^1H and ^{31}P Fourier Transform Magnetic Resonance Studies of the Conformation of Enzyme-Bound Propionyl Coenzyme A on Transcarboxylase†

Chien H. Fung,‡ Richard J. Feldmann, and Albert S. Mildvan*

ABSTRACT: The relaxation rates of the carbon-bound protons and of the three assigned phosphorus resonances of propionyl-CoA were measured in solutions of free propionyl-CoA and of the transcarboxylase–propionyl-CoA complex. In free propionyl-CoA, analysis of the $1/T_1$ values of 15 protons at 100 and 220 MHz and of $1/T_1$ and $1/T_2$ of the three phosphorus atoms at 40.5 MHz indicated free rotation of the propionyl region ($\tau_r \sim 3 \times 10^{-11}$ sec) but hindered motion of the remainder of the molecule with correlation times of $1\text{–}3.5 \times 10^{-10}$ sec, approaching the tumbling time of the entire molecule ($\tau_r = 6 \times 10^{-10}$ sec). The correlation times of the three phosphorus atoms were indistinguishable from those of their nearest neighbor protons. The effects of three homogeneous enzyme preparations with varying contents of Zn(II), Co(II), and Cu(II) on $1/T_1$ of 12 protons and 3 phosphorus atoms of propionyl-CoA were analyzed with the help of simultaneous equations to yield the individual contributions at the three metal sites. Only diamagnetic effects were detected on the relaxation rates of the three phosphorus atoms. From the diamagnetic effects it was calculated that the motions of the propionyl side chain and of the terminal pantetheine methylene protons were hindered on the enzyme by an order of magnitude ($\tau_r \sim 6 \times 10^{-10}$ sec) and that the phosphorus atoms were hindered by two orders of magnitude ($\tau_r \sim 1 \times 10^{-8}$ sec) over the τ_r values found in free propionyl-CoA, but that these τ_r values remained well below that of the entire protein molecule ($\tau_r = 6 \times 10^{-7}$ sec). The paramagnetic effects at the Co(II) site were analyzed with the previously determined τ_c value (Fung, C. H., Mildvan, A. S., and Leigh, J. S., Jr. (1974), *Biochemistry 13,* 1160) to yield distances from bound Co(II) to seven protons (6.5–8.7 Å) and lower limit distances to five additional protons (8.5–10.0 Å), and to the three phosphorus atoms (9.0–10.0 Å) of bound propionyl-CoA. Those protons nearest to Co(II) (6.5 Å) were the reaction center propionyl methylene protons. The 15 distances from Co(II), all of which were too great for direct coordination of any portion of the propionyl-CoA molecule, were used, with the help of a computer search among 46656 structures, to determine a unique conformation of propionyl-CoA at the active site of transcarboxylase which best fit the calculated distances and minimized van der Waals overlap. This conformation was U shaped about the bound Co(II), and was estimated to be ~22% unfolded with the adenine and thioester ends of propionyl-CoA approaching the second coordination sphere of Co(II). The Co(II) atom lies 4.5 Å above the plane defined by the adenine H(2), the pantetheine C-9, and the propionyl methylene carbon. At the inactive Cu(II) site, distances from the bound metal to six protons (7.0–8.9 Å) and lower limit distances to six additional protons (10.0–12.5 Å) and to the three phosphorus atoms (10.5–11.5 Å) of bound propionyl-CoA suggest a more open conformation. The present distances from Co(II) to the protons and phosphorus atoms of propionyl-CoA and previous distances to the protons and carbon atoms of pyruvate (Fung, C. H., Mildvan, A. S., and Leigh, J. S., Jr. (1974), *Biochemistry 13,* 1160) were used to position both substrates with respect to Co(II) at the active site.

Transcarboxylase from *Propionibacterium shermanii,* like other biotin enzymes (Moss and Lane, 1971; Wood and Utter, 1965) (EC 2.1.3.1), catalyzes two half-reactions (eq 1 and 2). The enzyme contains tightly bound divalent cobalt, zinc, and copper in a stoichiometry approximating 12 g-ions of metal per mol (790000 g) of enzyme or 2 g-ions of metal per mol of biotin (Fung et al., 1974). Using ^1H and ^{13}C Fourier transform nuclear magnetic resonance (NMR) techniques, it has been established that the substrate pyruvate binds near the bound Co(II) to form an active second sphere complex. The distances from the bound Co(II) to the carbon atoms and methyl protons of bound pyruvate were 5.0–6.3 Å as calculated from the paramagnetic effects of Co(II) on the relaxation rates ($1/T_{1p}$) of pyruvate nuclei (Fung et al., 1974), with the carbonyl carbon of pyruvate closest to the Co(II). Similarly, the interatomic distances between the bound Cu(II) and the carbon atoms and protons of pyruvate indicate a second sphere complex although the Cu(II) site is probably inactive (Fung et al., 1974). The proximity of the bound metal ions and pyruvate binding sites suggests that the metal ion may participate in the carboxylation of pyruvate (eq 2) by orienting an inner sphere water ligand or protein ligand so that it polarizes the carbonyl group of the pyruvate molecule by hydrogen bonding or protonation. From a steady-state kinetic analysis, Northrop (1969) has suggested that the propionyl-CoA and keto acid binding sites are near enough to each other to permit

† From the Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111 (C.H.F. and A.S.M.), and Division of Computer Research, National Institutes of Health, Bethesda, Maryland 20014 (R.H.F.). *Received July 10, 1975.* This work was supported by U.S. Public Health Service Postdoctoral Fellowship GM-53808 (C.H.F.), Grant BMS74-03739 from the National Science Foundation, U.S. Public Health Service Grants AM-13351, NIH CA-06927, and NIH RR-05539, and by an appropriation from the Commonwealth of Pennsylvania. In addition, 220-MHz NMR spectra were taken at the Middle Atlantic Regional NMR Facility which is supported by National Institutes of Health Grant RR542 at the University of Pennsylvania.

‡ Present address: Department of Medicine, Rutgers Medical School, Piscataway, New Jersey 08854.

the carboxybiotin ring to oscillate between these two substrate sites during transcarboxylation. Thus, the metal ion may also exert a paramagnetic effect on propionyl-CoA. From the paramagnetic effects on the relaxation rates of the individual protons and phosphorus nuclei of propionyl-CoA, the geometric arrangement and conformations of enzyme-bound propionyl-CoA can be deduced.

$$\text{E-biotin} + \text{(S)-}CH_3CH(COO^-)COSCoA \rightleftharpoons \text{E-biotin-}COO^- + CH_3CH_2COSCoA \quad (1)$$

$$\text{E-biotin-}COO^- + CH_3COCOO^- \rightleftharpoons \text{E-biotin} + {}^-OOCCH_2COCOO^- \quad (2)$$

Mieyal et al. (1974) have recently reported that approximately 65% of benzoyl-CoA molecules in D_2O at biological pH and temperature exist in a folded conformation in which the two bases, adenine and benzene, are stacked onto each other. Lee and Sarma (1974, 1975) have shown that CoA and acetyl-CoA in aqueous solution exist in a dynamic equilibrium of linear and hairpin-folded conformations. Conformations of large coenzymes free in solution are generally different from those of enzyme-bound coenzymes. For example, the structures of NADH and NAD^+ in solution as studied by fluorescence (Velick, 1961) and by NMR spectroscopy consist primarily of a folded structure in rapid equilibrium with an open form (Jardetzky and Wade-Jardetzky, 1966; Oppenheimer et al., 1971; Lee et al., 1973). Fluorescence (Velick, 1961), NMR (Sloan and Mildvan, 1974), and x-ray data (Chandrasekhar et al., 1973; Webb et al., 1973) have established that enzyme-bound NADH and NAD^+ are exclusively in an open form.

To study the conformation of propionyl-CoA on transcarboxylase, we have determined the paramagnetic effects of the tightly bound metal ions on the relaxation rates of the individual ^1H and ^{31}P nuclei of propionyl-CoA under the conditions of fast chemical exchange between free and enzyme-bound propionyl-CoA. As shown previously (Sloan and Mildvan, 1974) a set of distances from a single paramagnetic reference point to multiple nuclei of a bound substrate can be used to deduce the conformation of the bound substrate. The conformation so obtained agrees with that obtained by the independent method of x-ray diffraction (Branden et al., 1973).

A preliminary report of this work has been published (Mildvan et al., 1975).

Experimental Procedure

Materials and Methods. The growth of *Propionibacterium shermanii* 52W, purification of transcarboxylase from propionibacteria, and assay of the enzyme were according to the method of Wood et al. (1969) except that the concentrations of Co(II) and Zn(II) in the media were higher (Fung et al., 1974). Propionyl-CoA was purchased from P-L Biochemicals, Inc. For the assay of transcarboxylase, (*RS*)-methylmalonyl-CoA was synthesized from the transcarboxylase reaction of propionyl-CoA with oxalacetate in the presence of excess L-lactate dehydrogenase and NADH and was purified as described by Fung (1972). Ultra-pure Tris base was obtained from Mann Research Laboratory. L-Lactate dehydrogenase (360 units/mg) and L-malate dehydrogenase (1100 units/mg) were obtained from Boehringer und Soehne. All other compounds were reagent grade or of the highest purity commercially available.

The specific activities of the three preparations of transcarboxylase used in this study, TC-A, TC-B, and TC-C, were 33, 33, and 40 units/mg, respectively, and all migrated in gel electrophoresis as homogeneous enzymes (Fung et al., 1974). Metal analyses were done by atomic absorption spectroscopy using the Varian Techtron instrument after appropriate dilutions with metal-free distilled water. The protein concentrations were determined by the method of Warburg and Christian (1957). For the ^1H NMR experiments, aliquots of transcarboxylase stored at −70°C in 0.2 *M* phosphate buffer (pH 6.8) were thawed and then precipitated by addition of 2.5 volumes of saturated ammonium sulfate. The enzyme was recovered by centrifugation at 16000*g* for 10 min and was then taken up in a minimum volume of 0.15 *M* potassium phosphate buffer in D_2O (pH 6.60). The enzyme solution was equilibrated with 0.15 *M* potassium phosphate buffer in D_2O (pH 6.60) by passage through a column (30 × 1 cm) of Sephadex G-25 (medium) which had previously been equilibrated with this buffer at 0–4°. Transcarboxylase obtained from the gel filtration was transferred to a collodion bag and concentrated by vacuum dialysis as described by Fung et al. (1974). For the ^{31}P NMR experiments, the gel filtration was performed in 0.2 *M* phosphate buffer in H_2O (pH 6.60). However, the concentration of potassium phosphate buffer in the NMR tube was reduced to 0.1 *M* with D_2O. All enzyme preparations used for NMR experiments were treated with Chelex-100 resin and then transferred to an NMR tube as previously described (Nowak and Mildvan, 1970). No loss of the enzyme activity or of the tightly bound metal ions resulted from such treatment.

The longitudinal relaxation time (T_1) of water protons at 24.3 MHz was determined as previously described (Fung et al., 1974). The longitudinal (T_1) and transverse (T_2) relaxation times of the ^{31}P and ^1H resonances of propionyl-CoA, at 40.5 and 100 MHz, respectively, were determined by the Fourier transform method using the Varian XL-100-15-FT NMR spectrometer. The field was locked on the ^2H resonance of D_2O for both ^{31}P and ^1H studies. Broad band noise decoupling of the protons was used for the ^{31}P relaxation measurements, and continuous wave saturation of the water proton resonance was employed for the ^1H relaxation measurements. The field homogeneity spoil method of McDonald and Leigh (1973) was used for measuring T_1. The pulse sequence of 90°-τ-180°-τ originally introduced by Carr and Purcell (1954) for spin echo type experiments was employed for measuring the T_2 relaxation time, where τ is the time interval between the two pulses. The sample sizes were 0.4 ml for ^1H relaxation and 1.5 ml for ^{31}P relaxation studies of propionyl-CoA. During the NMR experiments, which lasted 5–7 hr at 25°, the loss of enzyme activity did not exceed 15%. The experimental data 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, as previously described (Fung et al., 1974).

Since each preparation of transcarboxylase contained different molar ratios of two paramagnetic metals, Co(II) and Cu(II), and a diamagnetic metal, Zn(II), the contributions of the bound Co(II), $(1/fT_{1p})_{Co}$, the bound Cu(II), $(1/fT_{1p})_{Cu}$, and the bound Zn(II), $(1/fT_{1d})_{Zn}$, to the total paramagnetic and diamagnetic effects on the longitudinal relaxation rate of the observed nucleus were determined by simultaneous equations of the form:

$$(1/T_1)_{obsd} - (1/T_1)_0 = (1/fT_{1d})_{Zn}([Zn]_i/[\text{ligand}]) + (1/fT_{1p})_{Cu}([Cu]_i/[\text{ligand}]) + (1/fT_{1p})_{Co}([Co]_i/[\text{ligand}]) \quad (3)$$

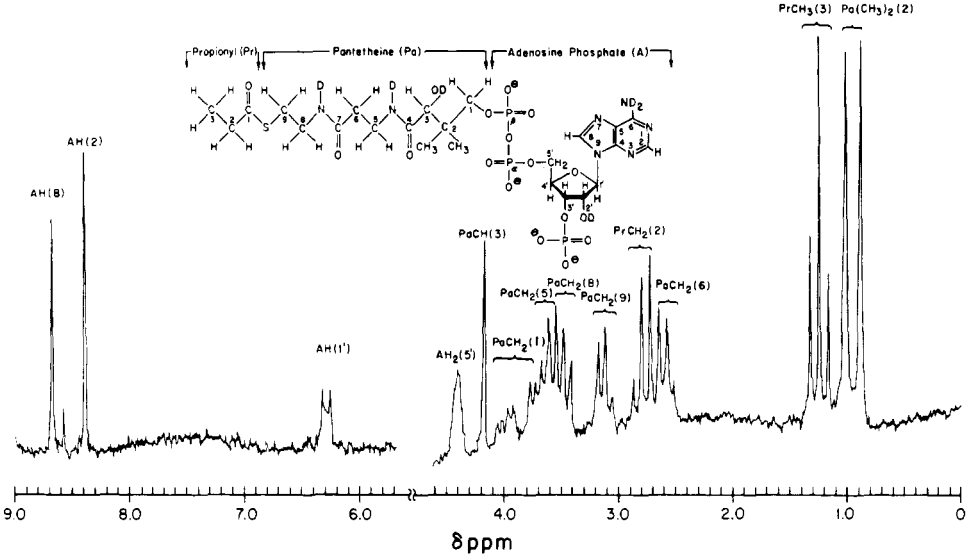

FIGURE 1: Fourier transform proton magnetic resonance spectrum of propionyl-CoA at 100 MHz. The system contained propionyl-CoA (10.0 mM) in 0.15 M potassium phosphate buffer (pH 6.62); temperature, 25°C. The scale is in ppm downfield from tetramethylsilane (Me_4Si). The spectrum shown is the result of 25 transients with a recycle time of 27 sec. The field was locked on the ^2H resonance of D_2O. The HDO peak which covers the AH(2'), AH(3'), and AH(4') resonances ranging from 4.60 to 5.70 ppm downfield from Me_4Si has been deleted.

for each enzyme preparation (Fung et al., 1974). In eq 3, $(1/T_1)_{obsd}$ is the longitudinal relaxation rate of a magnetic nucleus in the presence of enzyme, $(1/T_1)_0$ is the relaxation rate in its absence, f represents the normalization factor [metal ion]/[ligand], and $[Co]_i$, $[Cu]_i$, and $[Zn]_i$ represent the bound metal ion concentrations in a given experiment. Distances from the enzyme-bound Co(II) and Cu(II) to the individual protons and phosphorus atoms of propionyl-CoA were calculated from $(1/fT_{1p})_{Co} - (1/f_{1d})_{Zn}$ and $(1/fT_{1p})_{Cu} - (1/f_{1d})_{Zn}$, respectively, using the dipolar term of the Solomon-Bloembergen equation (Fung et al., 1974; Sloan and Mildvan, 1974).

The conformation of enzyme-bound propionyl-CoA which best fits the calculated distances to Co(II) at the active site was investigated by construction of stick and space filling models. The space filling model was confirmed by a computer search (Furie et al., 1974), carried out on the time sharing DEC PDP-10 computer at the National Institutes of Health which is equipped with two graphics display systems, an ADAGE AGT-30 graphics display computer and a DEC-340 graphics terminal. The coordinates for the structure of propionyl-CoA were synthesized from the known coordinates taken from x-ray crystallographic data for various fragments of the propionyl-CoA molecule. The coordinates for the nucleotide portion were generated from adenosine 5'-monophosphate (Kraut and Jensen, 1963) to which phosphoryl groups were attached at the 3'-hydroxyl and the 5'-phosphate groups using known bond angles and bond lengths. For the pantothenic portion of propionyl-CoA, portions of the coordinates for isocitrate from x-ray crystallographic data were used (Van der Helm et al., 1968). The coordinates for $(CH_2)_2$, CH_3, NHCO, and SCO were obtained from lysine (Wright and Marsh, 1962), alanine (Lehmann et al., 1972), asparagine (Kartha and de Vries, 1961), and spironolactone (Dideberg and Dupont, 1972), respectively. Thus the resulting molecule of propionyl-CoA had appropriate covalent bond lengths and bond angles. A series of possible conformations of propionyl-CoA was generated by simultaneous rotation of the molecule about the N(9)-C(1'), C(5')-O(5'), and C(1)-O(1) bonds in increments of 10°. In this search among $(36)^3$ or 46656 conformations, the computer printed out the coordinates of those structures which satisfied the experimentally determined distances within their experimental errors and which permitted ≤0.5 Å total van der Waals overlap of the atoms of enzyme-bound propionyl-CoA.

Results and Discussion

Proton NMR Spectrum and Proton Relaxation Rates of Propionyl-CoA. The assignments of the individual proton resonances of the Fourier transform ^1H spectra of the CoA portion of propionyl-CoA corresponding to its structure (Figure 1) were made by Mieyal et al. (1974) and by Lee and Sarma (1974). The additional methyl ($PrCH_3$, 1.25 ppm downfield from Me_4Si) and methylene ($PrCH_2$, 2.75 ppm downfield from Me_4Si) resonances of the propionyl group are a triplet and quartet, respectively (J = 7.6 Hz). The assignment of these two signals was further confirmed by decoupling. Decoupling experiments were also done to identify the coupled pairs of methylene resonances $PaCH_2(5)$-$PaCH_2(6)$ (J = 6.4 Hz) and $PaCH_2(8)$-$PaCH_2(9)$ (J = 6.0 Hz). The longitudinal relaxation rates of the protons of propionyl-CoA at 100 and 220 MHz are summarized in Table I.

Two striking features of the proton relaxation rates of propionyl-CoA were observed. First, the $1/T_1$ value for the protons of the two methyl groups on the pantetheine portion of the molecule ($PaCH_3(2)$ downfield and $PaCH_3(2)$ upfield) are an order of magnitude greater than $1/T_1$ of the propionyl methyl group. Second, the methylene protons located in the adenosyl and pantetheine regions of propionyl-CoA also relax an order of magnitude faster than the propionyl methylene protons. These data suggest a hindrance of rotation of those methyl and methylene protons in the internal portion of propionyl-CoA.

The longitudinal relaxation rates of the methylene and methyl protons at 100 and 220 MHz were analyzed according to the equation of Solomon for two interacting spins (Solomon, 1955):

$$\frac{1}{T_1} = \frac{D^6}{r^6}\left(\frac{\tau_r}{1+\omega_1^2\tau_r^2} + \frac{4\tau_r}{1+4\omega_1^2\tau_r^2}\right) \quad (4)$$

Table I: Analysis of the Longitudinal Proton Relaxation Rates of Propionyl-CoA.[a]

Resonance	$1/T_1$ (sec^{-1}) 100 MHz	$1/T_1$ (sec^{-1}) 220 MHz	$(1/T_1)$ 100 MHz/ $(1/T_1)$ 220 MHz)	τ_r[b] (sec × 10^{10})	r[b] (Å)
AH(8)	0.82	0.83	0.99	≤1.3	≤2.26
AH(2)	0.19	0.14	1.30	2.6 ± 0.5	3.21 ± 0.10
AH(1′)	0.50	0.45	1.10	1.5 ± 0.6	2.51 ± 0.14
AH_2(5′)	6.38	4.99	1.28	2.5 ± 0.5	1.77 ± 0.06
$PaCH_2$(1) downfield	3.50	3.51	1.00	1.9 ± 0.6[c]	
$PaCH_2$(1) upfield	3.63	3.55	1.02	1.9 ± 0.6[c]	
PaCH(3)	0.92	0.71	1.30	2.6 ± 0.5	2.45 ± 0.07
$PaCH_2$(5)	3.60	3.12	1.15	1.8 ± 0.6	1.86 ± 0.09
$PaCH_2$(8)	3.44	2.22	1.55	3.5 ± 0.5	2.05 ± 0.05
$PaCH_2$(9)	2.03	1.94	1.05	1.1 ± 0.4[c]	
$PrCH_2$(2)	0.65	0.61	1.06	0.34 ± 0.12[c]	
$PaCH_2$(6)	3.09	2.84	1.09	1.6 ± 0.6[c]	
$PrCH_3$(3)	0.31	0.28	1.08	0.17 ± 0.05[d]	
$PaCH_3$(2) downfield	3.33	2.93	1.14	1.7 ± 0.5	2.09 ± 0.09
$PaCH_3$(2) upfield	3.23	2.72	1.19	2.0 ± 0.5	2.16 ± 0.10

[a] Components present were propionyl-CoA (10.0 mM) and 0.15 M potassium phosphate buffer (pH 6.65); T = 25°. [b] Calculated from frequency dependence of $1/T_1$ using eq 4. [c] Calculated from eq 4 assuming average methylene interproton distance of 1.89 ± 0.10 Å. [d] Calculated from eq 4 assuming average methyl interproton distance of 2.13 ±0.10 Å. Nuclei are defined in Figure 1.

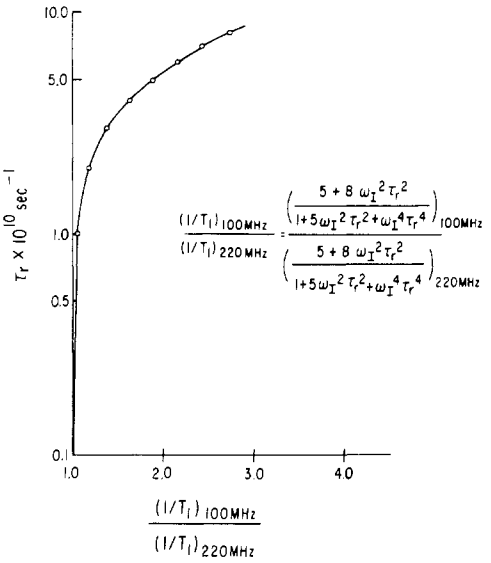

FIGURE 2: The rotational correlation time (τ_r) as a function of the ratio of longitudinal relaxation rates at two frequencies. The theoretical curve is generated from the relationship shown, which is derived from eq 4 in the text.

In eq 4 D is a product of physical constants numerically equal to 74.48 Å (sec)$^{-1/3}$ for two interacting protons and to 83.60 Å (sec)$^{-1/3}$ for three interacting protons (Werbelow and Marshall, 1973), r is the interproton distance in Å, and τ_r is the correlation time for interproton dipolar interaction which, in the present case, is dominated by internal rotation. Equation 4 yields the curve of Figure 2 which relates the frequency dependence of $1/T_1$ to the correlation time τ_r. From Figure 2 it may be seen that for protons with a $\tau_r \leq 1.3 \times 10^{-10}$ sec no frequency dependence of $1/T_1$ would be expected within our experimental error of ±7%. For those protons which showed a frequency dependence of $1/T_1$ beyond the ±7% error (Table I), τ_r was directly determined from Figure 2 and r was calculated from eq 4. The average interproton distances for the two methyl groups (2.13 ± 0.10 Å) and the three methylene groups (1.89 ± 0.10 Å) for which a frequency dependence of $1/T_1$ was observed (Table I) are in reasonable agreement with the expected values of 1.74 ± 0.04 Å from neutron diffraction data (Verbist et al., 1972).[1] These average r values were then used directly in the Solomon equation to calculate τ_r for those protons for which no frequency dependence of $1/T_1$ was detected.

The calculated values of τ_r (Table I) indicate that the internal methyl and methylene groups of propionyl-CoA rotate an order of magnitude more slowly than do the terminal groups. Moreover, the τ_r values of the internal protons approach the tumbling time of the entire propionyl-CoA molecule which is calculated from Stokes law to be 6.2×10^{-10} sec, suggesting that the internal protons may be highly immobilized by steric hindrance.

Although the chemical shifts of the two methyl groups of the pantetheine region differ by 0.13 ppm presumably due to differing positions with respect to the adenine ring (Lee and Sarma, 1975), their correlation times are long and approximately equal indicating comparable hindrance of rotation (Table I).

For the methine protons, the distances of ≤2.3–3.2 Å calculated from the simplest assumption of dipolar interaction with another single proton (Table I) are of the same order or greater than 2.4 Å, the sum of the van der Waals radii of two protons (Pauling, 1960).

The difference in the τ_r values for the two ring protons of adenine indicates that while the adenine ring may be immobilized as reflected in the long correlation time of AH(2), the AH(8) proton may be interacting with the rapidly rotating methyl or methylene protons of the propionyl group. This possibility is suggested by the observation of ring stacking in aromatic esters of CoA (Mieyal et al., 1974) and by small upfield shifts of the pantetheine protons of CoA derivatives suggesting the contribution of some folded conformations (Lee and Sarma, 1975).

Phosphorus Chemical Shifts and Relaxation Rates of Propionyl-CoA. The proton-decoupled phosphorus magnetic resonance spectrum of propionyl-CoA shown in Figure 3a

[1] A survey of interproton distances from neutron diffraction data by Dr. G. J. Williams of the Brookhaven National Laboratory yields a value of 1.75 Å (1.721–1.769 Å) for methylene groups and 1.74 Å (1.691–1.763 Å) for methyl groups.

consists of three phosphorus signals. The resonance having a chemical shift of 7.39 ppm downfield from 80% H_3PO_4 is assigned to the 3′-phosphoryl phosphorus resonance of propionyl-CoA. This assignment is strongly supported by the resonance position observed for 3′-AMP which appears at 8.10 ppm downfield from 80% H_3PO_4. The similarity of the coupling of the 5′-α- and 5′-β-pyrophosphate phosphorus nuclei (J = 20 Hz) and their difference in chemical shift ($\Delta\nu$ = 22 Hz) result in an AB pattern. To assign the resonances, double resonance experiments were carried out as shown in Figure 3b. The upfield doublet with a chemical shift of 6.68 ppm upfield from 80% H_3PO_4 was assigned to ^{31}P (5′-α) and the downfield doublet with a chemical shift of 6.14 ppm upfield from 80% H_3PO_4 was assigned to ^{31}P (5′-β). The evidence is the following: when the $AH_2(5')$ methylene protons were irradiated at their resonance frequency of 4.43 ppm downfield from Me_4Si, the amplitude of the ^{31}P (5′-α) doublet was greatly enhanced. Upon irradiation of the region of the $PaCH_2(1)$ methylene resonance at 3.78 ppm downfield from Me_4Si, the intensity of the other doublet, the ^{31}P (5′-β) resonance was markedly enhanced (Figure 3c). This double resonance experiment thus confirms the ^{31}P assignments of Lee and Sarma (1975) which were based on computer simulation of the undecoupled spectrum.

From eq 4 the relaxation rate of a phosphoryl phosphorus attached to a methylene group with the same correlation time should be 17.1–43.4 fold smaller than the relaxation rate of the methylene protons because of the greater distance between the phosphorus and the protons (2.5 ± 0.1 Å) and the 0.164-fold smaller D^6 value for phosphorus. Consistent with this view, the $1/T_1$ values of the 5′-α and 5′-β P atoms (Table II, experiment I) were found to be 38.0- and 16.7-fold smaller than the $1/T_1$ values of their respective adjacent methylene protons (Table I) indicating the τ_r values for the pyrophosphate portion of the propionyl-CoA to be similar to those of the adjacent methylene protons namely $\sim 2 \times 10^{-10}$ sec. The 1.27 ± 0.07-fold greater $1/T_1$ value of the 5′-β over the 5′-α phosphorus may well result from additional relaxation effects of the nearby pantetheine methyl groups. The slightly smaller $1/T_1$ value of the 3′-phosphorus may well result from the presence of only a single nearest neighbor proton rather than 2, from a small difference in distance, or a shorter τ_r. The larger $1/T_2$ values argue against a shorter τ_r.

Effects of Transcarboxylase Bound Metals on the Relaxation Rates of the Protons of Propionyl-CoA. Since transcarboxylase contains Co(II), Cu(II), and Zn(II) in various molar ratios at the pyruvate binding site (Fung et al., 1974), measurements of the effects of transcarboxylase bound metal ions on the proton relaxation rates of the nuclei of propionyl-CoA were carried out with three transcarboxylase preparations, namely, transcarboxylase A (TC-A), transcarboxylase B (TC-B), and transcarboxylase C (TC-C) containing differing proportions of the three metal ions (Table III). A typical Fourier transform proton spectrum of propionyl-CoA in the presence of transcarboxylase is shown in Figure 4. Each of the three different transcarboxylase preparations caused marked broadening of all of the proton resonances of propionyl-CoA, but no significant changes in the chemical shifts. As a result of this broadening of the resonances, the triplets of the $PaCH_2(5, 6, 8, \text{and } 9)$ and $PrCH_3(3)$ and the quartet of the $PrCH_2(2)$ are no longer well resolved (Figure 4). The strong HDO resonance although attenuated by irradiation rendered the measurements of the relaxation rates of the AH(2′, 3′, and 4′) and the $AH_2(5')$ resonances impossible. The $PaCH_2(1)$ resonance was also too broad and small to be measured (Figure 4). The AH(1′) doublet collapses to a singlet due to the irradiation of the HDO resonance under which the AH(2′) signal is hidden. All measurable proton relaxation rates ($1/T_1$) in the presence of the enzyme preparations are summarized in Table III, as the differences in the values of $1/T_1$ obtained in absence (Table I) and in presence of enzyme. The apparent increases in the proton relaxation rates

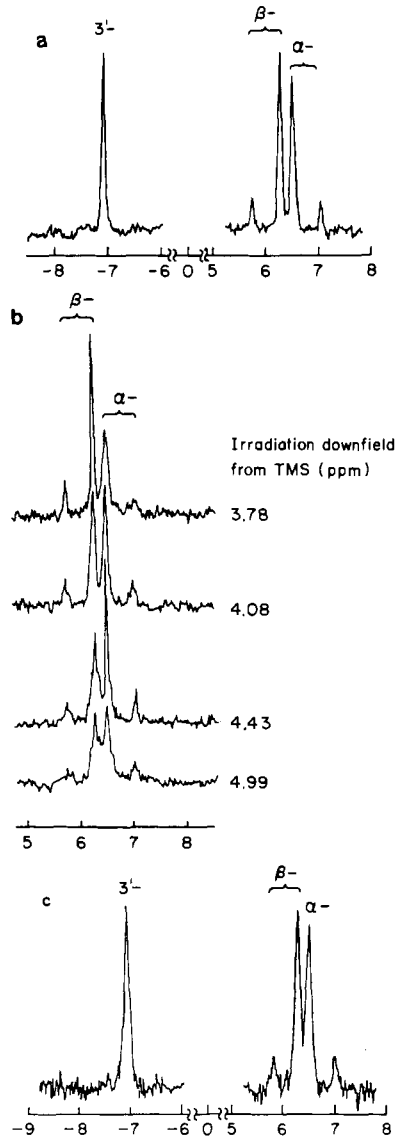

FIGURE 3: Fourier transform ^{31}P NMR spectra of propionyl-CoA at 40.5 MHz. (a) Proton noise decoupled Fourier transform ^{31}P NMR spectrum of propionyl-CoA. The system contained propionyl-CoA (4.0 mM) in 0.1 M potassium phosphate buffer (pH 6.6) containing 50% D_2O, for field frequency locking; T = 25°. The spectral range is from 8.5 ppm downfield from 80% H_3PO_4 to 7.8 ppm upfield from 80% H_3PO_4. The spectrum shown is the result of 150 transients with a recycle time of 32 sec. The noise decoupling was carried out at a bandwidth of 100 Hz and a power of 1 W. (b) Selective proton decoupling of the 5′-α-P and 5′-β-P resonances. The experimental conditions are as described in Figure 3a except that selective point irradiation of the ^1H resonances of propionyl-CoA was performed as indicated at 350 mW. (c) Proton noise decoupled Fourier transform ^{31}P NMR spectrum of propionyl-CoA (3.7 mM) in the presence of transcarboxylase. The system contained TC-B (32.9 mg/ml), 0.1 mM potassium phosphate buffer (pH 6.6) and 50% D_2O; temperature, 25°C. The spectrum shown is the result of 200 transients with a recycle time of 32 sec. The other conditions were as indicated in Figure 3a.

Table II: Effect of Transcarboxylases on the Relaxation Rates of the 3′ and Pyrophosphate ^{31}P Nuclei of Propionyl-CoA in the Transcarboxylase–Propionyl-CoA Complex.[a]

^{31}P Nucleus	I $(1/T_1)_0$ (sec^{-1})	II $(1/T_1)_{TC-A} - (1/T_1)_0$ (sec^{-1})	III $(1/T_1)_{TC-B} - (1/T_1)_0$ (sec^{-1})	IV $(1/T_1)_{TC-C} - (1/T_1)_0$ (sec^{-1})	$1/fT_{1d}$ (sec^{-1})	$1/fT_{2d}$ (sec^{-1})	$\tau_r \times 10^8$(sec)
5′-α	0.168 ± 0.005	0.072 ± 0.010	0.062 ± 0.011	0.093 ± 0.009	1.03 ± 0.05		1.3 ± 0.3[b]
5′-β	0.213 ± 0.009	0.008 ± 0.011	0.024 ± 0.013	0.004 ± 0.014			
3′	0.152 ± 0.008	0.060 ± 0.015	0.055 ± 0.014	0.086 ± 0.011	0.90 ± 0.12		0.66 ± 0.20[c]
	$(1/T_2)_0$ (sec^{-1})	$(1/T_2)_{TC-A} - (1/T_2)_0$ (sec^{-1})	$(1/T_2)_{TC-B} - (1/T_2)_0$ (sec^{-1})	$(1/T_2)_{TC-C} - (1/T_2)_0$ (sec^{-1})			
5′-α	0.75 ± 0.14	2.82 ± 0.51	6.39 ± 1.27	4.81 ± 0.78		61.4 ± 14.9	6.3 ± 2.5[d]
5′-β	0.60 ± 0.08	2.52 ± 0.56	4.96 ± 0.57	7.74 ± 1.42		64.0 ± 13.6	11.4 ± 6.7[d]
3′	0.85 ± 0.06	2.60 ± 0.28	6.29 ± 0.69	5.39 ± 0.50		61.8 ± 13.8	6.7 ± 2.6[d]

[a] Components present in experiment I were propionyl-CoA (4.0 m*M*). In experiment II were propionyl-CoA (5.0 m*M*) and TC-A (29.0 mg/ml) containing 263 ± 12 μ*M* Zn(II), 15 ± 2 μ*M* Cu(II), and 11 ± 2 μ*M* Co(II). In experiment III were propionyl-CoA (3.7 m*M*) and TC-B (32.9 mg/ml) containing 170 ± 7 μ*M* Zn(II), 21 ± 2 μ*M* Cu(II), and 91 ± 18 μ*M* Co(II). In experiment IV were propionyl-CoA (4.0 m*M*) and TC-C (31.2 mg/ml) containing 163 ± 10 μ*M* Zn(II), 32 ± 3 μ*M* Cu(II), and 177 ± 14 μ*M* Co(II). Other components present were 0.10 *M* Potassium phosphate buffer (pH 6.65), $T = 25°$. [b] Calculated from eq 4 using a D value of 61.85 Å $(sec)^{-1/3}$ and an r value of 2.5 ± 0.1 Å for the interactions of two protons with one phosphorus. [c] Calculated from eq 4 using a D value of 55.11 Å $(sec)^{-1/3}$ and an r value of 2.5 ± 0.1 Å for the interaction of one proton with one phosphorus. [d] Estimated upper limit from the T_1/T_2 ratio using eq 5.

Table III: Effects of Transcarboxylase Preparations on the Longitudinal Relaxation Rates of the Protons of Propionyl-CoA at 100 MHz.[a]

Resonance	I $(1/T_1)_{TC-A} - (1/T_1)_0$ (sec^{-1})	II $(1/T_1)_{TC-B} - (1/T_1)_0$ (sec^{-1})	III $(1/T_1)_{TC-C} - (1/T_1)_0$ (sec^{-1})
AH(8)	1.53	1.88	3.01
AH(2)	1.20	1.60	2.72
AH(1′)	1.30	1.71	2.30
PaCH(3)	1.40	1.42	1.73
$PaCH_2(5)$	<0.22	<0.09	<0.23
$PaCH_2(8)$	<0.39	<0.23	<0.06
$PaCH_2(9)$	0.53	0.91	1.25
$PrCH_2(2)$	0.63	1.44	2.20
$PaCH_2(6)$	<0.39	<0.39	<0.10
$PrCH_3(3)$	0.64	0.78	0.92
$PaCH_3(2)$ downfield	≤0.30	≤0.09	≤0.03
$PaCH_3(2)$ upfield	≤0.23	≤0.19	≤0.29

[a] Components present in experiment I were: propionyl-CoA (10.0 m*M*) and TC-A (38.3 mg/ml) which contained 327 ± 15 μ*M* Zn(II), 17 ± 2 μ*M* Cu(II), and 22 ± 21 μ*M* Co(II). In experiment II were propionyl-CoA (10.0 m*M*) and TC-B (38.3 mg/ml) containing 197 ± 9 μ*M* Zn(II), 27 ± 2 μ*M* Cu(II), and 106 ± 21 μ*M* Co(II). In experiment III were propionyl-CoA (11.87 m*M*) and TC-C (43.9 mg/ml) containing 218 ± 13 μ*M* Zn(II), 236 ± 19 μ*M* Co(II), and 43 ± 4 μ*M* Cu(II). Other components present are given in Table I. $T = 25°$. $(1/T_1)_0$ are the values obtained in absence of enzyme but under otherwise identical conditions (Table I).

in the presence of transcarboxylase (Tables I and III) are due to the paramagnetic effects of the bound Co(II) and Cu(II) and the diamagnetic effects at all active sites. Such diamagnetic effects are operative at the Zn(II) sites while both diamagnetic and paramagnetic effects are expected to be operative at the Co(II) and Cu(II) sites (Fung et al., 1974). For some protons, namely the 5, 6, and 8 CH_2 protons and the methyl protons of pantetheine, no effect of the three enzyme preparations on $1/T_1$ was detected within the experimental error. If we make the assumption that the observed corrected values of the $(1/T_1)_{TC-B}$ and $(1/T_1)_{TC-C}$ for the $PaCH_2(5)$, $PaCH_2(6)$, $PaCH_2(8)$, and $PaCH_3(2)$ upfield and downfield resonances are solely due to the Co(II) effect (Table III), the calculated upper limits of the $(1/fT_{1p})_{Co}$ for these protons are as follows: $PaCH_2(5)$, <13 sec^{-1}; $PaCH_2(6)$, <27 sec^{-1}; $PaCH_2(8)$, <16 sec^{-1}; $PaCH_3(2)$ downfield, <6 sec^{-1}; $PaCH_3(2)$ upfield, <18 sec^{-1}. For those protons which showed significant effects of the three enzyme preparations on their $1/T_1$ values, namely the H_2, H_8, and H_1' protons of adenosine, the 3 and 9 protons of pantetheine, and the methylene and methyl protons of the propionyl group, three simultaneous equations of the form of eq 3 were set up to solve for the paramagnetic and diamagnetic contributions. In all cases positive and self-consistent sets of values of $(1/fT_{1d})_{Zn}$, $(1/fT_{1p})_{Co}$, and $(1/fT_{1p})_{Cu}$ were found, which satisfied the measured $1/T_1$ value of each proton within its respective experimental error (Table IV). The effects on $1/T_1$ at the Zn site (Table IV) are all much larger than the diamagnetic effects observed in absence of the enzyme (Table I). By differentiating eq 4 with respect to τ_r and setting $d(1/T_1)d\tau_r = 0$ it may be shown that the maximal value of $1/T_1$ occurs at a $\tau_r = 0.3843/\omega_I = 6.11 \times 10^{-10}$ sec at 100 MHz. Using this value of τ_r and the range of interproton distances previously determined, maximal $1/fT_{1d}$ values of 10.1–5.7 sec^{-1} for the methyl group and 10.7–5.7 sec^{-1} for the methylene groups are calculated, in reasonable agreement with the observed values (Table IV). Hence the binding of propionyl-CoA to transcarboxylase may well have hindered the rotation of the propionyl side chain by more than an order of magnitude and the pantetheine methylene by a smaller factor (≥4). Similar effects were observed on the binding of substrate analogues to pyruvate kinase (Nowak and Mildvan, 1972a), although in the present case the τ_r values of the bound substrate are well below the tumbling time of transcarboxylase. From Stokes law, a value of $\tau_r = 5.9 \times 10^{-7}$ sec is calculated for a spherical protein of molecular weight 790000. For the methine protons of enzyme bound propionyl-CoA, the maximal calculated values for $1/fT_{1d}$ are 3.8–11.4 sec^{-1} for protons at a minimal distance of 2.26 Å. The measured values of 25–39 sec^{-1} (Table IV) are significantly greater, suggesting additional interactions possibly due to distant paramagnetic centers, and support the validity of subtracting the $1/fT_{1d}$ values observed at the Zn(II) site from those observed at the Co(II) and Cu(II)

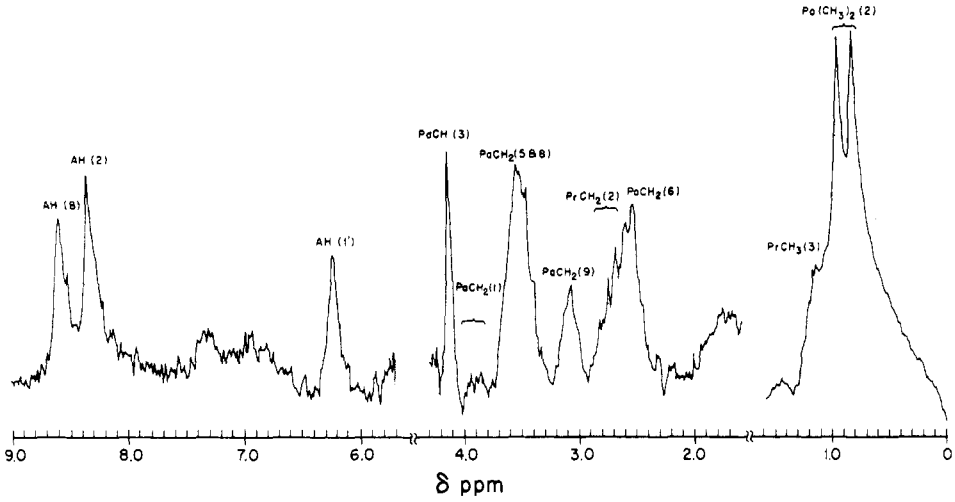

FIGURE 4: Fourier transform proton magnetic resonance spectrum of propionyl-CoA at 100 MHz in the presence of transcarboxylase. The system contained propionyl-CoA (10.0 m*M*) and TC-A (42.5 mg/ml) in 0.15 *M* potassium phosphate buffer (pH 6.62); temperature, 25°C. The spectrum shown is the result of 200 transients with a recycle time of 5.5 sec. For the tall methyl resonances between 0 and 1.6 ppm, the vertical gain was decreased by a factor of 0.40.

Table IV: The Contributions of the Transcarboxylase-Bound Co(II), Cu(II), and Zn(II) Sites to the Relaxation Rates of the Proton Resonances of Propionyl-CoA.[a]

Resonance	$(1/fT_{1p})_{Co}$	$(1/fT_{1p})_{Cu}$	$(1/fT_{1d})_{Zn}$	$(1/fT_{1p})_{Co} - (1/fT_{1d})_{Zn}$	$(1/fT_{1p})_{Cu} - (1/fT_{1d})_{Zn}$	%Error
AH(8)	106.7	92.1	33.9	72.8	58.2	9
AH(2)	104.9	57.5	25.0	79.9	32.5	10
AH(1')	86.7	52.3	34.3	52.4	18.0	3
PaCH(3)	54.3	55.6	39.0	15.4	16.6	5
$PaCH_2$(9)	46.4	83.8	9.7	36.7	74.1	4
$PrCH_2$(2)	114.1	40.3	9.6	104.5	30.7	10
$PrCH_3$(3)	32.4	7.6	14.9	17.5	<7.3	16

[a] $(1/fT_{1p})_{Co}$, $(1/fT_{1p})_{Cu}$, and $(1/fT_{1d})_{Zn}$ were calculated by simultaneous equations of the form of eq 3. The units of all of the relaxation rates are sec^{-1}.

sites for analysis of only the local paramagnetic effects.

Effect of Transcarboxylase-Bound Metals on the Relaxation Rates of the ^{31}P Nuclei of Propionyl-CoA in the Transcarboxylase-Propionyl-CoA Complex. Because of the difficulty in detecting small paramagnetic effects of an enzyme-bound metal on resonances which already have large relaxation rates in the unbound state, such as the methyl and methylene protons in the pantetheine portion of propionyl-CoA (Tables I and III) the effects of transcarboxylase on the ^{31}P relaxation rates were determined (Table II). When compared with $1/T_1$ relaxation rates of the ^{31}P nuclei of propionyl-CoA, there is no significant effect of any of the three transcarboxylase preparations on the ^{31}P (5′-β) resonance (Table II). Although the $1/T_1$ relaxation rates of the ^{31}P (5′-α) and ^{31}P (3′) signals are significantly increased when propionyl-CoA forms complexes with the enzyme, the magnitudes of the observed increases in the $1/T_1$ values with each of the three transcarboxylase preparations are very similar within experimental error (Table II, experiments II, III, and IV), despite large differences in the amounts of bound paramagnetic ions. Hence, these results indicate that transcarboxylase exerts only a diamagnetic effect on the ^{31}P (5′-α) and (3′) nuclei of propionyl-CoA. Similarly, the larger enzyme effects on the $1/T_2$ values of the phosphorus atoms of propionyl-CoA detected by line broadening (Figure 3c) and measured by pulse methods (Table II) do not correlate with the paramagnetic metal content of the three enzyme preparations, indicating predominantly diamagnetic effects on $1/T_2$. This view is supported by the agreement of the relaxation rates $1/fT_{1d}$ or $1/fT_{2d}$ for a given phosphorus atom in the three enzyme experiments when normalized by the ratio f = [total metal]/[propionyl-CoA]. The average $1/fT_{2d}$ values so determined are given in Table II.

Accordingly, τ_r values for the 3′-P and 5′-α-P of bound propionyl-CoA on the order of 10^{-8} sec were calculated (Table II) from the $1/fT_{1d}$ values, using eq 4 with appropriate values of r of 2.5 ± 0.1 Å and D of 55.11 Å $(sec)^{-1/3}$ and 61.85 Å $(sec)^{-1/3}$ for the dipolar interaction of phosphorus with 1 and 2 protons, respectively. Because in the limit of fast exchange, chemical shifts as well as dipolar effects contribute to the $1/fT_{2d}$ values (Swift and Connick, 1962) $1/fT_{2d}$ can be used only to estimate upper limits to τ_r. Such upper limits to τ_r were calculated from eq 5 (Solomon, 1955) which assumes only dipolar effects on $1/fT_{2d}$:

$$\frac{T_{1d}}{T_{2d}} = \frac{2\omega^4\tau_r^4 + 13\omega^2\tau_r^2 + 4}{8\omega^2\tau_r^2 + 5} \quad (5)$$

The upper limit values of τ_r from T_{1d}/T_{2d} are consistent with the absolute values of τ_r obtained from T_{1d} alone (Table II). The τ_r values of $\sim 10^{-8}$ sec indicate that the phosphoryl groups of enzyme-bound propionyl-CoA are more hindered in their motion by an order of magnitude than are the methylene and methyl protons of the bound

coenzyme ($\sim 6 \times 10^{-10}$ sec). The phosphoryl groups are not, however, totally immobilized, since the τ_r of the protein, as discussed above, is $\sim 6 \times 10^{-7}$ sec.

Calculation of Distances between the Paramagnetic Cations, Co(II) or Cu(II), and the Magnetic Nuclei (^1H and ^{31}P) in the Transcarboxylase–Propionyl-CoA Complexes. The distance between a paramagnetic metal ion and a magnetic nucleus may be calculated from the value of $1/fT_{1p}$ if the relaxation rate is not limited by the rate of chemical exchange ($1/\tau_M$). If the value of $1/fT_{1p}$ is exchange limited it may be used only to calculate an upper limit to the distance between the metal ion and the nucleus. As pointed out in detail elsewhere (Nowak and Mildvan, 1972b), when $1/fT_{1p}$ is much less than $1/fT_{2p}$, the former cannot be limited by chemical exchange, but is dominated by $1/T_{1M}$, the relaxation rate of a bound ligand. In the transcarboxylase–propionyl-CoA complex the AH(8), AH(2), AH(1′), PaCH(3), and two $PaCH_3(2)$ methyl resonances were chosen for $1/T_2$ measurements because these signals are singlets. The average maximum value of $1/f(T_{2p} + T_{2d})$ calculated from T_2 data (not shown) of the AH(2) and AH(1′) resonances in the presence of transcarboxylase is about 366 sec^{-1} which is 3.4 times greater than the largest value of $1/fT_{1p}$ (Table IV). Thus, $1/fT_{1p}$ contains a major contribution (≥71%) from $1/T_{1M}$, and may be used for distance calculations using the dipolar term of the Solomon–Bloembergen equation for paramagnetic effects on $1/T_1$ (Solomon and Bloembergen, 1956):

$$1/fT_{1p} = C^6/r^6[f(\tau_c)] \quad (6)$$

In eq 6 r is the metal ion–magnetic nucleus distance, C is a constant which depends on the spin state and average g value of the metal ion, and $f(\tau_c)$ is the correlation function which is given by:

$$f(\tau_c) = 3\tau_c/(1 + \omega_I^2\tau_c^2) + 7\tau_c/(1 + \omega_S^2\tau_c^2) \quad (6A)$$

In eq 6A 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 τ_c for the paramagnetic effect of Co(II) on propionyl-CoA in the transcarboxylase–propionyl-CoA complex is dominated by τ_S, the short electron spin relaxation time of high spin Co(II) (Fung et al., 1974). This value of τ_c was previously determined to be 2.2×10^{-12} sec from $1/fT_{1p}$ of the methyl protons of transcarboxylase bound pyruvate as a function of frequency (Fung et al., 1974). The value of C of 897 Å $(sec)^{-1/3}$ based on an electron spin quantum number $S = 3/2$ and an effective g value of 4.13 for high spin Co(II) (Fung et al., 1974) was used in eq 6 to calculate distances between bound Co(II) and the protons of bound propionyl-CoA. For those protons for which no paramagnetic effects of Co(II) were detected, within experimental error, lower limit distances ≥8.5 Å (Table V) were estimated from the error limits of the relaxation rates (Table III). Similar calculations using a C value of 664 Å $(sec)^{-1/3}$ for Co(II)–phosphorus interactions yield lower limit Co(II)–phosphorus distances ≥9.0 Å (Table V) from the error limits of the relaxation rates of phosphorus (Table II). All of the distances from enzyme-bound Co(II) to the protons and phosphorus atoms of bound propionyl-CoA (Table V) are too great for direct coordination of any portion of the propionyl-CoA molecule by the enzyme-bound Co(II). Interestingly, those protons closest to Co(II) are the reaction center propionyl methylene protons, one of which, the *pro-R* proton (Cheung et al., 1975) is removed during catalysis. Their distance from Co(II) of 6.5 ± 0.3 Å places them in the second or third coordination sphere of the enzyme-bound metal. The intervening ligands are probably not water molecules, as suggested by the lack of effect of propionyl-CoA at levels as great as 11.0 mM on $1/T_{1p}$ of water protons at 24.3 MHz in a titration of transcarboxylase (TC-B, 36.8 mg/ml) containing 102 μM Co(II), with propionyl-CoA.

Table V: Average Distances between Co(II) or Cu(II) and Nuclei of Propionyl-CoA in the Transcarboxylase–Propionyl-CoA Complex.[a]

Nucleus	Distances from Co(II) (Å)	Distances from Cu(II) (Å)
AH(8)	6.9 ± 0.3	8.6 ± 0.4
AH(2)	6.8 ± 0.3	8.0 ± 0.4
AH(1′)	7.2 ± 0.4	8.8 ± 0.5
PaCH(3)	8.3 ± 0.4	8.9 ± 0.4
$PaCH_2(9)$	7.7 ± 0.4	7.0 ± 0.4
$PrCH_2(2)$	6.5 ± 0.3	8.1 ± 0.4
$PrCH_3(3)$	8.7 ± 0.4	≥10.0
$PaCH_2(5)$	≥9.0	≥11.0
$PaCH_2(6)$	≥9.0	≥10.0
$PaCH_2(8)$	≥10.0	≥11.0
$PaCH_3(2)$ downfield	≥9.5	≥12.5
$PaCH_3(2)$ upfield	≥8.5	≥10.5
$^{31}P(5'\text{-}\alpha)$	≥9.0	≥10.5
$^{31}P(5'\text{-}\beta)$	≥10.0	≥11.5
$^{31}P(3')$	≥9.5	≥10.5

[a] Nuclei are defined in Figure 1.

For Cu(II)–proton distances on transcarboxylase a C value of 545 Å $(sec)^{-1/3}$ and a τ_c of 3.2×10^{-10} sec have previously been determined (Fung et al., 1974). For Cu(II)–phosphorus distances, the same τ_c value, the electron spin relaxation time of enzyme-bound Cu(II), and a C value of 403 Å $(sec)^{-1/3}$ adjusted for the gyromagnetic ratio of phosphorus were used (Mildvan and Engle, 1972).

The distance from enzyme-bound Cu(II) to the reaction center propionyl methylene protons of propionyl-CoA is 1.6 ± 0.5 Å greater than the corresponding distance from Co(II) (Table V). All of the other calculated distances from Cu(II) are also greater than those from Co(II) with the exception of PaCH(3) and $PaCH_2(9)$. As previously pointed out (Fung et al., 1974), the Cu(II) sites are probably inactive catalytically.

Conformation of Enzyme-Bound Propionyl-CoA. The distances from the bound Co(II) to the AH(8), AH(2), AH(1′), PaCH(3), $PaCH_2(9)$, $PrCH_2(2)$, and $PrCH_3(3)$ protons were used with a computer program to determine the optimum propionyl-CoA geometry, which satisfies the measured distances and which minimizes van der Waals overlap of the atoms of propionyl-CoA. A computer search among 46656 conformations of propionyl-CoA was carried out by simultaneous rotation of portions of the molecule about the N(9)–C(1′), C(5′)–O(5′), and C(1)–O(1) bonds in increments of 10° searching for those conformations with less than 0.5 Å total van der Waals overlap. We chose to rotate about these three bonds because rotation about the N(9)–C(1′) bond, the glycosidic linkage, would generate anti and syn adenine–ribose conformations and rotations around the C(5′)–O(5′) and C(1)–O(1) bonds of CoA would produce a large number of greatly different extended and folded conformations. The latter bonds were chosen for rotation over the pyrophosphate region because less variability may exist in pyrophosphate conformations on and

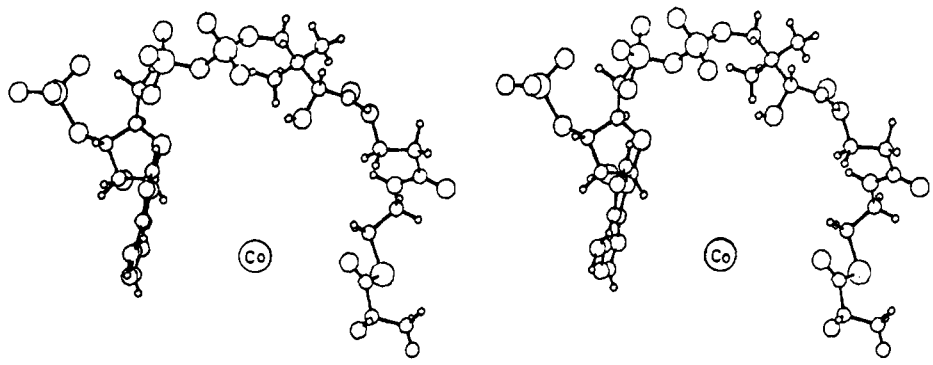

FIGURE 5: Conformation of propionyl-CoA at the active site of transcarboxylase which provides the best fit to the distances from Co(II). This conformation resulted from a computer search, and was drawn as a stereo pair by a CALCOMP plotter. The Co(II) atom is 4.54 Å above the plane defined by the adenine H(2), the pantetheine C-9, and the propionyl methyl carbon.

off enzymes (Webb et al., 1973). From the 46656 rotamers examined, the computer generated 21 conformations which were consistent with the seven Co(II)–proton distances of propionyl-CoA and which had a van der Waals overlap of ≤0.5 Å. The three-dimensional molecular structures for these 21 solutions were displayed and printed out stereoscopically. All showed a partially unfolded U shape about Co(II). Nineteen of these 21 conformations for the transcarboxylase-bound propionyl-CoA showed an anti adenine–ribose conformation. Only two conformations had a syn adenine–ribose relationship. These two indistinguishable syn conformations which differed by a total of only 0.0014 Å in their atomic coordinates could be ruled out since they required distances which were less than four of the eight lower limit distances from Co(II) to propionyl-CoA with a total deviation of 3.81 Å. By the same criteria, two anti structures with total deviations from lower limit distances to four atoms of 4.43 and 4.63 Å could be ruled out. The remaining 17 structures differed little in gross conformation, but could be ranked by tabulating the total deviation of the calculated distances from three of the observed lower limit distances, namely $PaCH_2(5)$, 5′-α-P, and 5′-β-P. Two "best fit" conformations differing in atomic coordinates by only 0.006 Å minimized these three deviations, hence the total deviation from the lower limit distances to a value of 3.38 Å. The mean deviation of these conformations from the eight lower limit distances is 3.38 Å/8 or 0.423 Å. One of these two indistinguishable "best fit" structures is shown in Figure 5.[2] The remaining 15 structures fell into six groups with total deviations from the 15 measured distances ranging from 3.73 to 4.94 Å.

The best-fit structure shows a partially unfolded U shape about Co(II), as do all of the structures that fit the seven absolute distances, and an anti adenine–ribose conformation (Figure 5). The Co(II) atom lies 4.54 Å above the plane defined by the adenine H(2), the pantetheine C-9, and the propionyl methylene carbon. The distance from the adenine H(2) proton to the propionyl methylene carbon, a measure of the extent of unfolding, is 9.9 Å as calculated from the atomic coordinates. In a completely folded structure this distance would be 3.2 Å while in a fully extended structure this distance would be 34 Å. The extent of unfolding of propionyl-CoA at the active Co(II) site of transcarboxylase, from the ratio $(9.9 - 3.2)/(34 - 3.2)$, is ~22%.

At the catalytically inactive Cu(II) site, where most of the metal–nuclei distances are greater by 1–2 Å, a somewhat more open conformation is suggested by model building. However, the local conformations of individual portions of propionyl-CoA at the Cu(II) site are indistinguishable from those at the Co(II) site.

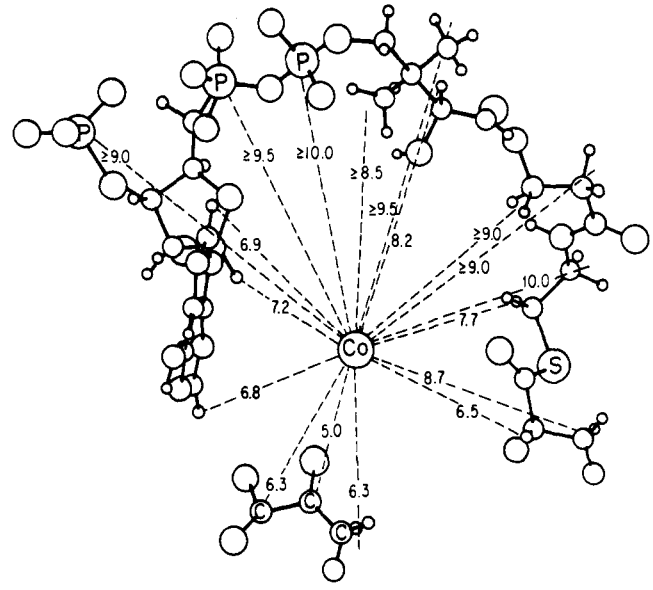

FIGURE 6: Arrangement of the substrates at the active site of transcarboxylase. The positions and conformations of the substrates are based on the indicated distances from enzyme-bound Co(II) to the protons and phosphorus atoms of propionyl-CoA (Table V) and to the protons and carbon atoms of pyruvate (Fung et al., 1974).

A computer search as described above yielded a pair of indistinguishable best-fit conformations[2] which fit the six distances from Cu(II) (Table V) with a van der Waals overlap of ~0.5 Å but which deviated from five of the nine lower limit distances by a total of 13.6 Å. The mean deviation from the lower limit distances for these conformations is 1.51 Å. Poorer fits of the Cu(II)–pyruvate distances than the Co(II)–pyruvate distances on transcarboxylase have previously been ascribed to variability in the position of Cu(II) (Fung et al., 1974). The best-fit conformation of propionyl-CoA at the Cu(II) site is 37% unfolded from the distance between the adenine H(2) and the propionyl methylene carbon of 14.6 Å.

At the catalytically active Co(II) site, in the best-fit structure (Figures 5 and 6) no atoms of bound propionyl-CoA are close enough to the metal for direct coordination. Atoms which are closest to Co(II) are the 6-amino nitrogen of adenine (5.29 Å), the adenine ring nitrogens (5.61–6.48

[2] Atomic coordinates will be provided on request.

Å), and the thioester oxygen (5.64 Å), all of which may be considered to be in the second or third coordination sphere of Co(II). At greater distances are the reaction center propionyl methylene carbon (6.86 Å), and the thioester sulfur (8.22 Å). Hence, as with pyruvate (Fung et al., 1974) (Figure 6), activation of propionyl-CoA by the metal, if occurring, must take place through intervening ligands. All of the measured distances from Co(II) to the substrate propionyl-CoA and pyruvate (Fung et al., 1974) are accommodated in the active site structure of Figure 6. As previously pointed out, transcarboxylase has ~6 biotins per mole (Fung et al., 1974) but ~12 divalent cations per mole (Fung et al., 1974) and pyruvate is known to interact with the active site metal ions to form a second sphere complex. Hence it remains to be established whether propionyl-CoA and pyruvate are near the same metal ion. If this were so, as depicted in Figure 6, distances of only ~10 Å are predicted between the propionyl group of bound propionyl-CoA and the protons and carbon atoms of bound pyruvate between which the carboxybiotin ring must migrate during catalysis. The next paper in this series, which uses a paramagnetic ester of CoA to determine the intersubstrate distance on transcarboxylase (Fung et al., 1976), shows this prediction to be correct.

Acknowledgments

We are grateful to Dr. R. K. Gupta, Miss B. Peticolas, Dr. G. McDonald, and Mr. J. Simmons for help with NMR instrumentation, to Dr. E. Melamud and Mr. R. Marc Oesterling for help with the computer analysis, to Dr. G. J. Williams of the Brookhaven National Laboratory for a survey of interproton distances from neutron diffraction data, and to Mr. S. H. Millmond for preparation of the propionibacteria.

References

Branden, C. I., Eklund, H., Nordstrom, B., Boiwe, T., Soderlund, G., Zeppezauer, E., Ohlsson, I., and Akeson, A. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2439.

Carr, H. Y., and Purcell, E. M. (1954), *Phys. Rev. 94,* 630.

Chandrasekhar, K., McPherson, A., Jr., Adams, M. J., and Rossman, M. G. (1973) *J. Mol. Biol. 76,* 503.

Cheung, Y. F., Fung, C. H., and Walsh, C. (1975), *Biochemistry 14,* 2981.

Dideberg, O., and Dupont, L. (1972), *Acta Crystallogr., Sect. B 28,* 3014.

Fung, C. H. (1972), Ph.D. Thesis, Case Western Reserve University.

Fung, C. H., Gupta, R. K., and Mildvan, A. S. (1976), *Biochemistry,* following paper in this issue.

Fung, C. H., Mildvan, A. S., and Leigh, J. S., Jr. (1974), *Biochemistry 13,* 1160.

Furie, B., Griffen, J. H., Feldmann, R. J., Sokoloski, A., and Schechter, A. N. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2833.

Jardetzky, O., and Wade-Jardetzky, N. G. (1966), *J. Biol. Chem. 241,* 85.

Kartha, G., and de Vries, A. (1961), *Nature (London) 192,* 862.

Kraut, J., and Jensen, L. H. (1963), *Acta Crystallogr. 16,* 79.

Lee, C., Eichner, R. D., and Kaplan, N. O. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1593.

Lee, C.-H., and Sarma, R. H. (1974), *FEBS Lett. 43,* 271.

Lee, C.-H., and Sarma, R. H. (1975), *J. Am. Chem. Soc. 97,* 1225.

Lehmann, M. S., Koetzle, T. F., and Hamilton, W. C. (1972) *J. Am. Chem. Soc. 94,* 2657.

McDonald, G. G., and Leigh, J. S., Jr. (1973), *J. Magn. Resonance 9,* 358.

Mieyal, J. J., Webster, L. T., and Siddiqui, U. A. (1974), *J. Biol. Chem. 249,* 2633.

Mildvan, A. S., and Engle, J. L. (1972), *Methods Enzymol. 26C,* 654.

Mildvan, A. S., Fung, C. H., and Feldmann, R. J. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 690 (Abs.).

Moss, J., and Lane, M. D. (1971), *Adv. Enzymol. Relat. Areas Mol. Biol. 35,* 321.

Northrop, D. B. (1969), *J. Biol. Chem. 244,* 5808.

Nowak, T., and Mildvan, A. S. (1970), *J. Biol. Chem. 245,* 6057.

Nowak, T., and Mildvan, A. S. (1972a), *Biochemistry 11,* 2813.

Nowak, T., and Mildvan, A. S. (1972b), *Biochemistry 11,* 2819.

Oppenheimer, N. J., Arnold, L. J., and Kaplan, N. O. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 3200.

Pauling, L. (1960), The Nature of the Chemical Bond, 3rd ed, Ithaca, N.Y., Cornell University Press, p 257.

Sloan, D. L., and Mildvan, A. S. (1974), *Biochemistry 13,* 1711.

Solomon, I. (1955), *Phys. Rev. 99,* 559.

Solomon, I., and Bloembergen, N. (1956), *J. Chem. Phys. 25,* 261.

Swift, T. J., and Connick, R. E. (1962), *J. Chem. Phys. 37,* 307.

Van der Helm, D., Glusker, J. P., Johnson, C. K., Minkin, J. A., Burow, N. E., and Patterson, A. L. (1968), *Acta Crystallogr., Sect. B 24,* 578.

Velick, S. F. (1961), in Light and Life, McElroy, W. D., and Glass, B., Ed., Baltimore, Md., John Hopkins Press, p 108.

Verbist, J. F., Lehmann, M. H., Koetzle, T. F., and Hamilton, W. C. (1972), *Acta Crystallogr. Sect. B 28,* 3006.

Warburg, O., and Christian, W. (1957), *Methods Enzymol. 3,* 451.

Webb, L. E., Hill, E. J., and Banaszak, L. J. (1973), *Biochemistry 12,* 5101.

Werbelow, L. G., and Marshall, A. G. (1973), *J. Am. Chem. Soc. 95,* 5132.

Wood, H. G., Jacobson, B., Gerwin, B. I., and Northrop, D. B. (1969), *Methods Enzymol. 13,* 215.

Wood, H. G., and Utter, M. F. (1965), in Essays in Biochemistry, Vol. I, Campbell, P. N., and Greville, G. D., Ed., New York, N.Y., Academic Press, p 1.

Wright, D. A., and Marsh, R. E. (1962), *Acta Crystallogr. 15,* 54.