Frequency and Temperature Dependence of the Proton Relaxation Rates of Solvent and Substrate Interaction with Isocitrate Dehydrogenase Bound Mn(II)†

Joseph J. Villafranca* and Roberta F. Colman

ABSTRACT: The longitudinal ($1/pT_{1p}$) and transverse ($1/pT_{2p}$) proton relaxation rates of water have been measured as a function of frequency (6–48 MHz) and temperature (3–35°) for three complexes of TPN-dependent isocitrate dehydrogenase isolated from pig heart. All three complexes, namely, the binary isocitrate dehydrogenase–Mn(II) complex and the ternary isocitrate dehydrogenase–Mn(II)–α-ketoglutarate and isocitrate dehydrogenase–Mn(II)–isocitrate complexes, showed a maximum in their respective $1/pT_{1p}$ values between 6 and 48 MHz. The explanation for this observation is that the correlation time which modulates the electron-nuclear dipolar interaction is in part due to the electron spin relaxation time. At 48 MHz the rate of rotation of the macromolecular complex is approximately equal to the electron spin relaxation rate and was included in the analysis of the water relaxation rates. An analysis of the number of rapidly exchanging water molecules in the primary coordination sphere of isocitrate dehydrogenase bound Mn(II) led to the conclusion that there were two water molecules in the binary isocitrate dehydrogenase–Mn(II) complex. For both of the ternary complexes (with α-ketoglutarate or isocitrate) the number of water molecules was reduced to ~1.0 at 25°. These data strongly indicate that one water coordination site on isocitrate dehydrogenase bound Mn(II) is displaced upon formation of the ternary complexes as suggested earlier by Villafranca and Colman ((1972), *J. Biol. Chem. 247*, 209). Isocitrate dehydrogenase enhances the effect of Mn(II) on the $1/T_1$ and $1/T_2$ proton relaxation rates of α-ketoglutarate-*3,3-d*$_2$ at 100 MHz. The correlation time for this interaction at 100 MHz is the rotation time (2×10^{-8} sec at 22°) of the complex. These data were used in the Solomon–Bloembergen equation for $1/T_1$ to calculate a Mn(II) to $-CH_2-$ (carbon 4) distance of 6.3 ± 0.2 Å. One possible complex consistent with this distance, and the fact that one water molecule is displaced on the isocitrate dehydrogenase bound Mn(II), involves coordination of the keto moiety of α-ketoglutarate in the ternary complex. The role of the metal ion in this reaction could therefore be to stabilize an enolate intermediate during the decarboxylation of oxalosuccinate to form α-ketoglutarate. Distances between Mn(II) and the protons of isocitrate were obtained at 220 MHz for the binary complex and implicate bidentate coordination. Since this binary complex is the kinetically significant form of the substrate for the dehydrogenation reaction (Colman, R. F. (1972), *J. Biol. Chem. 247*, 215), these data are consistent with the aforementioned conclusion that formation of the ternary isocitrate dehydrogenase–Mn(II)–isocitrate complex from the binary isocitrate dehydrogenase–Mn(II) complex involves displacement of a water ligand.

The ability of native isocitrate dehydrogenase to bind Mn(II) was established by Villafranca and Colman (1972) using several techniques. A dissociation constant of 45 μM was obtained for the enzyme–Mn(II) complex. This value was unchanged in the presence of TPN^+ or TPNH, α-ketoglutarate, or bicarbonate. In contrast, the dissociation constant is decreased to 2 μM in the presence of isocitrate. The nuclear magnetic resonance (nmr) (Villafranca and Colman, 1972) and kinetic (Colman, 1972) data therefore suggested that a ternary enzyme–Mn(II)–isocitrate complex forms. The exact nature of the complex of isocitrate dehydrogenase (ICDH)–Mn(II) with its substrates, however, is not known and an investigation by nmr techniques was continued to determine the nature of metal ion and substrate sites on the enzyme.

$$\text{ICDH} + \text{Mn(II)} \rightleftharpoons \text{ICDH–Mn(II)} \quad (1)$$

$$\text{ICDH} + \text{isocitrate–Mn(II)} \rightleftharpoons \text{ICDH}\begin{matrix}\diagup \text{isocitrate} \\ \\ \diagdown \text{Mn(II)}\end{matrix} \quad (2)$$

Experimental Section

Materials. Pig heart TPN-dependent isocitrate dehydrogenase was purified as previously described (Colman, 1968). In all calculations the molecular weight is assumed to 58,000. All coenzymes and substrates were purchased from Sigma Chemical Co. The buffer used to dissolve the enzyme was 0.1 M TEA-Cl[1] (pH 7.5) containing 0.1 M Na_2SO_4 and 10% glycerol.

Mn(II) Binding Experiments by Epr. The concentration of free Mn(II) was determined from the peak heights of the epr spectra obtained on a JEOL epr spectrometer equipped with a variable-temperature attachment; 35-μl samples were placed in quartz capillaries and allowed to come to thermal equilibrium for 5 min prior to measurement of the signal. The temperature was maintained to ±1°.

Measurements of Prr of Water and Substrate Protons. The longitudinal, $1/T_1$, and transverse, $1/T_2$, relaxation rates of

† From the Departments of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802 (J. J. V.), and the University of Delaware, Newark, Delaware 19711 (R. F. C.). *Received November 13, 1973.* This is paper III in the series entitled "Role of Metal Ions in Reaction Catalyzed by Pig Heart Triphosphopyridine Nucleotide-Dependent Isocitrate Dehydrogenase." This work was supported by NSF Grant No. GB 34291, USPHS Grant No. 5R01-AM 17552 and a grant from the Research Corporation.

[1] Abbreviations used are: nmr, nuclear magnetic resonance; epr, electron paramagnetic resonance; prr, proton relaxation rate; TEA, triethanolamine.

solvent protons were measured on an NMR-Specialties variable-frequency pulsed nmr spectrometer. Measurements were made from 6 to 48 MHz andf rom 3 to 35°. Relaxation rates of substrate protons were measured at 100 MHz on a Varian XL-100-15 spectrometer and a Fourier Transform JEOL-PS-100-FT pulsed spectrometer. We wish to thank Dr. A. S. Mildvan for the use of his Varian spectrometer. Longitudinal relaxation rates were measured using a 180°-τ-90° pulse sequence (with the exception of data taken on the Varian spectrometer when the progressive saturation technique was used (Mildvan and Cohn, 1970). Transverse relaxation rates were measured for substrate protons by measuring the width at half-height and for solvent protons by the Meiboom–Gill (1958) modification of a Carr-Pursell pulse train 90° (x'), τ, 180° (y'), τ, (echo), τ, 180° (y'), τ, (echo), τ, etc.

The paramagnetic contribution to the observed relaxation rate of solvent protons, $1/T_{1p}$, is obtained by subtracting the $1/T_1$ value for a solution of enzyme and buffer in the absence of Mn(II) from the $1/T_1$ value of a solution of buffer, enzyme, and Mn(II). The amount of free Mn(II) can be obtained for each solution at each temperature from an epr measurement as described above. The contribution due to bound Mn(II), $(1/T_{1p})_b$, is calculated from eq 3 and 4, where subscripts b, f, and t

$$1/T_{1p} = \frac{[Mn]_f}{[Mn]_t T_{1p}(h)} + \frac{[ICDH\text{–}Mn]}{[Mn]_t T_{1p}(i)} \quad (3)$$

$$(1/T_{1p})_b = n/(T_{1m} + \tau_m) \quad (4)$$

refer to bound, free, and total Mn(II), h and i to the relaxation rate of the free and bound Mn(II) complexes ($1/T_{1p}$(i) becomes equal to $(1/T_{1p})_b$ therefore), n is the number of water molecules interacting with bound Mn(II), T_{1m} is the relaxation time of water molecules in the first coordination sphere, and τ_m is their residence time. When substrates are added eq 3 becomes

$$1/T_{1p} = \frac{[Mn]_f}{[Mn]_t T_{1p}(h)} + \frac{[ICDH\text{–}Mn]}{[Mn]_t T_{1p}(i)} + \frac{[Mn\text{–}S]}{[Mn]_t T_{1p}(j)} + \frac{[ICDH\text{–}Mn\text{–}S]}{[Mn]_t T_{1p}(k)} \quad (5)$$

and the $(1/T_{1p})_b$ value corresponding to $1/T_{1p}$(k) is used to calculate n and τ_m for the ternary complex of enzyme, Mn(II), and substrate. Analogous equations are used to calculate the value of $1/T_{2p}$ for the various bound species. For Mn(II) bound to proteins the chemical shift mechanism for $1/T_{2p}$ discussed by Swift and Connick (1962, 1964) does not apply and the results in this paper provide more evidence for justifying the use of equations similar to 3 and 4 for $1/T_{2p}$.

In eq 4 and 6, the $(1/T_{1p})_b$ and $(1/T_{2p})_b$ are related to (1) the longitudinal $(1/T_{1m})$ and transverse $(1/T_{2m})$ relaxation rates of solvent (or substrate) protons in a paramagnetic complex, (2) the residence time of the molecule, τ_m, and (3) the number of molecules interacting with the bound metal ion, n.

$$(1/T_{2p})_b = n/(T_{2m} + \tau_m) \quad (6)$$

The Solomon–Bloembergen (1955, 1956) equations for the electron-nuclear dipole–dipole interaction of $1/T_{1m}$ and $1/T_{2m}$ are

$$1/T_{1m} = C\left[\frac{3\tau_c}{1 + \omega_I^2\tau_c^2} + \frac{7\tau_c}{1 + \omega_s^2\tau_c^2}\right] \quad (7)$$

$$1/T_{2m} = C\left[2\tau_c + \frac{1.5\tau_c}{1 + \omega_I^2\tau_c^2} + \frac{6.5\tau_c}{1 + \omega_s^2\tau_c^2}\right] \quad (8)$$

where ω_I and ω_s are the nuclear and electronic Larmor frequencies respectively; C is $(2/15)S(S + 1)\gamma_I^2 g^2\beta^2 r^{-6}$ where S is the electronic spin angular momentum in h units, g is the electronic g value (approximated by 2.0), γ_I is the nuclear gyromagnetic ratio, β is the Bohr magneton, and r is the metal ion–nuclear distance. In eq 7 and 8 the hyperfine term has been neglected and this simplification will be justified later.

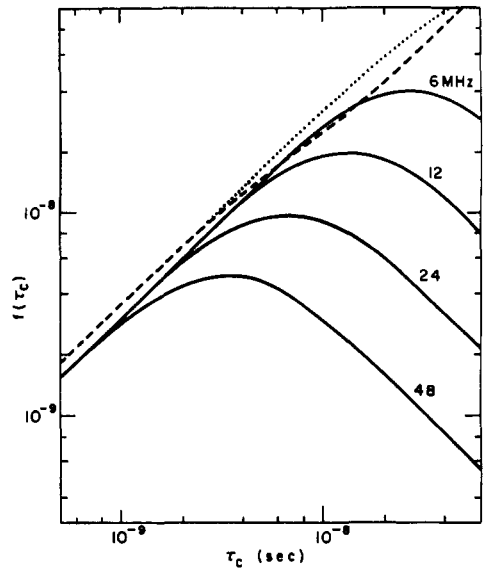


FIGURE 1: Function of τ_c *vs.* the correlation time. Solid lines represent the curves for the function in brackets in eq 7 at values of 6, 12, 24, and 48 MHz. The broken lines represent the curves for the function in brackets in eq 8 at 6 MHz (····) and 24 MHz (----). The function for the solid curves is proportional to $1/T_{1m}$ and for the broken lines is proportional to $1/T_{2m}$.

A common correlation time, τ_c, which is the time constant for reorientation of the interacting nuclear and electronic spins is given by

$$\tau_c^{-1} = \tau_m^{-1} + \tau_r^{-1} + \tau_s^{-1} \quad (9)$$

where τ_r is the time for random reorientation of the tumbling macromolecular complex. τ_s is the electron spin relaxation time which should be the longitudinal electron spin relaxation time for eq 7 and the transverse electron spin relaxation time for eq 8 (Reuben *et al.*, 1970). We are assuming that these relaxation times are equal for the macromolecular complex since they are equal for hydrated Mn(II) (Bloembergen and Morgan, 1961).

It is necessary for an evaluation of the pertinent correlation time for eq 7 and 8 to study the effect of frequency on the $1/T_{1p}$ and $1/T_{2p}$. Figure 1 shows a plot of τ_c *vs.* the functions in brackets on the right-hand side of eq 7 and 8 for some of the frequencies used in this study.

For a value of τ_c of 10^{-9} sec, the $1/T_{1p}$ would be equal for frequencies in the range of 6–48 MHz and the correlation time could only be estimated to within one order of magnitude. For a correlation time of 10^{-8} sec, the $1/T_{1p}$ values will be different in the frequency range of 6–48 MHz and the T_{1p}/T_{2p} ratio will be different for each frequency. Under these conditions a good estimation of the correlation time can be obtained from a replot of T_{1p} *vs.* frequency. Under these conditions eq 7 and 8 reduce to

$$1/T_{1m} = C\left[\frac{3\tau_c}{1 + \omega_I^2\tau_c^2}\right] \quad (10)$$

$$1/T_{2m} = C\left[2\tau_c + \frac{1.5\tau_c}{1 + \omega_I^2\tau_c^2}\right] \quad (11)$$

Anticipating the analysis of the prr of Mn(II) bound to isocitrate dehydrogenase the $1/T_{1p}$ data are frequency depen-

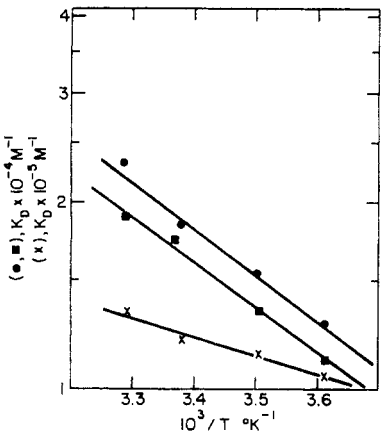


FIGURE 2: Effect of temperature on the dissociation constants of complexes involving isocitrate dehydrogenase, Mn(II), and substrates: (◑) isocitrate dehydrogenase–Mn(II) complex; (■) isocitrate dehydrogenase–Mn(II)–α-ketogluarate; and (x) isocitrate dehydrogenase–Mn(II)–isocitrate. The composition of each sample can be found in the legend to Figures 3, 6, and 7.

dent but the data at 6 MHz are lower than the 12-MHz data. This situation could only arise if the correlation time itself was frequency dependent. Only the electron spin relaxation time, τ_s, can be frequency dependent.

For the analysis of the isocitrate dehydrogenase data, both τ_s and τ_r are of the same order of magnitude in the 6–48-MHz range which makes this analysis much more complex than the analysis of pyruvate kinase by Reuben and Cohn (1970). A brief outline of the data analysis will be given here and a complete analytical solution to the problem will be given elsewhere (J. J. Villafranca and R. Viola, manuscript in preparation).

The rotational time, τ_r, can be reasonably approximated by a Stokes law calculation (eq 12) where M is the molecular

$$\tau_r = (M)\bar{v}\eta/RT \tag{12}$$

weight of the macromolecule $\bar{v}$ is the partial specific volume (0.744 was used), η is the viscosity of the solution, R is the gas constant, and T is the temperature in degrees Kelvin.

According to Bloembergen and Morgan (1961) the electron spin relaxation rate is given by

$$1/\tau_s = B\left[\frac{\tau_v}{1 + \omega_s^2\tau_v^2} + \frac{4\tau_v}{1 + 4\omega_s^2\tau_v^2}\right] \tag{13}$$

where $B = 12C^2/5S(S + 1)\hbar^2$. C is a constant defined by Bloembergen and Morgan (1961) and τ_v is the time constant for symmetry distortions of the complex. Equation 13 can be approximated by

$$1/\tau_s = 5B\tau_v/(1 + 2.5\omega_s^2\tau_v^2) \tag{14}$$

Using $\tau_c^{-1} = \tau_r^{-1} + \tau_s^{-1}$ and substituting the right-hand side of eq 14 for τ_s^{-1} gives

$$\tau_c = \frac{\tau_r + 2.5\omega_s^2\tau_v^2\tau_r}{1 + 2.5\omega_s^2\tau_v^2 + 5B\tau_v\tau_r} \tag{15}$$

When eq 15 is substituted into eq 10 the expression is very complex. However, using a combination of eq 15 and 10 and the same approach as outlined in the appendix of the paper by Reuben and Cohn (1970), initial estimates of τ_v, B, and C can be obtained. The final expression which one is manipulating to get these estimates was solved by a computer program written by Springer and deMaine (1972) followed by iterations of τ_v, B, and C to give the least error between the calculated $(1/T_{1p})_b$ and the actual values. We are gratefully indebted to Dr. deMaine of the Computer Science Department of The Pennsylvania State University for aid in using his program.

This procedure outlined above was used to analyze the $(1/T_{1p})_b$ data at all temperatures with the assumption that τ_v and τ_r obey exponential relationships of the form

$$\tau = \tau^0 \exp(E_a/RT) \tag{16}$$

Once τ_c was determined, T_{1m} and T_{2m} were calculated from eq 10 and 11 and these values were used in eq 4 and 6 to determine values of n and τ_m. This procedure works best, however, when the T_{1p}/T_{2p} ratio is large which is the case for 12, 24, 35, and 48 MHz in our analysis. It was assumed that τ_m obeyed Eyrings equation for a chemical rate process

$$1/\tau_m = (kT/h)\exp\left[-\frac{\Delta H^{\ddagger}}{RT} + \frac{\Delta S^{\ddagger}}{R}\right] \tag{17}$$

The omission of the hyperfine term from eq 7 and 8 (the form of this term can be found in Mildvan and Cohn (1970)) is justified for our data analysis since it predicts that at the lowest frequency which we used (6 MHz), T_{1p}/T_{2p} should be greater than 7/6. Within our experimental error this was not the case and the hyperfine contribution to the relaxation rates can be neglected.

Results and Discussion

Apparent Dissociation Constants for Isocitrate Dehydrogenase Complexes. In order to determine the amount of Mn(II) distributed among all the species present in solutions containing isocitrate dehydrogenase, Mn(II), and substrates (at each temperature), an epr experiment was performed using samples identical with the ones used for the prr determinations. From this epr experiment the amount of free Mn(II) could be measured as described in Methods.

With the binary isocitrate dehydrogenase–Mn(II) system the buffer does not appreciably complex Mn(II), so the apparent dissociation constant (at the same range of temperatures which were used for the prr study) was determined directly. These results are given in Figure 2.

For the solutions which contained isocitrate dehydrogenase, Mn(II), and either isocitrate or α-ketoglutarate the analysis was not straightforward. Published dissociation constants for binary isocitrate dehydrogenase–substrate complexes (Colman, 1969), the binary isocitrate dehydrogenase–Mn(II) complex determined above, ternary isocitrate dehydrogenase–Mn(II)–substrate complexes (Villafranca and Colman, 1972), and binary Mn(II)–substrate complexes (Villafranca and Colman, 1972, and references therein) were used at 25°. The temperature dependence of the dissociation constant for the binary Mn(II)–substrate complexes was determined since this had not been reported and these data in combination with the temperature dependence of the dissociation constant for the isocitrate dehydrogenase–Mn(II) complex were used to analyze the dissociation constant of Mn(II) from the ternary isocitrate dehydrogenase–Mn(II)–substrate complexes. The epr technique was used to determine the amount of free Mn(II) at each temperature.

In Figure 2 plots of K_D for the ternary complexes involving α-ketoglutarate and isocitrate are given as a function of $1/T$. The presence of α-ketoglutarate does not tighten the binding of Mn(II) appreciably but the presence of isocitrate does. This confirms and expands the previous results of Villafranca and Colman (1972).

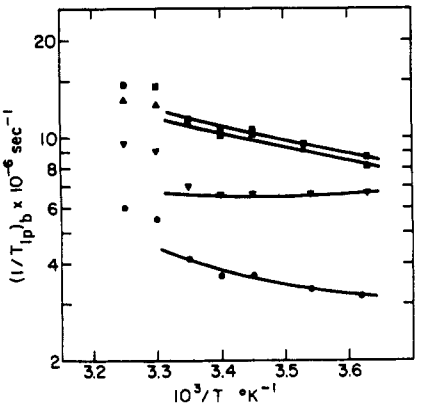


FIGURE 3: The longitudinal prr, $(1/T_{1p})_b$, of solvent due to the isocitrate dehydrogenase–Mn(II) complex as a function of the reciprocal absolute temperature. Solutions of isocitrate dehydrogenase (0.121 mM) and $MnCl_2$ (0.061 mM) in 0.1 M TEA-Cl (pH 7.5) containing 0.1 M Na_2SO_4 and 10% glycerol. The various frequencies used are (▼) 6 MHz, (▲) 12 MHz, (■) 24 MHz, and (●) 48 MHz.

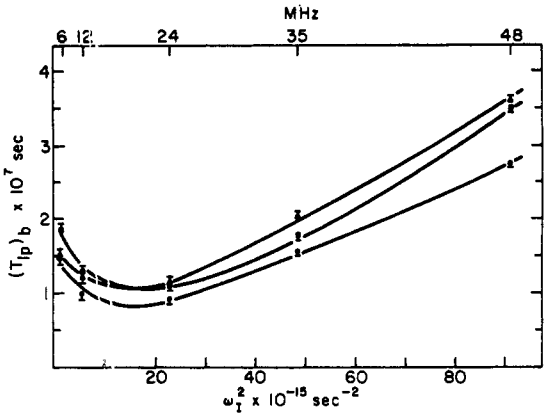


FIGURE 4: The longitudinal proton relaxation time $(T_{1p})_b$ of various complexes of isocitrate dehydrogenase plotted against the square of the proton Larmor precession frequency. All data are at 21°: (●) isocitrate dehydrogenase–Mn(II); (■) isocitrate dehydrogenase–Mn(II)–α-ketoglutarate; (▲) isocitrate dehydrogenase–Mn(II)–isocitrate. Error bars for each frequency are listed as half flags for clarity.

Temperature and Frequency Dependence of Prr of Solvent for Isocitrate Dehydrogenase–Mn(II). The $(1/T_{1p})_b$ for solutions containing isocitrate dehydrogenase and Mn(II) was determined in the temperature range 3–35° and at 6, 12, 24, and 48 MHz (Figure 3). Data in a more limited temperature range (15–25°) were taken at 35 MHz and are not shown in Figure 3. As mentioned in Methods, the $(1/T_{1p})_b$ was calculated using eq 3 for each temperature. The data in Figure 2 were used to calculate the amount of free and complexed Mn(II) in the temperature range of the prr study.

The values of $(1/T_{1p})_b$ increase at 12, 24, and 48 MHz but decrease slightly or have a zero slope at 6 MHz as the temperature increases. This behavior can occur for two reasons. Case 1, if the rate of exchange of water molecules, τ_m, is of the same order of magnitude as T_{1m} in eq 4. $1/\tau_m$ always has a positive temperature dependence and this effect would be largest at the highest values of $(1/T_{1p})_b$. This trend is observed at 24 MHz. Case 2, if the correlation time was $\sim 10^{-8}$ sec then from Figure 1 one can see that the temperature dependence of $1/T_{1m}$ would have a negative slope for 24 and 48 MHz, a positive slope for 6 MHz, and go through a maximum for 12 MHz. All of these features are found except the maximum at 12 MHz and as a first approximation one cannot decide between cases 1 and 2. The 6-MHz data could be at a maximum if the slope in Figure 3 is considered zero.

The second feature of the $(1/T_{1p})_b$ data is that the values at 12 and 24 MHz are similar and that the value at 6 MHz is lower than predicted from Figure 1. This means that the correlation time is at least in part frequency dependent and therefore must have a contribution from τ_s. This is seen more clearly in Figure 4 where the data are plotted as $(T_{1p})_b$ *vs.* ω_I^2. This plot represents a rearrangement of eq 10.

The third feature of the data in Figure 3 is the discontinuity in the $(1/T_{1p})_b$ values at the two highest temperatures. This feature of the data is reproducible and does not result from denaturation of the enzyme as determined from a measurement of enzymic activity after the prr experiments. One possible explanation of the higher values of $(1/T_{1p})_b$ is that they reflect another form of isocitrate dehydrogenase which is apparent only at the higher temperatures; although this is the first time that evidence for such a form has been presented. Pyruvate kinase is an example of a protein which shows a temperature dependent transformation between two forms as shown by prr of solvent protons (Reuben and Cohn, 1970) and by epr of bound Mn(II) (Reed and Cohn, 1973). The data at the two highest temperatures were neglected in the analysis of the prr data for the binary isocitrate dehydrogenase–Mn(II) complex.

The values of B, τ_v, τ_s, τ_r, and T_{1m} were determined as outlined in the Experimental Section. In Table I are listed the values of B and τ_v which give the best fit to the data. The value of B (which is a constant containing terms in S and the zero field splitting of Mn(II) (Bloembergen and Morgan, 1961)) is not appreciably different from the value for free Mn(II), but the value of τ_v (the time constant for solvent impact on the Mn(II) complex) is about seven times longer. The activation energy for τ_v is about 1.6 kcal/mol as expected for the solvent impact process. It must be pointed out, however, that the use

TABLE I: Constants for Water in the First Sphere of Mn(II).

Constants	Mn(II)[a]	ICDH–Mn(II)[c]	ICDH–Mn(II)–Isocitrate	ICDH–Mn(II)–α-Ketoglutarate[b]
B (rad/sec)^2	0.10×10^{20}	$(0.11 \pm 0.005) \times 10^{20}$	$(0.12 \pm 0.01) \times 10^{20}$	$(0.13 \pm 0.01) \times 10^{20}$
τ_v, sec (21°)	2.1×10^{-12}	15.0×10^{-12}	6.5×10^{-12}	8.0×10^{-12}
E_v, kcal/mol	2.0	1.55 ± 0.05	1.90 ± 0.10	0.74×0.10
τ_m, sec (21°)	3.0×10^{-7}	$(1.0 \pm 0.2) \times 10^{-7}$	$(0.6 \pm 0.1) \times 10^{-7}$	$(0.43 \pm 0.10) \times 10^{-7}$
E_m, kcal/mol	13.5	3.7 ± 0.3	4.75 ± 0.50	4.0 ± 0.4
n	6	2.05 ± 0.10	0.91 ± 0.08	1.3 ± 0.1

[a] From Collingwood and White (1973). [b] For the best fit to all the parameters the value of E_m is 4.0 for the temperature range 21–35°; the value of E_m from 3 to 21° is 2.8 ± 0.1 kcal/mol and the value of n is 2.2 at 3°. [c] ICDH, isocitrate dehydrogenase.

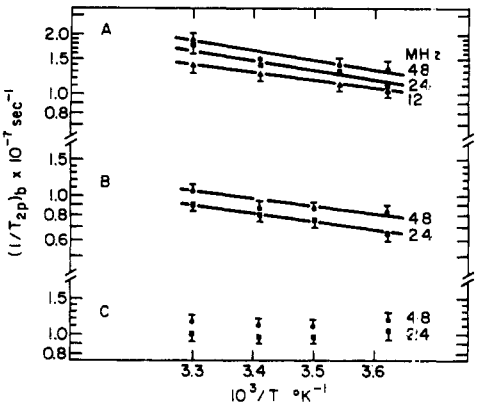


FIGURE 5: The transverse prr, $(1/T_{2p})_b$, of solvent due to the isocitrate dehydrogenase-Mn(II) complexes as a function of the reciprocal absolute temperature. Conditions are the same as for Figure 3: (▲) 12 MHz, (■) 24 MHz, and (●) 48 MHz; (A) isocitrate dehydrogenase-Mn(II); (B) isocitrate dehydrogenase-Mn(II)-isocitrate; (C) isocitrate dehydrogenase-Mn(II)-α-ketoglutarate. The lines drawn through the data points are the theoretical curves calculated from eq 6.

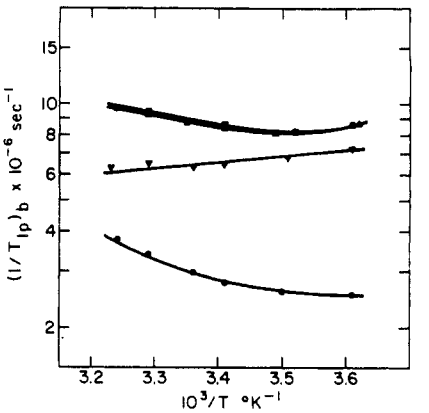


FIGURE 6: The longitudinal prr, $(1/T_{1p})_b$, of solvent due to the isocitrate dehydrogenase-Mn(II)-isocitrate complex as a function of the reciprocal absolute temperature. Solutions were the same as described in Figure 3 but also contained 0.303 mM isocitrate. The various frequencies used are (▼) 6 MHz, (▲) 12 MHz, (■) 24 MHz, and (●) 48 MHz.

of eq 14 to describe the τ_s process for *bound* Mn(II) is only an empirical approach to the problem, but since the data are fit very well, the use of this equation is justified. Hopefully other workers will provide a more rigorous theory for protein-bound Mn(II).

In Table II are listed the values for $1/\tau_s$ and $1/\tau_r$ used to calculate τ_c. This value and a value of $r = 2.8$ Å were used to calculate T_{1m} and T_{2m} from eq 10 and 11. Values of τ_m and n were then derived from eq 4 and 6 and are listed in Table I for one temperature. The $(1/T_{2p})_b$ values used to calculate τ_m over the entire temperature range are given in Figure 5A.

All of the constants listed in Tables I and II represent the best fit to all parameters. The values of $(T_{1m} + \tau_m)/n$ and $(T_{2m} + \tau_m)/n$ all represent a good fit to the experimentally determined pT_{1p} (equivalent to $(T_{1p})_b$ with p = [isocitrate dehydrogenase–Mn(II)]/(H_2O)) and pT_{2p} ($(T_{2p})_b$) data.

From this analysis it seems that there are *two* water molecules in the first hydration shell of isocitrate dehydrogenase bound Mn(II) which are exchanging approximately three times faster than in free Mn(II). Since τ_m and n are calculated from both $(T_{1p})_b$ and $(T_{2p})_b$ data the limiting factor is the accuracy in the relaxation data. The error in T_1 determinations is ±3% and in T_2 determinations is ±10%. The combined error for τ_m is 10–20% since it is the average value derived from T_1 and T_2 data taken at a number of frequencies (at each temperature).

The value of n is very sensitive to the value of r and the best fit was obtained with $r = 2.8$ Å. With $r = 2.95$ Å, the value of n is 3.0 which is also a reasonable number. The choice of $n = 2.0$ was based on the consideration that an enhancement of 11.0 was reported by Villafranca and Colman (1972) at 24 MHz, 21° for isocitrate dehydrogenase–Mn(II). This enhancement (ratio of $(1/T_{1p})_b/(1/T_{1p})_f$) predicts a value of $n = 1.0$ (Mildvan and Cohn, 1970). From our data in Table II, one can see that τ_m and T_{1m} are nearly equal at 21° (for 24 MHz) and thus the observed $(1/T_{1p})_b$ is half the expected value for $n = 2.0$.

From the analysis of these data one can see that any calculation of n based on relaxation data which are taken at one frequency may not be correct. Even if one takes data at various frequencies, a temperature dependence must also be performed to accurately evaluate the contribution of τ_m to the total relaxivity.

Temperature and Frequency Dependence of Prr of Solvent for Isocitrate Dehydrogenase–Mn(II)–Isocitrate. The $(1/T_{1p})_b$

TABLE II: Comparison of Calculated and Experimental Relaxation Times.

Complex[a]	ν (MHz)	$1/\tau_s$[b] × 10^{-9} (sec^{-1})	$1/\tau_c$[c] × 10^{-9} (sec^{-1})	T_{1m}[d] × 10^6 (sec)	$(T_{1m} + \tau_m)/n$[e] × 10^6 (sec)	pT_{1p} × 10^6 (sec)	T_{2m}[f] × 10^6 (sec)	$(T_{2m} + \tau_m)/n$[e] × 10^6 (sec)	pT_{2p} × 10^6 (sec)
ICDH–Mn(II)[g]	6	0.57	0.61	0.210	0.147	0.148	0.176	0.130	0.148
	12	0.31	0.36	0.123	0.104	0.099	0.104	0.095	0.081
	24	0.12	0.16	0.101	0.094	0.096	0.058	0.073	0.073
	48	0.035	0.079	0.410	0.242	0.260	0.038	0.069	0.069
ICDH–Mn(II)–isocitrate	6	0.31	0.35	0.096	0.156	0.159	0.083	0.144	0.131
	12	0.24	0.28	0.077	0.137	0.126	0.073	0.133	0.132
	24	0.14	0.18	0.076	0.136	0.130	0.057	0.117	0.121
	48	0.069	0.11	0.278	0.338	0.360	0.043	0.103	0.116

[a] All values are for 21°. [b] Calculated from eq 14 with B and τ_v from Table I. [c] Calculated from $1/\tau_c = 1/\tau_s + 1/\tau_r$ where $1/\tau_r = 0.44 \times 10^8$ sec^{-1} at 21°. [d] Calculated from eq 10 with $r = 2.8$ Å. [e] $\tau_m = 0.96 \times 10^{-7}$ sec and $n = 2.1$ for ICDH–Mn(II) and $\tau_m = 0.60 \times 10^{-7}$ sec and $n = 1.0$ for ICDH–Mn(II)–isocitrate. [f] Calculated from eq 11 with $r = 2.8$ Å. [g] ICDH, isocitrate dehydrogenase.

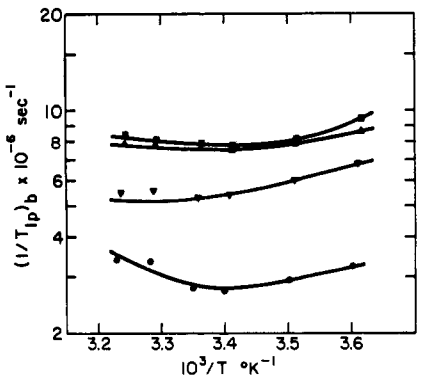


FIGURE 7: The longitudinal prr, $(1/T_{1p})_b$, of solvent due to the isocitrate dehydrogenase–Mn(II)–α-ketoglutarate complex as a function of the reciprocal absolute temperature. Solutions were the same as described in Figure 3 but also contained 3.03 mM α-ketoglutarate: (▼) 6 MHz, (▲) 12 MHz, (■) 24 MHz, (●) 48 MHz.

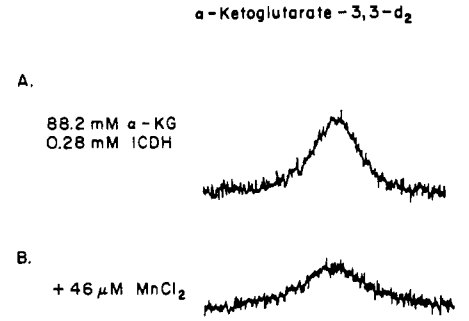


FIGURE 8: The effect of isocitrate dehydrogenase–Mn(II) on the nmr spectrum of the C-4 protons of α-ketoglutarate-*3,3-d*$_2$ at 100 MHz. The sample was prepared as described in the footnotes to Table III. Spectra (10–15) were run at various power levels and the relaxation rates calculated as described in Methods: (A) isocitrate dehydrogenase and α-ketoglutarate in 99.5% D_2O in the amouts indicated; (B) addition of $MnCl_2$ to A.

(Figure 6) and $(1/T_{2p})_b$ (Figure 5) for solutions containing isocitrate dehydrogenase, Mn(II), and isocitrate were determined over the same temperature and frequency range as in the previous section.

In Tables I and II are listed all of the calculated constants for the ternary complex formed with isocitrate present. The value of τ_v is *ca.* three times larger than for free Mn(II) and is *ca.* two times smaller than for the binary isocitrate dehydrogenase–Mn(II) complex. The value of B has increased ~10% upon going from the binary to ternary complexes while the activation energy for τ_v has also changed slightly.

The most dramatic change upon going from the binary to ternary complexes is the change in the number of water molecules. This value has been reduced by one water molecule which could represent the displacement of a water molecule by a substrate hydroxyl or carboxyl group. There is the possibility, however, that upon binding of the substrate, isocitrate, a protein conformational change takes place and another protein ligand displaces a water molecule. A further discussion of this point will be deferred to later in this paper.

From the data in Table II, one can see why the plot in Figure 4 curves upward. For both the isocitrate dehydrogenase–Mn(II) and isocitrate dehydrogenase–Mn(II)–isocitrate complexes the value of $1/\tau_s$ changes with frequency. This introduces a frequency dependence into the $1/\tau_c$ value which accounts for the upward curvature at 6 and 12 MHz. Since τ_v is different for the binary and ternary complexes, this shifts the position of the "minimum" in the data (in Figure 4) and also results in the nearly equal values of $(1/T_{1p})_b$ at 6 MHz. Thus, if the titrations reported by Villafranca and Colman (1972) using the prr technique were conducted at 6 MHz, no decrease in the $1/T_{1p}$ values would have been seen. The titration data were conducted at 24 MHz, however, where an appreciable difference is noted. This brings out another important feature in the use of nmr and the prr of solvent to determine dissociation constants; the lack of a decrease in the prr when titrating with a substrate or inhibitor may be the result of changes in the value of τ_c between the binary and ternary complexes and this effect may mask the decrease in the number of water molecules upon going from a binary to ternary complex.

The agreement between the calculated $(T_{1m} + \tau_m)/n$ and $(T_{2m} + \tau_m)/n$ values and the $pT_{1p}((T_{1p})_b)$ and $pT_{2p}((T_{2p})_b)$ data is very good although the data have a slightly greater error than for the binary complex. As with the analysis for the binary complex, a value of $r = 2.8$ Å for the one remaining water molecule gives the best overall fit to the data.

Temperature and Frequency Dependence of Prr of Solvent for Isocitrate Dehydrogenase–Mn(II)–α-Ketoglutarate. In Figure 7 is the $(1/T_{1p})_b$ data for a solution containing isocitrate dehydrogenase, Mn(II), and α-ketoglutarate. The relationship between the 6-, 12-, 24-, and 48-MHz data (Figures 4 and 7) is the same as in Figures 3 and 6 but the features of the temperature dependence of the $(1/T_{1p})_b$ data are different. From 3 to 21° (3.6–3.4 on the $1/T$ *abscissa*), the temperature dependencies for all frequencies decrease. This observation cannot be easily rationalized upon comparison with Figure 1. The data for 6, 12, and 24 MHz may fit in the region of $\tau_c \simeq 5 \times 10^{-9}$ sec with a large contribution from τ_s, but the predicted value for 48 MHz is too far in error.

The data in Figure 7 do, however, fit the predicted temperature dependence of $1/T_{1m}$ for $\tau_c \simeq 10^{-8}$ sec in the temperature range 21–35° (3.4–3.25 in Figure 7). When the data were analyzed as described previously for the isocitrate dehydrogenase–Mn(II) and isocitrate dehydrogenase–Mn(II)–isocitrate complexes, the number of water molecules in the ternary isocitrate dehydrogenase–Mn(II)–α-ketoglutarate complex went from 2.2 to 1.3 (from 3 to 21°) and then remained ~1.3 from 21 to 35°. This reduction in the number of water molecules from 3 to 21° in this ternary complex would give a positive slope for all frequencies. It is possible that the mode of binding of α-ketoglutarate to the enzyme–Mn(II) complex is different in the low and high temperature regions. At low temperatures, the α-ketoglutarate molecule may not interact directly with the metal ion, *i.e.*, the number of water molecules in the ternary isocitrate dehydrogenase–Mn(II)–α-ketoglutarate complex is not significantly different from that in the binary isocitrate dehydrogenase–Mn(II) complex. In contrast, in the temperature range above 21°, the carboxyl or carbonyl group of α-ketoglutarate displaces one water ligand on the isocitrate dehydrogenase bound Mn(II). At an intermediate temperature (~15°), both modes of binding of α-ketoglutarate may contribute equally giving an apparent n value of ~1.7.

Paramagnetic Effects of Mn(II) and Isocitrate Dehydrogenase–Mn(II) on the Protons of α-Ketoglutarate-3,3-d$_2$. The proton nmr spectrum of α-ketoglutarate-*3,3-d*$_2$ is a singlet (Figure 8) due to the $-CH_2-$ protons at C-4. The substitution of two deuterons at C-3 introduces a slight broadening of the proton resonance due to H–D coupling which is unresolved in the spectrum in Figure 8. The coupling constant is expected to be small ($J \sim 0.1$ Hz for D–C–C–H) and was neglected in this analysis since the paramagnetic effect of the five unpaired electrons of Mn(II) is the dominant relaxation process.

In Table III are listed the paramagnetic contributions to the longitudinal $(1/pT_{1p})$ and transverse $(1/pT_{2p})$ relaxation rates of

TABLE III: Paramagnetic Effects of Mn(II) and Isocitrate Dehydrogenase–Mn(II) on the Relaxation Rates of the Protons of α-Ketoglutarate-*3,3-d*$_2$.[a]

Complex[b]	$1/pT_{1p}$[c] × 10^{-3} (sec^{-1})	$1/pT_{2p}$ × 10^{-3} (sec^{-1})	ϵ_1[d]	ϵ_2
Mn(II)–α-keto-glutarate	1.01 ± 0.05	3.00 ± 0.40		
ICDH–Mn(II)–α-ketoglutarate[e]	1.56 ± 0.07	16.4 ± 1.1	1.5	5.5

[a] α-Ketoglutarate-*3,3-d*$_2$ was prepared by dissolving monopotassium α-ketoglutarate in D_2O and heating at 100° for 15 min in a sealed tube. Complete replacement of the two protons at C-3 was obtained. [b] A microcell in a 5-mm tube contained 0.085 ml of ICDH (0.28 mM) and α-ketoglutarate-*3,3-d*$_2$ (88.2 mM) in 0.1 M Tris-Cl (pD 7.5) buffer containing 0.3 M NaCl; T = 22°, 99.5% D_2O. [c] $MnCl_2$ was added to the solution described in *b*. p = [ICDH–Mn(II)–αKG]/[αKG]$_t$ = 5.33 × 10^{-4}. [d] The enhancement is defined as the ratio of $1/pT_{1p}$ (or $1/pT_{2p}$) of the ternary complex divided by $1/pT_{1p}$ (or $1/pT_{2p}$) of the binary complex. [e] ICDH, isocitrate dehydrogenase.

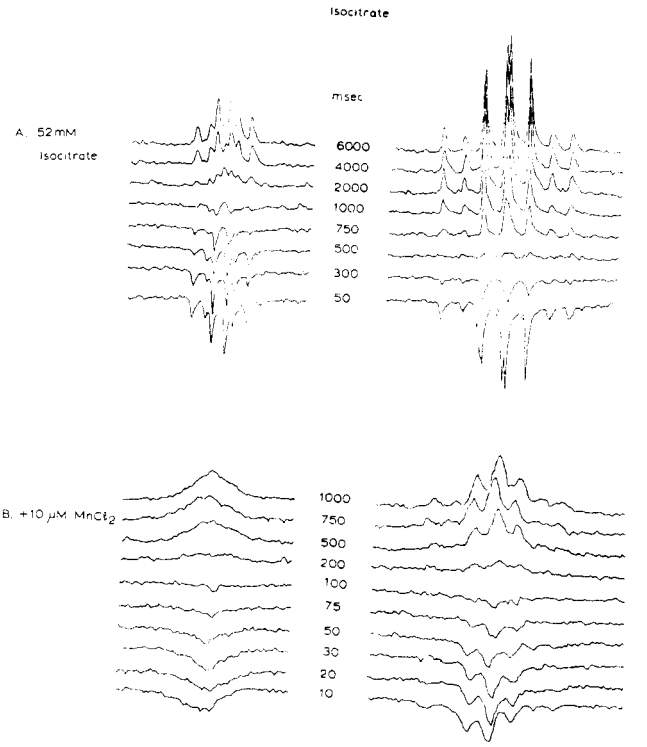


FIGURE 9: The effect of Mn(II) on the nmr spectrum of the protons of isocitrate at 220 MHz. These experiments were performed using a Varian HA-220 spectrometer in the Fourier transform mode with a 180°-τ-90° pulse sequence: (A) a 0.5-ml solution contained 52 mM isocitrate in 0.01 M Tris-Cl (pD 7.5) in D_2O; T = 18°. The spectrum at the left is for the H–C–CO_2^- proton and to the right is for the CH_2 protons. The H–C–OH proton is not shown. The values in msec are the τ values which were used; (B) an addition of $MnCl_2$ (10 μM) was made to solution A. The 220-MHz data were obtained at the University of Pennsylvania. We wish to thank Dr. Mildred Cohn and Dr. John Leigh for these spectra.

the CH_2 protons. In the ternary complex with isocitrate dehydrogenase, Mn(II), and α-ketoglutarate-*3,3-d*$_2$ the $1/pT_{1p}$ value is enhanced by 1.5 and the $1/pT_{2p}$ value is enhanced by 5.5 (at 22°). This trend in enhancement for the longitudinal and transverse relaxation rates is expected for a τ_c value of $\geq 5 \times 10^{-9}$ sec at 100 MHz.

In order to calculate a distance between the CH_2 protons and the isocitrate dehydrogenase bound Mn(II) the correlation time must be known. Rearrangement of eq 10 gives

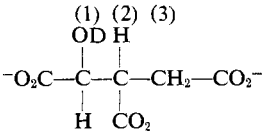
$$r^6 = C'(T_{1m})[3\tau_c/(1 + \omega_I^2\tau_c^2)] \quad (18)$$

where $C' = Cr^6$. From the correlation times derived from the solvent relaxation experiments a value for τ_c can be determined for 100 MHz. The value of τ_c becomes equal to the value of τ_r (2×10^{-8} sec at 22°) since the predicted value of τ_s at 100 MHz is $\sim 10^{-6}$ sec (using eq 14 and the values of τ_v and B in Table I).

The calculation of r requires that the value of τ_m (the lifetime of α-ketoglutarate-*3,3-d*$_2$ in the ternary complex) is known (or a good estimate of this value known). The reason for this is apparent from eq 4 (where $pT_{1p} = (T_{1p})_b$ since the calculation of r is based on $1/pT_{1p} = 1/T_{1m}$.

The value of τ_m can be estimated as follows: with the value of $\tau_c = 2 \times 10^{-8}$ sec, the ratio of T_{1m}/T_{2m} can be calculated from eq 10 and 11 without the value of r being used. This value is ~100 and, if τ_m is negligible for both $1/pT_{1p}$ and $1/pT_{2p}$, this should be the ratio found for the experimental data. The pT_{1p}/pT_{2p} ratio is ~10 which means that the $1/pT_{2p}$ is exchange limited. This allows one to make the good approximation that $1/pT_{2p} \simeq 1/\tau_m$. The $1/\tau_m$ value thus calculated is a lower limit which means that τ_m is about a 10% contribution to T_{1m}. After this correction is made to the $1/pT_{1p}$ data a value of 6.3 ± 0.2 Å is obtained for the CH_2 to Mn(II) distance in the isocitrate dehydrogenase–Mn(II)–α-ketoglutarate ternary complex. A further discussion of these data will be presented later in this paper.

Paramagnetic Effect of Mn(II) on the Protons of Isocitrate at 220 MHz. The proton nmr spectrum of isocitrate at 220 MHz is very complex but accurate $1/T_1$ data can be obtained using the Fourier transform technique (Figure 9). The H–C–OD proton is a doublet due to coupling with the adjacent H–C–CO_2^- proton. The protons labeled 2 and 3 are an ABC



multiplet with the additional complication that proton number 2 is split by proton 1. T_1 data are easily obtained for this ABCX system since all protons are in "groups" of multiplets.

The data for the binary Mn(II)–isocitrate complex are presented in Figure 9 and Table IV, and from these data distances between the protons and complexed Mn(II) can be obtained. The τ_c value was calculated from eq 12 for the small molecule complex of Mn(II)–isocitrate ($\tau_c = 8 \times 10^{-11}$ sec).

Implications of Prr Data on Mechanism of Isocitrate Dehydrogenase. The pig heart TPN-dependent isocitrate dehydrogenase has been shown to bind (per 58,000 molecular weight) one molecule each of isocitrate, TPN, and Mn(II) (Colman, 1968; Villafranca and Colman, 1972). Previous prr work (Villafranca and Colman, 1972) gave suggestive evidence that ternary complexes were formed with isocitrate dehydrogenase–Mn(II) and isocitrate or α-ketoglutarate. The dissociation constants obtained from this prr work agreed with kinetically obtained K_m values. Other kinetic experiments (Colman, 1972) suggested that the formation of the ternary complex with isocitrate could occur by two pathways; one involving the binary

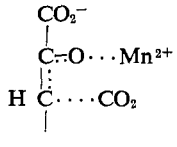
isocitrate–Mn(II) complex binding to free isocitrate dehydrogenase and the other involving isocitrate binding to the binary isocitrate dehydrogenase–Mn(II) complex. For both of these situations the presence of TPN was not obligatory. In this paper we have reported data on the ternary complexes (with isocitrate or α-ketoglutarate) which implicate the enzyme-bound metal ion as a coordination site for one or more ligand groups of the substrates.

The frequency dependence of the prr for all three complexes which were studied exhibit the same qualitative behavior (Figure 4). In each case the correlation time, τ_c, for the dipolar interaction of the relaxation rates has a contribution from a frequency dependent electron spin relaxation time, τ_s. This frequency dependence leads to a complication in the calculation of the number of water molecules in each complex but the data are fit quite well by the use of the Solomon–Bloembergen–Morgan scheme. The details and interpretation of the, τ_v, process for bound Mn(II) are not well understood, however.

For each complex, the $(1/T_{1p})_b$ and $(1/T_{2p})_b$ data have significant contributions from τ_m. This is most apparent for the data at 12 and 24 MHz and results in an upward trend in these data from ~20 to 35°. The full analysis is also complicated by the fact that at 24–48 MHz, the correlation time has a significant contribution from the rotational reorientation time of the macromolecular complex, τ_r. This represents the first analysis of a Mn(II)-activated enzyme where this factor was of significance in the data analysis.

The binary isocitrate dehydrogenase–Mn(II) complex has two to three water molecules in the primary coordination shell which would suggest three to four ligands from the enzyme (assuming the bound Mn(II) has the same coordination number as free Mn(II)). The ternary complexes with isocitrate and α-ketoglutarate have one water molecule in the primary hydration shell. For both substrates this may represent mono- or bidentate chelation to the Mn(II) depending upon whether one or two water molecules have been replaced.

From the data on the prr of the CH_2 protons of α-ketoglutarate-*3,3-d*$_2$, one distance was obtained. If it is assumed that the C-2 carbonyl (and/or C-1 carboxyl) of this substrate is coordinated to the Mn(II), then the C-4 CH_2 would be 6–7 Å from the Mn(II). The coordination of the C-2 carbonyl is attractive from a mechanistic viewpoint since the Mn(II) could serve as an "electrophilic sink" to stabilize an enolate intermediate of α-ketoglutarate. The stabilization of this enolate, formed during the decarboxylation of the β-keto acid, oxalosuccinate, may account for the catalysis by metal ions of the nonenzymatic decarboxylation (Ochoa, 1948) as well as the metal ion requirement of isocitrate dehydrogenase.



The exchange rate of α-ketoglutarate from the ternary complex, $1/\tau_m \gtrsim 1.6 \times 10^4\ sec^{-1}$, sets a lower limit for the diffusion of this substrate into the complex. Using $1/\tau_m = k_{off}$ and $K_D = k_{off}/k_{on}$ with $K_D \sim 3 \times 10^{-4}$ M (Villafranca and Colman, 1972) gives $k_{on} \sim 5 \times 10^9\ M^{-1}\ sec^{-1}$ which is close to the upper limit for diffusion of a small molecule. The k_{off} is ~500 times the turnover number of isocitrate dehydrogenase (~30 sec^{-1}).

The Mn(II) to proton distances in the binary isocitrate–Mn(II) complex are consistent with bidentate chelation to the C-1 carboxyl and C-2 carbonyl of this substrate. The ternary complex with isocitrate could not be studied since the K_D for isocitrate from the ternary complex is ~2 μM (Villafranca and Colman, 1972) and would be exchange limited. As mentioned before, the kinetic analysis of isocitrate dehydrogenase (Colman, 1972) was consistent with the association of isocitrate–Mn(II) and enzyme in certain concentration ranges of substrate and Mn(II). If this is true, then the binary complex studied herein may retain the same chelation pattern in the ternary complex. This seems reasonable since isocitrate dehydrogenase binds Mn(II) and isocitrate independently but the presence of isocitrate tightens the binding of Mn(II).

TABLE IV: Paramagnetic Effect of Mn(II) on the Longitudinal Relaxation Rate of the Protons of Isocitrate at 220 MHz.[a]

Complex	Sec^{-1} $1/T_1$	$1/T_{1p}$	$1/pT_{1p}$	r (Å)
Isocitrate				
H–C–OD	0.69			
H–C–CO_2^-	0.69			
–CH_2–	1.38			
Mn(II)–isocitrate				
H–C–OD	5.89	5.20	27,000	3.7
H–C–CO_2^-	4.50	3.81	19,700	3.9
–CH_2–	4.62	3.24	16,900	4.0

[a] Conditions of this experiment are given in the legend to Figure 9. $1/T_{1p} = 1/T_1$ (plus Mn(II)) $- 1/T_1$ (minus Mn(II)). $p = 1.92 \times 10^{-4}$. The values of r were obtained from eq 7 with $\tau_c = 8 \times 10^{-11}$ sec and $1/pT_{1p} = 1/T_{1m}$.

Evidence has been presented that the transfer of a proton is involved in the rate-determining step of the isocitrate dehydrogenase reaction (Colman and Chu, 1969) and it was proposed that an enzyme functional group may be involved in the abstraction of a proton from the α-hydroxyl group of isocitrate. In analogy to the Cannizzaro reaction (Jencks, 1969) the α hydrogen of an alkoxide ion should be easily removed as hydride ion, resulting in the transfer of hydrogen to TPN. Coordination by Mn(II) of the hydroxyl of isocitrate and the alkoxide ion which is generated may facilitate the transfer of a proton in the initial stages of the reaction. The carbonyl group of the resultant oxalosuccinate would also be coordinated to the Mn(II) and the decarboxylation assisted as mentioned above.

Further studies of the substrates of isocitrate dehydrogenase by ^{13}C nmr are being pursued along with a study of the quaternary complexes with TPN^+ and TPNH.

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Electron and Nuclear Magnetic Resonance Studies of the Interaction of Pyruvate with Transcarboxylase†

Chien-Hung Fung,* Albert S. Mildvan, and John S. Leigh, Jr.

ABSTRACT: Electron paramagnetic resonance studies of the tightly bound cobalt in transcarboxylase at ~12°K are consistent with high-spin Co(II) in a distorted octahedral environment with g values = 7.2, 3.1, 2.7, and 2.3 and A = 0.027 cm^{-1}. Formation of the transcarboxylase–Co(II)–pyruvate and oxalate ternary complexes sharpens the electron paramagnetic resonance (epr) spectra. The interaction of [1-^{13}C]- and [2-^{13}C]pyruvate with Co(II)–transcarboxylase has been examined by measurements of the longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates of the enriched carbon atoms at 25 MHz, and the methyl protons at 100 and 220 MHz. The frequency dependence observed for $1/T_1$ of the methyl protons of pyruvate in the transcarboxylase complex permits estimation of the correlation time for the Co(II)–pyruvate dipolar interaction as 2.2×10^{-12} sec. Using this correlation time and the average g value of 4.16, the distances between Co(II) and the carbon atoms and methyl protons of pyruvate calculated from $1/T_{1p}$ (5.0–6.3 Å) are consistent with a second sphere complex in which the bound cobalt lies ~1 Å out of the plane of the pyruvate carbon atoms and 1.3 Å closer to the carbonyl carbon than to the carboxyl carbon or the methyl protons. From the correlation time for the transcarboxylase–Co(II)–H_2O dipolar interaction estimated from the frequency-dependent proton relaxation rate of water (6.6×10^{-12} sec), assuming a Co(II) to water proton distance of 2.75 Å from crystallographic data, it is calculated that 2 rapidly exchanging water ligands are coordinated to the enzyme-bound Co(II). This value decreases to 1 in the pyruvate and oxalate complexes presumably due to occlusion of the metal site. In addition to cobalt and zinc (Northrop, D. B., and Wood, H. G. (1969a), *J. Biol. Chem. 244*, 5801), transcarboxylase also contains tightly bound Cu(II) as determined by epr and atomic absorbtion spectroscopy. The total metal content (Co + Zn + Cu) is 12 ± 1 g-atoms per 790,000 g of enzyme, or 2 metal ions/biotin. Like Co(II), the bound Cu(II) is near the pyruvate binding site as judged by the distances between the copper and the bound pyruvate molecule (5.7–8.2 Å) but Cu(II) appears to form a kinetically incompetent ternary complex as judged by the lack of change in the proton relaxation rate of water with pyruvate or oxalate, the specific activity, and other criteria. These results are consistent with those previously reported for the pyruvate carboxylase–Mn(II)–pyruvate complex (Fung *et al.* (1973), *Biochemistry 12*, 620) and indicate that metallobiotin enzymes form second sphere enzyme–metal–ligand–substrate bridge complexes in which an unidentified ligand, possibly water, intervenes between the metal and the substrate. The catalytic role of second sphere complexes in these enzymes is discussed.

Transcarboxylase (EC 2.1.3.1), a metallobiotin enzyme containing tightly bound cobalt and zinc (Northrop and Wood, 1969a; Ahmad *et al.*, 1972), catalyzes the reversible transfer of the carboxyl group of (*S*)-methylmalonyl-CoA to pyruvate to form propionyl-CoA and oxalacetate. In common with other biotin enzymes, the reaction sequence occurs in two steps as shown in the following two half-reactions (Wood *et al.*, 1963):

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

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

From a steady-state kinetic analysis, Northrop (1969) has suggested the presence of two independent substrate binding sites—one for the CoA esters in the first partial reaction, one for the keto acids in the second partial reaction—and that these two partial reactions are linked by oscillation of the biotin ring between these two substrate sites during transcarboxylation. The enzyme was found to increase the transverse relaxation rate of the methyl protons of pyruvate, sug-

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