyrolidinyl-1-oxy CoA thioester), has been shown by competition studies using electron paramagnetic resonance (EPR) and nuclear magnetic resonance (NMR) to bind specifically to the propionyl-CoA binding sites of transcarboxylase. Titrations indicate 0.7 ± 0.2 binding site for R·CoA per enzyme-bound biotin with a dissociation constant of 0.33 ± 0.12 m*M*. Propionyl-CoA binds to this site with a 1.3-fold lower dissociation constant as detected by competition. These dissociation constants are in reasonable agreement with kinetically determined inhibitor constants of CoA and propionyl-CoA (D. B. Northrop (1969), *J. Biol. Chem. 244,* 5808). The binding of R·CoA to these sites causes a sixfold enhancement of the paramagnetic effect of this spin-label on $1/T_1$ of water protons. The formation of a ternary transcarboxylase–R·CoA–pyruvate complex is suggested by the failure of pyruvate to displace R·CoA from the tight site and is established by the paramagnetic effects of enzyme-bound R·CoA on the relaxation rates of the protons and ^{13}C atoms of enzyme-bound pyruvate. From the paramagnetic effects of R·CoA on the relaxation rates of the methyl protons of pyruvate at 40.5 and 100 MHz, and on the ^{13}C-enriched carbonyl and carboxyl carbon atoms of pyruvate at 25.1 MHz, a correlation time of 7 nsec and distances from the bound nitroxide radical to the methyl protons, the carbonyl, and carboxyl carbon atoms of bound pyruvate of 7.9 ± 0.7, 10.3 ± 0.8, and 12.1 ± 0.9 Å, respectively, are calculated. These distances establish the close proximity of the CoA ester and keto acid sites on transcarboxylase. Together with the previously determined distances from the enzyme-bound Co(II) to the methyl protons and 2 carbon atoms of bound pyruvate and to 12 protons and 3 phosphorus atoms of bound propionyl-CoA, the present distances are used to derive a composite model of the bound substrates in the overall transcarboxylation reaction. In this model the distance from the methyl carbon of pyruvate and the methylene carbon of propionyl-CoA, between which the carboxyl transfer takes place is only ~7 Å. Depending on the detailed mechanism of the carboxyl transfer, the distance through which the carboxybiotin must migrate is therefore between 0 and 7 Å. Hence the major role of the 14-Å arm of carboxybiotin is not to permit a large carboxyl migration but, rather to permit carboxybiotin to traverse the gap which occurs at the interface of three subunits and to insinuate itself between the CoA and keto acid sites.

Magnetic resonance studies have shown that two substrates of transcarboxylase, propionyl-CoA (Fung et al., 1976) and pyruvate (Fung et al., 1974; Northrop and Wood, 1969), bind near the enzyme-bound paramagnetic ions, Co(II) and Cu(II). Because transcarboxylase contains ~12 metal ions per mole (Fung et al., 1974) and the stoichiometry of substrate binding is unknown, it is not clear whether both substrates bind near the same or different metal ions. The binding of both substrates near the same metal ion would indicate proximity of the active sites for carboxylation of both propionyl-CoA and pyruvate by the carboxybiotinyl subunit in the intact enzyme, despite the observation that each of these half-reactions takes place on different isolated subunits (Chuang et al., 1975).

Clarification of this point requires a determination of the distance between two substrates bound to an enzyme. Such distance measurements can be made by magnetic resonance methods when a paramagnetic analogue of one of the two substrates is available (Mildvan and Weiner 1969a; Mildvan et al., 1972; Gupta et al., 1976). A paramagnetic ester of CoA has previously been prepared and characterized, namely 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyl-1-oxy CoA thioester, symbolized R·CoA (Weidman et al., 1973):



R · CoA

The present paper shows that R·CoA is an analogue of propionyl-CoA on transcarboxylase, and examines the stoichiometry and affinity of R·CoA binding as well as the paramagnetic effects of enzyme-bound R·CoA on the relaxation rates of the protons and carbon atoms of pyruvate. The results establish the proximity of the two substrate sites on the intact enzyme and indicate that they bind near the same metal ion. From a composite model of bound propionyl-CoA, pyruvate, and carboxybiotin on transcarboxylase, the mechanism of the carboxyl transfer reaction and the role of the 14-Å long arm of carboxybiotin are discussed. A preliminary report of this work has been published (Fung et al., 1975).

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Experimental Section

Materials

R·CoA, prepared and purified as previously described (Weidman et al., 1973), was generously provided by Dr. S. W. Weidman. [1-^{13}C]- and [2-^{13}C]pyruvic acids (70 atom %) were synthesized separately from Na^{13}CN and acetic acid and from [1-^{13}C]acetic acid and NaCN, respectively, by the method of Calvin et al. (1949) and were purified as described by Fung et al. (1973). Unlabeled pyruvic acid, purchased from Eastman, was purified by vacuum distillation as described previously (Mildvan and Scrutton, 1967). Transcarboxylases from *Propionibacterium shermanii* 52W grown on a medium enriched with either $Co(NO_3)_2$ (200 μM) or $ZnCl_2$ (50 μM) were purified to homogeneity as described by Wood et al. (1969). Propionyl-CoA was purchased from PL Biochemicals. Ultra pure Tris base was obtained from Mann Research Laboratory. L-Lactate dehydrogenase and L-malate dehydrogenase used for assaying pyruvate and transcarboxylase activity, respectively, were purchased from Boehringer und Soehne. All other chemicals were reagent grade or of the highest purity commercially available.

Methods

The concentration of R·CoA was determined using the extinction coefficient 16000 M^{-1} cm^{-1} at 260 nm (Weidman et al., 1973). Metal and biotin analyses, protein determination, preparation of transcarboxylase for nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) measurements, and measurements of proton relaxation rates of water at 24.3 MHz were carried out as described previously (Fung et al., 1974). The T_1 values of the protons of pyruvate at 40.5 and 100 MHz, and of the enriched 1-^{13}C and 2-^{13}C atoms of pyruvate at 25.1 MHz were determined by the rate of signal reappearance after demagnetization of the sample as described by McDonald and Leigh (1973). Transverse relaxation rates of the protons of pyruvate at 100 MHz were determined by pulsed methods as previously described (Fung et al., 1974, 1976). The experimental data (usually 10–20 data points) collected from the nuclear relaxation measurements were analyzed by a computer least-squares fit to an exponential function to yield $1/T_1$ or $1/T_2$ with errors of 5 and 10%, respectively. Relaxation studies of the pyruvate methyl protons at 40.5 MHz were carried out on a Varian XL-100-15-FT spectrometer by lowering the field to 9.51 kG. The 40.5-MHz channel is normally used for ^{31}P NMR studies at a magnetic field of 23.5 kG together with a heteronuclear field/frequency lock on deuterium at a fixed frequency of 15.4 MHz. Lithium as a saturated solution of LiCl in D_2O, which resonates at 15.7 MHz at the lower magnetic field, was used for field frequency locking (Gupta, 1975). The choice of lithium as a lock nucleus was based on sensitivity and solubility. The sample tube assembly consisted of two parts, a 12-mm o.d. outer tube (Wilmad Glass Co., Buena, N.J., Catalog No. 516-0) which contained the LiCl solution and a 5-mm o.d. inner tube (Wilmad Glass Co., Catalog No. 520) which contained the sample. Experiments could also be done using a 10-mm o.d. inner sample tube inserted in a 12-mm outer lock tube with the Varian pulsed lock system. However, the sensitivity of the 5 mm o.d. inner tube was sufficient for our purposes. The LiCl solution was lyophilized three times to remove traces of H_2O and the lithium resonance was broadened with 20 mg/ml of $CuCl_2$ for improving the steady state lock signal. Sample volumes were normally 0.4 ml and a Teflon plug was used as a vortex suppressor. The lock resonance frequency was provided by the Gyrocode decoupler, which was used as transmitter (Ellis et al., 1973). The appropriate Gyrocode matrix was A3, A4, A5; B1, B3, B5; D2. The deuterium lock preamplifier was retuned to 15.7 MHz. The signal-to-noise ratio obtained for proton NMR at 40.5 MHz was approximately half of that at 100 MHz.

EPR Measurements. The EPR spectra of 30–40-μl samples of solutions containing R·CoA were obtained in quartz capillaries using a Varian E-4 EPR spectrometer. The temperature was controlled at 22 ± 1° by equilibration with gaseous nitrogen. The binding of R·CoA to transcarboxylase was studied, as previously described (Weidman et al., 1973), by monitoring the peak to peak amplitude of the high-field resonance which measures the concentration of free R·CoA. Solutions of equal concentrations of R·CoA in the presence of buffer alone were used as standards. The EPR spectrum of enzyme-bound R·CoA was observed with the use of a tenfold higher gain and a tenfold higher modulation amplitude of 6.3 G.

The enzyme–R·CoA binding data from the EPR studies were analyzed by a Scatchard plot as previously described (Villafranca and Mildvan, 1971; Miziorko and Mildvan, 1974) to yield the number of R·CoA binding sites (n) and the dissociation constant, K_D, of the binary enzyme–R·CoA complex.

Analysis of the Nuclear Relaxation Data. The binding of R·CoA to transcarboxylase causes an enhancement of the effect of the nitroxide radical on the proton relaxation rate of water. The observed enhancement (ϵ^*) of the proton relaxation rate in a solution of transcarboxylase and R·CoA is the weighted average of the enhancements of free R·CoA (ϵ_f) and of bound R·CoA (ϵ_b) on transcarboxylase (Mildvan and Weiner, 1969b; Weidman et al., 1973):

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

The subscripts f, b, and t in eq 1 refer to the concentrations of free, bound, and total R·CoA, respectively. ϵ_f is equal to 1 by definition. The values for the fractions of the bound and free R·CoA were obtained from EPR measurements permitting the calculation of ϵ_b using eq 1.

The paramagnetic contributions to the longitudinal $1/T_{1p}$ and transverse $1/T_{2p}$ relaxation rates of the protons and carbon atoms of pyruvate were normalized by the factor f, the concentration ratio of paramagnetic species to ligand. As pointed out elsewhere (Mildvan and Cohn, 1970; Mildvan and Engle, 1972), when not limited by chemical exchange, $1/fT_{1p}$ is equal to $1/T_{1M}$, the relaxation rate of the bound ligand, and may be used to calculate the distance (r) between a paramagnetic center and the ligand nuclei from the dipolar term of the Solomon–Bloembergen equation (eq 2):

$$r(\text{Å}) = C[T_{1M}f(\tau_c)]^{1/6} \qquad (2)$$

In eq 2, C is a constant which depends on the nature of the paramagnetic center and the magnetic nucleus undergoing relaxation. For nitroxide radicals interacting with protons and ^{13}C atoms the C values are 539 and 340 Å (sec)$^{-1/3}$, respectively (Mildvan and Engle, 1972). In eq 2 $f(\tau_c)$ is the correlation function which is given by:

$$f(\tau_c) = 3\tau_c/(1 + \omega_I{}^2\tau_c{}^2) + 7\tau_c/(1 + \Omega_S{}^2\tau_c{}^2) \qquad (2A)$$

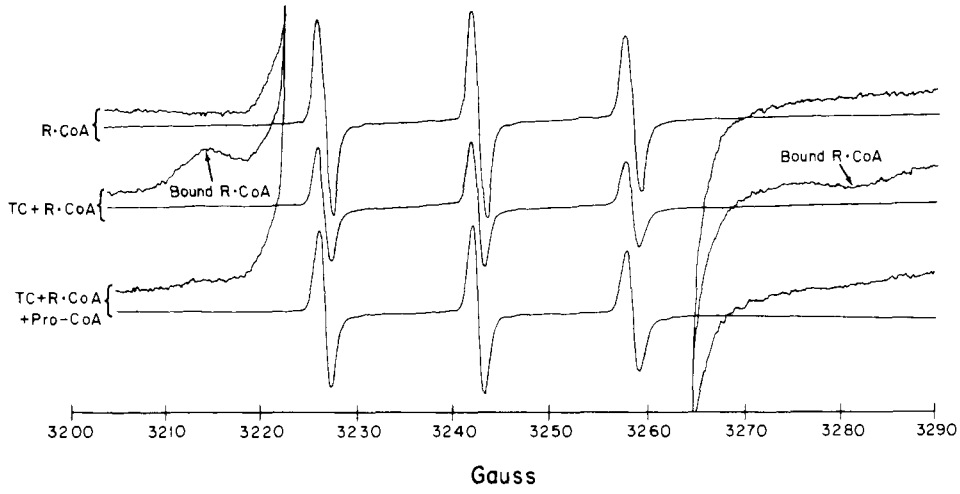

FIGURE 1: Paramagnetic resonance spectra of free R·CoA and of the binary transcarboxylase–R·CoA complex. The upper curves, labeled R·CoA, were run on a solution containing 0.19 m*M* R·CoA, 0.15 *M* Tris-Cl buffer (pH 6.8), and 0.22 *M* KCl, $T = 22°$, at low gain (150) and modulation amplitude (0.63 G). The outer regions of the spectrum from 3205 to 3290 G were also run at tenfold higher gain (1500) and modulation amplitude (6.3 G). The middle curves, labeled TC + R·CoA, contained in addition to the above components, 73.6 mg/ml of transcarboxylase (TC) containing 0.45 m*M* biotin, 0.62 m*M* Zn(II), 0.03 m*M* Co(II), and 0.07 m*M* Cu(II). The lower curves, labeled TC + R·CoA + Pro-CoA, contained, in addition to the above components, 1.9 m*M* propionyl-CoA. Other EPR settings and conditions were as follows: frequency = 9.145 GHz; microwave power = 10 mW; time constant = 0.3 sec; scan rate = 25 G/min; $T = 22°$.

In eq 2A, ω_I and ω_S are the Larmor angular precession frequencies for the nuclear and electron spins, respectively, and τ_c is the correlation time for the dipolar interaction. The correlation time was determined by three independent methods all of which agreed within experimental error. First, the frequency dependence of the effect of enzyme-bound R·CoA on $1/T_{1p}$ of the methyl protons of pyruvate at 40.5 and 100 MHz was used in equations 2 and 2A to calculate τ_c. Second, making the reasonable assumption that $1/T_{2p}$ of the methyl protons of pyruvate was not exchange limited and contained no hyperfine contribution, the T_{1p}/T_{2p} ratio of these protons was used in eq 3 to calculate τ_c (Mildvan and Engle, 1972):

$$\tau_c = \frac{1}{\omega_I}\left[\tfrac{3}{2}\left(\frac{T_{1M}}{T_{2M}} - \frac{7}{6}\right)\right]^{1/2} \qquad (3)$$

A third estimate of τ_c was made from the widths of the broadened resonances in the EPR spectrum due to bound R·CoA.

Results

Binding of R·CoA to the Zn-Enriched Transcarboxylase as Studied by EPR Spectroscopy. The EPR spectrum of R·CoA (Figure 1) like those of other nitroxide spin-labels (Hamilton and McConnell, 1968) consists of three resonance lines due to splitting by the nitrogen nucleus. The properties of R·CoA and its binding to pig heart citrate synthase have been well characterized by Weidman et al. (1973). The presence of the Zn-enriched transcarboxylase produced a marked decrease in the amplitudes of the three resonance lines of free R·CoA (Figure 1), indicating the binding of R·CoA to transcarboxylase to form a binary complex. Increasing both the receiver gain and the modulation amplitude of the EPR spectrometer by factors of 10 reveals the broad EPR signals of transcarboxylase-bound R·CoA at 3215 and 3282 G (Figure 1). The addition of a tenfold excess of propionyl-CoA (1.9 m*M*) to a solution of R·CoA (0.19 m*M*) and transcarboxylase (0.45 m*M* biotin sites) displaced 76 ± 4% of the bound R·CoA as estimated by the decreases in the amplitudes of the resonances of enzyme-bound R·CoA and the concomitant increases in the amplitudes of the resonances of free R·CoA (Figure 1). From these data it is calculated that propionyl-CoA binds to transcarboxylase with a dissociation constant 1.32-fold lower than that of R·CoA. In contrast, the presence of 5.5 m*M* pyruvate, which is seven times the K_M of pyruvate (Northrop, 1969), has no effect on the EPR spectrum of free or bound R·CoA. These results show that R·CoA binds specifically to the propionyl-CoA sites of transcarboxylase. When transcarboxylase containing 1.25 m*M* Zn(II), 53 μM Co(II), and 123 μM Cu(II) is replaced by an equal concentration of transcarboxylase containing 705 μM Zn(II), 766 μM Co(II), and 138 μM Cu(II), little or no difference in the amplitude or width of the EPR lines due to free R·CoA (≤7%) is detected (not shown), indicating essentially equal affinities of the two enzyme preparations for R·CoA. Moreover, no difference in the amplitude or width of the resonances due to bound R·CoA is detected (≤7%) (not shown) indicating no dipolar interaction between the bound Co(II) or Cu(II) and the bound R·CoA. From the theoretical equation for such interactions (Leigh, 1970) as applied to Co(II) nitroxide systems (Drott et al., 1974) it is estimated, from our error level, that the bound Co(II) is ≥9.0 Å from the nitroxide of bound R·CoA.

To measure the dissociation constant of the transcarboxylase–R·CoA complex, the enzyme was titrated with increasing concentrations of R·CoA, measuring the concentration of free R·CoA by EPR. Since in the presence of enzyme the largest change in resonance amplitude of free R·CoA relative to the standard was observed for the high-field line, the changes in the resonance height of the high-field line were assumed to indicate the concentration of free R·CoA (Weidman et al., 1973). The EPR data (Table I) when presented in the form of a Scatchard plot (Figure 2) yielded a biphasic curve indicating more than one class of binding sites for R·CoA on transcarboxylase. The data were fit to eq 4 for two thermodynamically noninteracting classes of R·CoA binding sites (Villafranca and Mildvan, 1971) by computer, as described by Miziorko and Mildvan (1974):

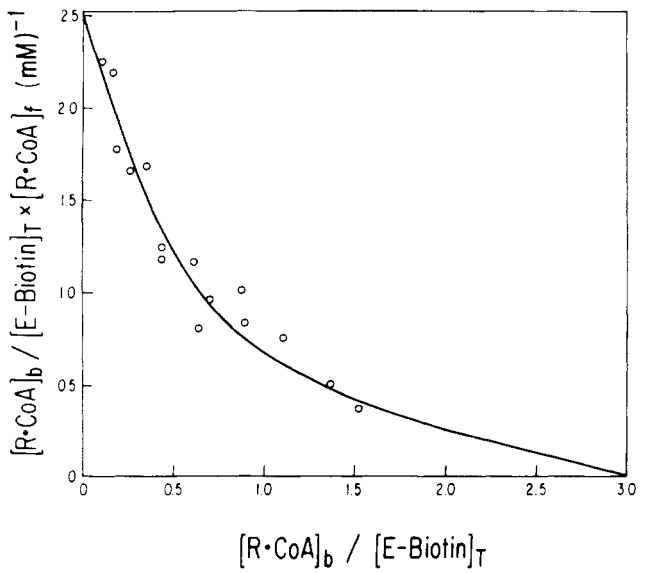

FIGURE 2: Scatchard plot of R·CoA binding to transcarboxylase. All data points are in Table I. Theoretical curve which is fit to the data with a mean deviation of ±10.4% is derived by assuming $n_1 = 0.7$, $K_1 = 0.33$ mM; $n_2 = 2.3$, $K_2 = 8.0$ mM.

$$\frac{[\text{R·CoA}]_{b1} + [\text{R·CoA}]_{b2}}{[\text{E-biotin}]_t[\text{R·CoA}]_f} = \frac{n_1}{K_1} + \frac{n_2}{K_2} + \frac{(n_1 + n_2)[\text{R·CoA}]_f}{K_1K_2} - \left[\frac{1}{K_1} + \frac{1}{K_2} + \frac{[\text{R·CoA}]_f}{K_1K_2}\right]\frac{[\text{R·CoA}]_{b1} + [\text{R·CoA}]_{b2}}{[\text{E-biotin}]_t} \quad (4)$$

In eq 4, n_1 is the number of R·CoA sites with a dissociation constant K_1, and n_2 is the number of sites with a dissociation constant K_2. The theoretical curve of Figure 2 which fits the experimental data points with a mean deviation of 10.4% was generated using the n and K values for R·CoA given in Table II which also gives the errors. The number of tight binding sites for R·CoA is indistinguishable from the number of bound biotin molecules.[1] Moreover, propionyl-CoA displaces R·CoA from this site (Figure 1). Hence the tight binding sites for R·CoA probably represent the active sites for carboxylation of propionyl-CoA. The dissociation constant of R·CoA from this class of sites measured directly (0.33 ± 0.12 mM) and that of propionyl-CoA (0.25 ± 0.10 mM) from the same class of sites determined by the displacement of R·CoA (Table II) are of the same order as the K_1 values of CoA (0.6 mM) and of propionyl-CoA (0.4 mM) as determined by steady-state kinetics (Northrop, 1969).

PRR Studies of Binding of R·CoA to Transcarboxylase. The longitudinal molar relaxivity of water protons due to the presence of R·CoA, obtained from 15 measurements at 24.3 MHz and 22°C was found to be 342 ± 17 M^{-1} sec^{-1} in 0.15 M of Tris-Cl buffer (pH 6.8) containing 0.22 M KCl (Table I). This value is higher than reported previously, 230 ± 24 M^{-1} sec^{-1} (Weidman et al., 1973), probably due to the greater purity of present samples of R·CoA and to the slightly lower temperature of the present experiments. The binding of R·CoA to transcarboxylase causes an enhancement of the effect of the nitroxide radical on the longitudinal relaxation rate of water protons (Table I). The observed enhancement factor ϵ^* decreases with increasing concentration of R·CoA due to the greater fraction of free R·CoA. When corrected for the effect of free R·CoA, using EPR data and eq 1, the enhancement parameter due to bound R·CoA (ϵ_b) is calculated. The value of ϵ_b shows a small decrease with increasing occupancy of the R·CoA binding sites between 0.07 and 1.52 bound R·CoA molecules per biotin (Table I). When ϵ_b was analyzed as a function of the distribution of bound R·CoA between tight and weak binding sites (Table I), a significant difference in ϵ_b for the two classes of sites ($p = 0.02$) was detected (Table II). Although ϵ_b for the weak binding sites is not accurately determined because of the low occupancy of these sites, its value is smaller than ϵ_b for the tight sites, indicating a different correlation time or a diminished access of solvent protons to R·CoA at the weak sites.

Table I: Binding to Transcarboxylase and Enhancement of the Paramagnetic Effects of R·CoA on the Proton Relaxation Rate of Water.[a]

$[\text{R·CoA}]_t$ (μM)	$[\text{E-Biotin}]_t$ (μM)	$[\text{R·CoA}]_b/[\text{E-Biotin}]_t$	$[\text{R·CoA}]_f/[\text{R·CoA}]_t$	$1/T_{1p}$ (sec^{-1})	ϵ^*	ϵ_b
67	450	0.067	0.556	0.100	3.4	6.3
91	436	0.103	0.504	0.150	4.1	7.2
143	411	0.183	0.474	0.175	3.7	6.7
188	450	0.187	0.556	0.190	3.0	5.4
273	436	0.365	0.419	0.290	2.8	5.3
560	447	0.465	0.628	0.515	2.7	5.6
563	450	0.433	0.654	0.500	2.5	5.4
818	434	0.677	0.641	0.640	2.4	4.9
1059	424	0.632	0.747	0.740	2.1	5.5
1059	421	0.793	0.685	0.730	2.1	4.5
1286	409	1.029	0.673	0.860	2.0	4.1
1500	398	1.075	0.715	0.950	2.1	4.9
1895	379	1.106	0.779	1.140	1.8	4.6
3130	313	1.371	0.863	1.555	1.5	4.7
4500	240	1.521	0.919	2.030	1.4	5.3

[a] Other components present were 0.15 M Tris-Cl buffer (pH 6.8) and 0.22 M KCl, $T = 22°$. $[\text{R·CoA}]_f/[\text{R·CoA}]_t$ was determined by EPR as described in the text. The paramagnetic contribution to the relaxation rate ($1/T_{1p}$) was calculated as $(1/T_1) - (1/T_1^0)$ where $(1/T_1)$ and $(1/T_1^0)$ are the relaxation rates of the same solution with and without R·CoA. The observed enhancement (ϵ^*) is calculated from the ratio $[(1/T_1)^* - (1/T_1^0)^*]/[(1/T_1) - (1/T_1^0)]$, where the starred terms indicate the presence of transcarboxylase. ϵ_b was calculated from ϵ^* and $[\text{R·CoA}]_f/[\text{R·CoA}]_t$ using eq 1.

Table II: Dissociation Constants and Enhancement Parameters of Transcarboxylase Complexes.[a]

Complex	n	K_D (mM)	ϵ_b
TC–R·CoA	0.7 ± 0.2	0.33 ± 0.12	6.4 ± 0.9
	2.3 ± 1.0	8.0 ± 3.0	2.5 ± 2.2
TC–propionyl-CoA	0.7 ± 0.2	0.25 ± 0.10	

[a] The ϵ_b values for the two classes of sites were determined by correcting the ϵ_b values of Table I for the distribution of bound R·CoA using the indicated n and K_D values. The ϵ_b values are given ±2 standard errors. The difference in ϵ_b values is statistically significant with a $p = 0.02$ (Draper and Smith, 1966).

[1] The deviation of the value of n_1 (0.7 ± 0.2) from unity may be related to the fact that the specific activity of the homogeneous Zn(II)-transcarboxylase preparation used in this titration (33 units/mg) was 87 ± 9% of that of three other preparations of transcarboxylase also purified to homogeneity in this laboratory at about the same time, and 73% of the highest specific activity we have observed (45 units/mg) (Fung et al., 1974).

Table III: Longitudinal and Transverse Relaxation Rates of the Methyl Protons of Pyruvate at 100 and 40.5 MHz in the Presence of Transcarboxylase and R·CoA.[a]

Expt	[E-Biotin] (m*M*)	[Pyruvate] (m*M*)	[R·CoA] (m*M*)	[ProCoA] (m*M*)	100 MHz				40.5 MHz	
					$1/T_1$ (sec^{-1})	$1/fT_{1p}$ (sec^{-1})	$1/T_2$ (sec^{-1})	$1/fT_{2p}$ (sec^{-1})	$1/T_1$ (sec^{-1})	$1/fT_{1p}$ (sec^{-1})
1	0.260	29.4			0.93 ± 0.03		7.7 ± 0.4		0.95 ± 0.03	
2	0.260	29.4	0.200[b]		1.09 ± 0.03	117 ± 44	10.6 ± 0.6	2130 ± 735	1.47 ± 0.04	382 ± 51
3	0.257	29.1	0.198	10.0	0.94 ± 0.03				0.96 ± 0.03	

[a] Other components present were 0.15 *M* Tris-Cl buffer (pH 6.6), 0.22 *M* KCl, and 95% D_2O, $T = 25°$. [b] 20% of the added R·CoA was bound to transcarboxylase as detected by EPR spectroscopy. $1/fT_{1p}$ and $1/fT_{2p}$ were calculated using the normalization factor $f = [R·CoA]_b/[pyruvate]$.

Table IV: Longitudinal Relaxation Rates of the Carbonyl ^{13}C and the Carboxyl ^{13}C of Pyruvate at 25.1 MHz, in the Presence of Transcarboxylase and R·CoA.[a]

Expt	[E-Biotin] (m*M*)	[[1-^{13}C]- and [2-^{13}C]-Pyruvate] (m*M*)	$[R·CoA]_t$ (m*M*)	$[R·CoA]_b$ (m*M*)	Carbonyl ^{13}C (sec^{-1})		Carboxyl ^{13}C (sec^{-1})	
					$1/T_1$	$1/fT_{1p}$	$1/T_1$	$1/fT_{1p}$
1	0.250	35.7			0.17 ± 0.01		0.080 ± 0.003	
2	0.248	34.0	1.090	0.176	0.23 ± 0.01	12 ± 3	0.100 ± 0.003	4 ± 1
3	0.244	33.4	2.961	0.213	0.24 ± 0.01	11 ± 3	0.115 ± 0.003	5 ± 1
4[b]	0.239	32.9	0.118	0.017	0.18 ± 0.01		0.080 ± 0.003	

[a] Other conditions were as described in Table I except that 15% D_2O was added for field-frequency locking. $T = 25°$. $1/fT_{1p}$ was calculated as described in Table III. [b] Experiment 4 was derived from experiment 3 by addition of 57.4 m*M* Tris ascorbate (pH 6.8) to the solution of experiment 3. The concentrations of $[R·CoA]_t$ and $[R·CoA]_b$ were decreased to the levels indicated in this table by reduction of nitroxide radical to an amine, as detected by EPR.

The magnitude of ϵ_b for the tight transcarboxylase–R·CoA complex (Table I) is 1.9-fold greater than that of the citrate synthase–R·CoA complex ($\epsilon_b = 3.4$, Weidman et al., 1973), is of the same order as that found for a paramagnetic iodoacetamide nitroxide derivative covalently bound to yeast alcohol dehydrogenase ($\epsilon_b = 6$, Sloan and Mildvan, 1974), but is considerably less than the ϵ_b value for a spin-labeled analogue of NAD^+ bound to alcohol dehydrogenase ($\epsilon_b = 13$, Mildvan and Weiner, 1969b).

The Paramagnetic Effects of Transcarboxylase-Bound R·CoA on the Longitudinal ($1/fT_{1p}$) and Transverse ($1/fT_{2p}$) Relaxation Rates of the Methyl Protons of Pyruvate at 40.5 and 100 MHz. Since the tight binding site of R·CoA corresponds to the CoA ester site of transcarboxylase, R·CoA may be used as a paramagnetic probe to determine the distance between the CoA ester and keto acid substrate sites of the enzyme. Table III summarizes the paramagnetic effects of transcarboxylase-bound R·CoA on the relaxation rates of the methyl protons of pyruvate. In the absence of R·CoA the longitudinal relaxation rate ($1/T_1$) of the methyl protons is independent of frequency. The binding of R·CoA to transcarboxylase causes a small but reproducible increase in $1/T_1$ of the methyl protons at 100 MHz. This increase becomes greater when measured at 40.5 MHz (Table III). The addition of a saturating concentration of propionyl-CoA to the system reduces the $1/T_1$ relaxation rates of the methyl protons to the values obtained in absence of R·CoA, indicating that the paramagnetic effects on pyruvate are due to interactions at the substrate sites on transcarboxylase, and that free R·CoA has no significant effect on $1/T_1$ at these concentrations.

For the methyl protons of pyruvate, the paramagnetic effect of bound R·CoA on the normalized transverse relaxation rate ($1/fT_{2p}$) is 18 times greater than its effect on the normalized longitudinal relaxation rate ($1/fT_{1p}$) measured at 100 MHz (Table III). As discussed in detail elsewhere (Nowak and Mildvan, 1972), this observation, together with the finding of a frequency dependence of $1/fT_{1p}$ of the methyl protons (Table III) indicates that the values of $1/fT_{1p}$ are not exchange limited and may be used in eq 2 to calculate distances between the unpaired electron of R·CoA and the protons of pyruvate.

Paramagnetic Effects of Transcarboxylase-Bound R·CoA on the Longitudinal Relaxation Rates of the Carbonyl and Carboxyl Carbon Atoms of Pyruvate. In a solution of transcarboxylase and pyruvate, separately enriched with ^{13}C in the carboxyl and carbonyl carbon atoms to avoid ^{13}C spin–spin coupling (Fung et al., 1973, 1974), the addition of R·CoA causes small but easily measurable increases in $1/T_1$ of the enriched carbon atoms of pyruvate (Table IV). These effects, which are 2.5 ± 0.5-fold greater on the carbonyl than on the carboxyl carbon atoms, were abolished by the reduction of 96% of the nitroxide of R·CoA to an amine, using excess ascorbate (Table IV). The effect of reduction indicates that the increases in $1/T_1$ of the carbon atoms of pyruvate are due to the paramagnetic nitroxide of R·CoA rather than to diamagnetic steric effects on the motion of bound pyruvate. When normalized by the concentration of bound R·CoA and pyruvate, the $1/fT_{1p}$ values of the carboxyl and carbon atoms of pyruvate (Table IV) are 200-fold lower than $1/fT_{2p}$ of the protons of pyruvate (Table III). Hence the $1/fT_{1p}$ values of the carbon atoms are not limited by chemical exchange and may therefore be used in eq 2 for distance calculations.

Determination of the Correlation Time for the Dipolar Effect of Bound R·CoA on the Protons of Bound Pyruvate on Transcarboxylase. In order to use eq 2 for determining distances from R·CoA to pyruvate one must evaluate τ_c, the

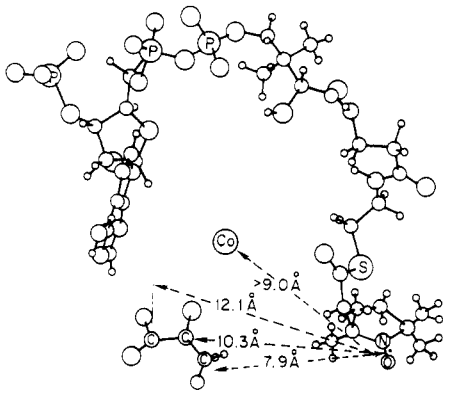

FIGURE 3: Spatial arrangement and conformations of pyruvate and R·CoA about the Co(II) site in ternary transcarboxylase-pyruvate-R·CoA complex. The atomic positions and conformations of the substrates are based on the indicated distances from the nitroxide radical to the methyl protons and carbonyl and carboxyl carbon atoms of pyruvate as well as previously determined distances from Co(II) to 10 protons and 3 ^{31}P nuclei of the CoA portion of propionyl-CoA (Fung et al., 1976) and to 3 nuclei of pyruvate (Fung et al., 1974).

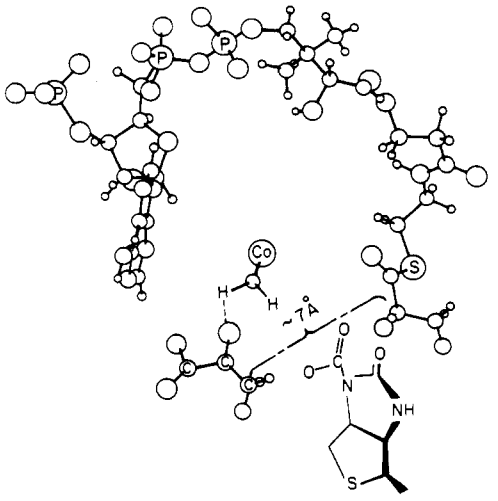

FIGURE 4: Spatial arrangement and conformations of pyruvate, propionyl-CoA, and carboxybiotin at the active site of transcarboxylase derived from Figure 3. The distance between the methyl carbon of pyruvate and the propionyl methylene carbon of propionyl-CoA between which the carboxyl group is transferred is ~7 Å.

correlation time for dipolar interaction. As summarized in Table V, the correlation time for the dipolar effect of bound R·CoA on the methyl protons of bound pyruvate was calculated from the frequency dependence of $1/fT_{1p}$ by making the extreme assumptions that τ_c was independent of frequency or that τ_c was maximally dependent on frequency, i.e., proportional to the square of the frequency (Bloembergen and Morgan, 1961; Nowak et al., 1973). The extreme values of τ_c differed by less than a factor of 2 and were essentially indistinguishable from an independent calculation of τ_c using the T_{1p}/T_{2p} ratio of the methyl protons of pyruvate in eq 3. The range of values of τ_c of 7 ± 5 nsec (Table V) thus obtained are significantly shorter than the tumbling time of transcarboxylase (620 nsec) estimated from Stokes law, or the residence time of pyruvate (≥140 nsec) or of R·CoA (≥3030 nsec) estimated from their respective dissociation constants (Fung et al., 1974 and Table II), indicating that τ_c is dominated by τ_s, the electron spin relaxation time of bound R·CoA. The calculated values of τ_c (Table V) are consistent with the electron spin relaxation time of

Table V: Determination of the Correlation Time and Distances between the Nitroxide Radical of Bound R·CoA and the Methyl Protons and Carbonyl and Carboxyl Carbon Atoms of Pyruvate in Transcarboxylase–R·CoA–Pyruvate Complex from the Observed Relaxation Rates.

τ_c at 23.5 kG		Distances from R·CoA to Pyruvate Nuclei (Å)		
Method	Value (nsec)	H_3C	$^{13}C{=}O$	$^{13}COO^-$
ω_I[a]	4.0 ± 1.6	8.5 ± 0.6	10.2 ± 0.7	12.0 ± 0.8
ω_I[b]	7.3 ± 2.9	7.7 ± 0.6	10.4 ± 0.7	12.2 ± 0.8
T_{1p}/T_{2p}	8.0 ± 4.1	7.6 ± 0.7	10.4 ± 0.8	12.1 ± 0.9
	Av	7.9 ± 0.7	10.3 ± 0.8	12.1 ± 0.9

[a] From $1/fT_{1p}$ of protons at 40.5 and 100 MHz assuming no frequency dependence of τ_c. [b] From $1/fT_{1p}$ of protons at 40.5 and 100 MHz assuming maximal frequency dependence of τ_c.

~10 nsec estimated from the line width of the EPR spectrum of bound R·CoA in the transcarboxylase R·CoA–pyruvate complex (Figure 1).

Distances between Enzyme-Bound R·CoA and the Magnetic Nuclei of Bound Pyruvate on Transcarboxylase. The $1/fT_{1p}$ values of the methyl protons (Table III) and of the carbonyl and carboxyl carbon atoms of pyruvate (Table IV) were used in eq 2, together with the correlation time, to calculate the distances from R·CoA to pyruvate on transcarboxylase (Table V). Since the protons and carbon atoms of pyruvate interact with the same unpaired electron, it is appropriate to use τ_c determined for the R·CoA–proton interaction to calculate distances to the carbon atoms as well (Fung et al., 1974).

The distances from bound R·CoA to the methyl protons (7.9 ± 0.7 Å), the carbonyl carbon (10.3 ± 0.8 Å), and the carboxyl carbon of bound pyruvate (12.1 ± 0.9 Å) establish the proximity of the binding sites for both substrates and indicate that they interact with the same metal ion (Figure 3). The distance from Co(II) at the active site to the nitroxide radical (Figure 3) is ~10 Å, consistent with our failure to detect a dipolar electron–electron interaction by EPR.

Making use of these facts, together with the previously determined distances from Co(II) to the methyl protons and two carbon atoms of pyruvate (Fung et al., 1974) and to 12 protons and three phosphorus atoms of bound propionyl-CoA (Fung et al., 1976), a composite model of the active site may be constructed (Figure 4). In this model the distance from the methyl carbon of pyruvate to the methylene carbon of propionyl-CoA, between which the carboxyl transfer takes place, is ~7 Å (Figure 4).

Discussion

The present studies of the binding of R·CoA to transcarboxylase have indicated approximately one tight binding site for CoA esters per biotin residue[1] or six per 790000 daltons of transcarboxylase. These tight binding sites appear to be specific for esters of CoA since the R·CoA radical is displaced by propionyl-CoA but not by pyruvate. The simultaneous binding of R·CoA and pyruvate to transcarboxylase is established by the paramagnetic effects of bound R·CoA on the protons and carbon atoms of bound pyruvate (Tables III and IV). These findings are in accord with kinetic studies of the partial reactions (Northrop and Wood, 1969):

$$\text{E-biotin} + (S)\text{-}CH_3CH(COO^-)COSCoA \rightleftharpoons \text{E-biotin-}COO^- + CH_3CH_2COSCoA \quad (5)$$

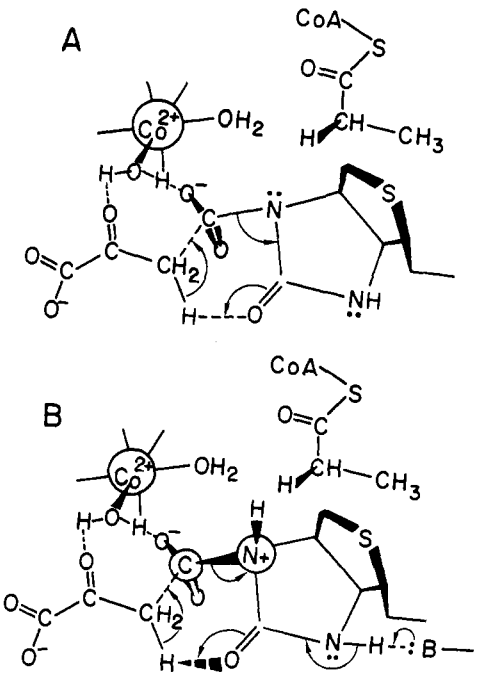

FIGURE 5: Alternative mechanisms for carboxylation of pyruvate on transcarboxylase and other metallobiotin enzymes. Mechanism A, in which tautomerization of the carboxyl bearing nitrogen of carboxybiotin takes place, is modified from that originally proposed for pyruvate carboxylase (Mildvan et al., 1966) taking into account the second sphere metal complexes generally found for such enzymes (Fung et al., 1973, 1974, and 1976). Mechanism B involves prior protonation of the carboxyl bearing nitrogen of carboxybiotin and tautomerization of the opposite nitrogen. Although written as concerted mechanisms for parsimony, intermediates have not been ruled out.

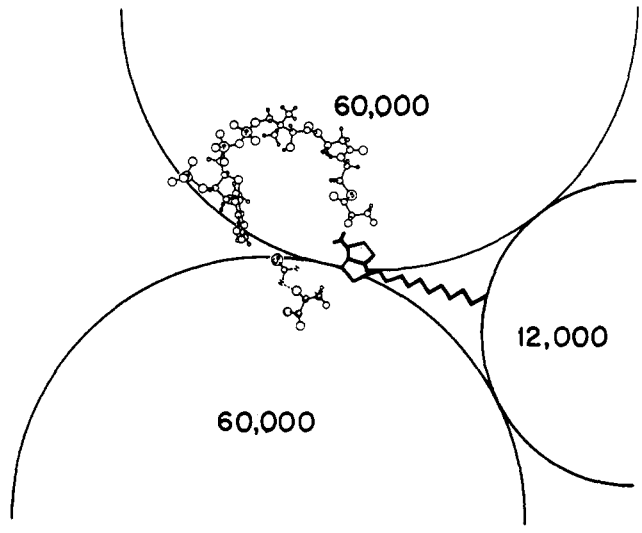

FIGURE 6: Diagram to scale of transcarboxylase subunits showing the role of the 14-Å arm of carboxybiotin. The protomers, of indicated molecular weight (Ahmad et al., 1975), are assumed to be spherical. The conformations of pyruvate and propionyl-CoA are from the work of Fung et al. (1974, 1976).

$$\text{E-biotin-COO}^- + \text{CH}_3\text{COCOO}^- \rightleftharpoons \text{E-biotin} + {}^-\text{OOCCH}_2\text{COCOO}^- \quad (6)$$

and of the overall reaction of transcarboxylase (Northrop, 1969):

$$\text{(S)-CH}_3\text{CH(COO}^-\text{)COSCoA} + \text{CH}_3\text{COCOO}^- \rightleftharpoons \text{CH}_3\text{CH}_2\text{COSCoA} + {}^-\text{OOCCH}_2\text{COCOO}^- \quad (7)$$

which indicate separate and independent binding sites for CoA compounds and for α-keto acids and the operation of a hybrid ping-pong kinetic scheme. Moreover, reactions 5 and 6 have been shown to be catalyzed by different subunits of transcarboxylase, the former by a 12S subunit and the latter by a 5S metallosubunit while biotin is covalently attached to a third 1.3S subunit (Chuang et al., 1975). Thus the overall reaction (eq 7) involves the interaction of three different subunits. The ping-pong nature of the transcarboxylase reaction and of all other biotin-enzyme reactions has long been thought to involve a step in which carboxybiotin, which is located at the end of a 14-Å long flexible arm, migrates between different active sites (Green, 1963; Mildvan et al., 1966; Gregolin et al., 1968; Northrop and Wood, 1969). The close proximity of the active site for the carboxylation of propionyl-CoA and of pyruvate found in the present studies (Table V, Figures 3 and 4) indicates that the overall transcarboxylation reaction (eq 7) takes place at the interface of three subunits such that the distance of carboxyl transfer, hence the maximal distance for carboxybiotin migration, is only ~7 Å (Figure 4). The minimum distance for carboxybiotin migration is estimated by assuming, as depicted in Figure 4, that carboxybiotin is located between the methyl carbon of pyruvate and the methylene carbon of propionyl-CoA. In this case no motion of 1′-*N*-carboxybiotin is permitted, since the van der Waals thickness of the conjugated ureido carboxy system (3.4 Å) and the van der Waals radii of the methyl (2.0 Å) and methylene groups (2.0 Å) (Pauling, 1960) of the substrates sum to 7.4 Å.

The detailed mechanism previously proposed for metallobiotin enzymes (Mildvan et al., 1966) modified in Figure 5A to take into account the observed second sphere complexes of pyruvate (Fung et al., 1973, 1974) and of propionyl-CoA (Fung et al., 1976) involves carboxyl transfer parallel to the plane of the ureido ring. Hence this mechanism would require maximal migration (~7 Å) of the carboxybiotin intermediate. An alternative mechanism, which cannot be ruled out on the basis of electronic considerations, involves prior protonation of the ureido ring nitrogen, tautomerization involving the 3′-N, and carboxyl transfer perpendicular to the ureido ring (Figure 5B). This mechanism could operate either with maximal carboxybiotin migration (~7 Å) or with carboxybiotin immobilized between the two substrates (Figure 4). Although the latter mechanism is less likely on steric grounds, neither of the mechanisms of Figure 5 can be excluded and the direction of carboxy transfer in relation to biotin is unknown.

We therefore conclude that the carboxybiotin intermediate on transcarboxylase migrates over a distance of, at most 7 Å, and possibly not at all. Hence the major role of the 14-Å arm of carboxybiotin (Figure 6) is to place the transferred carboxyl group at the end of a long probe, permitting it to traverse the gap which occurs at the interface of three subunits, and to insinuate itself between the CoA and α-keto acid sites.

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