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Structure–Function Relationships in TPN-Dependent Isocitrate Dehydrogenase. II. Determination of the Paramagnetic Relaxation Rates of Water Protons in Complexes of Enzyme, Mn(II), Substrates, Cofactors, and Inhibitors†

Ronald S. Levy and Joseph J. Villafranca*

ABSTRACT: Longitudinal and transverse water proton relaxation rates (PRR) were determined for complexes of isocitrate dehydrogenase containing various combinations of coenzymes, substrates, and substrate analogues. Titrations performed by following the decrease in the PRR suggest that the substrate analogue oxalylglycine and the nucleotides TPN and TPNH bind near, but not directly to, the metal ion site while the possibility for direct Mn(II) coordination is much greater for the substrates α-ketoglutarate and isocitrate. TPN and TPNH both decrease the PRR when added to enzyme–Mn(II) but for different reasons. The addition of TPNH weakens the affinity of isocitrate dehydrogenase for Mn(II) and the decrease in the PRR is due to release of Mn(II) from the enzyme. TPN apparently alters the conformation about enzyme–Mn(II), resulting in a decrease in the PRR since Mn(II) binding is not affected when TPN binds to the enzyme. These experimental results are confirmed by the EPR experiments in the previous paper (Levy, R. S., and Villafranca, J. J. (1977), *Biochemistry 16* (preceding paper in this issue)). The K_D value for oxalylglycine, a competitive inhibitor of the enzyme, is ~500 μM. The K_D value is changed to ~220 μM in the presence of TPNH. Similarily the K_D value for α-ketoglutarate is lowered from ~590 μM in the presence of TPN and HCO_3^- to ~90 μM by the presence of TPNH and HCO_3^-. The above data demonstrate that TPNH has a synergistic effect on the binding of substrate. Also, the presence of substrate and TPNH increase the binding of Mn(II) to the enzyme since no free Mn(II) is detected by EPR under the conditions of the titration. Longitudinal ($1/T_{1p}$) and transverse ($1/T_{2k}$) PRR data were obtained as a function of frequency and temperature for complexes of Mn(II), isocitrate dehydrogenase, substrates, cofactors, and inhibitors. Computer fits to the data demonstrate that the correlation times that modulate the PRR are in the range (0.5–1.0) $\times 10^{-8}$ s. Contributions to τ_C arise from both the rotational tumbling time of the macromolecular complex and the electron spin relaxation time of Mn(II). The above data provide further evidence for the previously reported role of Mn(II) (Villafranca, J. J., and Colman, R. F. (1974), *Biochemistry 13,* 1152) as an electrophilic center on the enzyme to bind substrate and perhaps stabilize the enzyme-bound enolate of α-ketoglutarate.

The behavior of the paramagnetic contribution to the longitudinal proton relaxation rates ($1/T_{1p}$) of water during titration of Mn(II) complexes of TPN-dependent isocitrate dehydrogenase with substrates, coenzymes, or substrate analogues can provide information concerning the relative dissociation constants for the titrating species. Observations of the fluctuations in these relaxation rates when the temperature and NMR[1] frequency are varied can also provide information concerning the nature of the rate processes which modulate the dipolar electron–nuclear interaction. Other important parameters such as the number of water molecules present in the primary coordination sphere of Mn(II) and the time constants for physical parameters such as solvent exchange can also be approximated under favorable conditions.

The manganous ion is particularly suitable as a probe for magnetic resonance studies because it has a long electron spin relaxation time. Consequently, the electron paramagnetic resonance spectrum is observable at room temperature and the water proton relaxation rate (PRR) enhancement is large when Mn(II) binds to a macromolecule.

A temperature and frequency dependence study for the ternary complexes of isocitrate dehydrogenase with isocitrate and α-ketoglutarate has been conducted by Villafranca and Colman (1974). The correlation time, τ_c, for the complexes was found to be frequency dependent, with a large contribution from both the rotational and electron spin relaxation times. The number of water molecules directly bound to Mn(II) appeared

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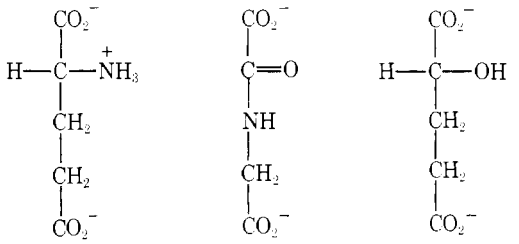
[1] Abbreviations used: PRR, proton relaxation rates; rf, radio frequency; NMR, nuclear magnetic resonance.

to decrease from two to one, in going from the binary enzyme-manganese complex to the ternary complex with either substrate. Preliminary titration of such ternary complexes with TPN or TPNH produced essentially no change in the observed PRR values (Villafranca and Colman, 1972).

The determination of the temperature-frequency dependence pattern for quaternary enzyme complexes with nucleotides is a natural extension of these preliminary investigations. Such an investigation would be a necessary prerequisite to any attempts to elucidate the enzymatic mechanism via structural determinations of the various enzyme complexes. Along with PRR titration data for binary and ternary enzyme complexes with both forms of the coenzyme, the results of these relaxation rate studies augment EPR investigations for the complexes reported in the previous paper (Levy and Villafranca, 1977).

In addition, similar determinations of relaxation rate behavior were conducted for three dicarboxylic substrate analogues, L-glutamate (I), oxalylglycine (II), and L-α-hydroxyglutarate (III), that are structurally similar to the natural enzymatic species isocitrate and α-ketoglutarate. Comparisons of the binding affinities of the natural substrates and their analogues may be instructive in the elucidation of the structural requirements for the binding of substrates as well as in the determination of the mechanism of the enzymatic reaction.



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Experimental Section

Isolation and Purification of Isocitrate Dehydrogenase. TPN-linked isocitrate dehydrogenase was purified according to previously published techniques (Levy and Villafranca, 1977). The specific activity of homogeneous enzyme samples was ~29 μmol min^{-1} (mg of enzyme)$^{-1}$. The purified enzyme was stored in liquid nitrogen in the standard buffer system employed throughout this study (0.1 M NaCl-0.1 M triethanolamine-10% glycerol, pH 7.7).

Determination of Enzyme Activity. The activity of isocitrate dehydrogenase was determined at 25 °C, using the standard assay mixture and techniques described earlier (Colman, 1968), with the exception that $MnCl_2 \cdot 4H_2O$ was substituted for $MnSO_4$. The specific activity of the enzyme samples used in the temperature-frequency experiments never decreased by more than 10% during the period of data accumulation.

Preparation of Oxalylglycine (Na Salt). Oxalylglycine was synthesized by combining glycylglycine and sodium nitrate according to the method of Viscontini (1946). The white powder was recrystallized from methanol, and its identity was verified by recording the ^{13}C spectrum of a 0.1 M aqueous solution. The spectrum was recorded on a JOEL PS-100FT spectrometer. The chemical shifts were 42.5 (methylene), 163.9 (C1 carboxyl), 165.1 (C5 carboxyl), and 175.5 (carbonyl) ppm downfield from Me_4Si.

PRR Titration Procedures. PRR data for titration experiments were obtained at 24 MHz using a modified NMR Specialties variable frequency pulsed spectrometer equipped with a SEIMCO broad band receiver system. Temperatures were maintained at 20 ± 1 °C by passing N_2 through a Dewar containing dry ice-ethanol and heating to the desired temperature. Samples were temperature equilibrated for several minutes prior to T_1 determinations. Longitudinal relaxation rates ($1/T_1$) were determined using the Carr-Purcell (1954) 180°-τ-90° pulse sequence, where τ is the time interval between the two pulses. All relaxation rates were reproducible within ±2%.

At an enzyme concentration of 0.35 mM, sufficient Mn(II) was added to maintain the free Mn(II) concentration around 10% (using the previously reported dissociation constant of 45 μM for the binary complex (Villafranca and Colman, 1972)). In order to eliminate both diamagnetic and volume corrections for each titrant addition, two solutions were prepared that were identical in composition with the exception of the titrating species itself.

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).

Temperature and Frequency Dependence Studies. Longitudinal PRR values were determined at 6, 12, 24, and 48 MHz over a 1-25 °C temperature range using the techniques described above. Detailed discussions describing the theoretical basis for the interpretation of PRR data are presented elsewhere (Villafranca and Colman, 1974; Reuben et al., 1970). Transverse relaxation rates ($1/T_2$) were measured at 24 and 48 MHz using the Meiboom-Gill (1958) modification of a Carr-Purcell pulse train, 90°, τ, 180°, [τ, (echo), τ, 180°, τ, (echo), τ, etc.

Substrate and analogue concentrations were selected in order to achieve a 60% or greater saturation of the enzyme. Enzyme and manganese concentrations were 0.33 and 0.05 mM, respectively.

Fifty-microliter samples were placed in 5-mm quartz NMR tubes which were plugged with greased rubber septa in order to achieve an air-tight seal. Each sample tube was stored in ice before and after relaxation rate measurements in order to minimize solvent evaporation and to maximize enzyme stability.

In order to obtain paramagnetic proton relaxation rate values ($1/T_{1p}$, $1/T_{2p}$) for a specific enzyme-manganese complex, the observed relaxation rates were corrected for diamagnetic and unbound paramagnetic (Mn-containing) complexes. Concentrations of all of the possible species were determined for each solution and adjusted for the specific temperature at which the T_1 or T_2 value was determined. For example, diamagnetic relaxation contributions to measured $1/T_1$ values were eliminated by subtracting $1/T_1$ values for enzyme-buffer solutions alone. Such relaxation effects vary only slightly over the temperature range studied and are essentially invariant at frequencies in excess of 2 MHz.

The resulting value is the total $1/T_{1p}$ value for all of the paramagnetic species in solution:

$$1/T_{1p_{(total)}} = \frac{[Mn]_f}{[Mn_t]T_{1p(h)}} + \frac{[ICDH\text{-}Mn]}{[Mn]_t T_{1p(i)}} + \frac{[Mn\text{-}S]}{[Mn]_t T_{1p(j)}} + \frac{[ICDH\text{-}Mn\text{-}S]}{[Mn]_t T_{1p(k)}}, \text{etc.} \quad (1)$$

where *h*, *i*, *j*, and *k* represent the different Mn complexes in the numerator of each term, and ICDH = isocitrate dehydrogenase.

In order to quantitatively determine the individual $1/T_{1p}$

values, the concentration of each manganese complex was determined by using the CRAMS (Chemical Reaction Analysis and Modeling System) computer program (Butler and de-Maine, 1975). The program determined the final concentration of individual species by solving the simultaneous equilibrium equations for all of the possible enzyme complexes.

For each temperature, the final, corrected $1/T_{1p}$ values obtained at the four different frequencies for the particular enzyme complex in question were computer fit to functions which estimated several of the physical parameters for each of the eight systems (Villafranca et al., 1976; Levy, 1976). These parameters were then adjusted by an iterative procedure to yield the best fit (smallest % error) between calculated and observed $1/T_{1p}$ values at a given temperature.

Results

PRR Titrations of Oxalylglycine Complexes. The first system studied was the enzyme complex of oxalylglycine, an inhibitor of isocitrate dehydrogenase. It is the most effective inhibitor of the three analogues studied in this work (Levy, 1976). The K_D for this analogue can be determined from a PRR titration. A smooth curve was obtained for a plot of $1/T_{1p}$ vs. log[oxalylglycine]. The concentration of inhibitor corresponding to the midpoint of the curve provides an initial estimate of the K_D value. It is important to note that all K_D values thus determined represent upper limits to the actual values, since the total $1/T_{1p}$ value for the system represents contributions from every Mn-containing species in the system. Conditions were arranged so that the major contribution of the $1/T_{1p}$ values was from the particular species of interest.

Oxalylglycine was used as the titrating species for solutions of enzyme-Mn(II) and enzyme-Mn(II)-TPNH. In the TPNH-containing solutions, two ratios of TPNH:enzyme were used, 1:9 and 2:1 (saturating). A plot of the latter titration is shown in Figure 1.

In each of the oxalylglycine titrations, initial additions of titrant resulted in small increases in the measured relaxation rates. With further additions of the inhibitor, the rates peaked and then decreased regularly. These observed rates can be related to kinetic and structural parameters of the specific Mn(II)-enzyme complex according to the following relationship:

$$\left(\frac{1}{T_{1p}}\right)_b = \frac{pq}{T_{1M} + \tau_M} \qquad (2)$$

where p equals [Mn(II)-complex]/[H_2O], q equals the effective number of rapidly exchanging H_2O molecules in the first coordination sphere of Mn(II) and T_{1M} equals the relaxation time for water protons in the first coordination sphere. The subscript b indicates the $1/T_{1p}$ value corresponding to the particular Mn-containing species under consideration.

The small initial increase in the $1/T_{1p}$ values could represent an increase in Mn(II) bound to the enzyme or a configurational change in the Mn(II) coordination sphere such that the coordinated water molecules are more readily accessible to exchange with the solvent. This phenomenon could also be caused by the shift in equilibrium between the enzyme-Mn(II) and the enzyme-Mn(II)-oxalylglycine conformations, which could be due to a change in conformation of Mn(II) on the enzyme surface in order to accommodate oxalylglycine binding.

Previously published results (Villafranca and Colman, 1974) suggest that enzyme-bound manganese contains approximately two exchangeable water molecules. Displacement of one of these by oxalylglycine should result in approximately a 50% decrease in observed $1/T_{1p}$ values. Even if three waters were present, a 33% decrease should be observed. In all titrations with oxalylglycine, the total decrease in $1/T_{1p}$ values was ~18%. Direct coordination of the inhibitor to manganese therefore seems incompatible with the experimental results; also, EPR studies show almost no change in the bound manganese signal upon addition of the inhibitor.

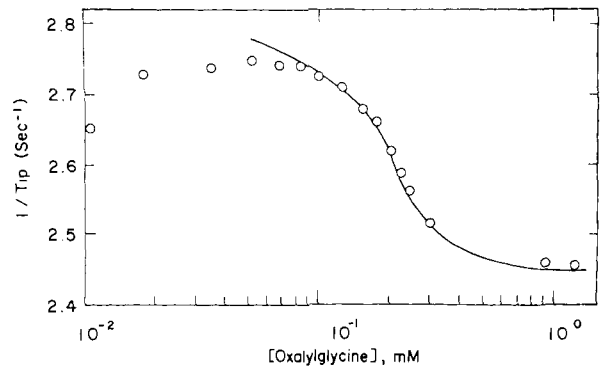

FIGURE 1: Paramagnetic proton relaxation rates at 24 MHz plotted as a function of oxalylglycine concentration at 20 °C. Solution contains 0.35 mM enzyme, 0.05 mM Mn, and 0.71 mM TPNH. Values plotted for the relaxation rates include the $1/p$ factor, where p = [Mn(II)]/[H_2O]. All relaxation rates have been multiplied by 10^{-6}.

The K_D values calculated from the oxalylglycine titration curves were a function of the concentration of the reduced nucleotide. Titrations in the absence of TPNH gave a K_D value for oxalyglycine of ~500 μM. When TPNH was present in a 1:9 ratio vs. enzyme, the value dropped to~320 μM. In solutions containing a 2:1 TPNH to enzyme ratio, the K_D was ~220 μM. Thus TPNH appears to promote tighter binding of oxalylglycine to the enzyme. The latter K_D value agrees well with the kinetically determined K_I value of 150 μM reported by Cleland and Northrup (1974) in the presence of saturating TPNH. The K_I value was determined for the Mg(II)-activated enzyme that has been shown to bind substrates more tightly than does enzyme activated by Mn(II) (Cleland et al., 1974). The fact that the oxalylglycine binding affinity for the enzyme is approximately unchanged despite changing the metal ion cofactor provides additional evidence for an inhibitor binding site near, but not at, the metal ion site.

Titrations of α-Ketoglutarate Complexes. Titrations of enzyme-Mn(II) complexes with an enzyme-Mn(II)-α-ketoglutarate solution generally displayed a smooth decrease in $1/T_{1p}$ values. For example, the titration curve obtained for an enzyme-Mn(II)-TPN solution was smooth and regular (Figure 2), while that for an enzyme solution containing TPNH was much more complex. This was expected, since a solution of α-ketoglutarate, HCO_3^-, and TPNH will lead to the formation of an equilibrium mixture. The $1/T_{1p}$ value at the end of a titration with α-ketoglutarate was dependent upon the components of the solution. Addition of TPN lowered the final $1/T_{1p}$ value ~20–25% and bicarbonate addition lowered the resulting value another 6–8%.

The large decrease in the measured relaxation rates upon formation of quaternary complexes of enzyme-Mn(II)-α-ketoglutarate and TPN or TPNH is consistent with the suggestion that α-ketoglutarate is near to, or directly coordinated to, the metal ion. Reagent concentrations were nearly identical in the experiments with the two nucleotides, and the relaxation rates decreased 33–35% overall. The uniformity of this decrease in the two trials suggests that α-ketoglutarate binds in a similar fashion at the end of the titration regardless of the type of nucleotide present in such complexes.

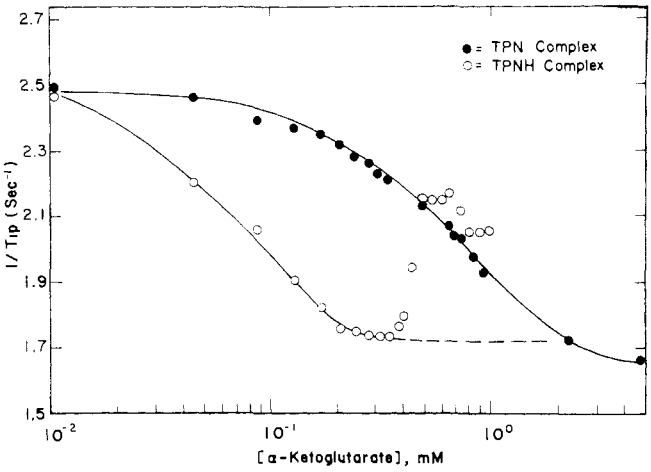

FIGURE 2: Paramagnetic proton relaxation rates at 24 MHz plotted as a function of α-ketoglutarate concentration at 20 °C. Solutions contain 0.35 mM enzyme, 0.05 mM Mn, 0.72 mM nucleotide, and 7.00 mM bicarbonate. Values plotted for the relaxation rates include the $1/p$ factor and have been multiplied by a factor of 10^{-6}.

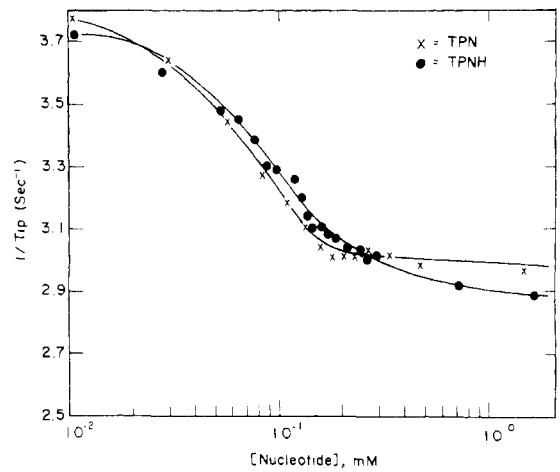

FIGURE 3: Paramagnetic proton relaxation rates at 24 MHz plotted as a function of nucleotide concentration at 20 °C. Solutions contain 0.35 mM enzyme and 0.05 mM Mn. Values plotted for the relaxation rates include the $1/p$ factor and have been multiplied by a factor of 10^{-6}.

Although the magnitude of the decrease in relaxation rates is similar, the rate of decrease with respect to α-ketoglutarate concentration is markedly different. The approximate dissociation constant for α-ketoglutarate of ~590 μM calculated from the data with TPN present is roughly sevenfold larger than the K_D value determined for the TPNH complex (~90 μM). The discrepancy in the dissociation constants may actually be even greater since the measured $1/T_{1p}$ values represent a sum total of the values for each Mn(II)-enzyme species in the solution. Since the TPNH-containing sample represents an equilibrium mixture, a larger number of species would be expected, and the actual K_D value for the α-ketoglutarate in this complex may be smaller. The difference in K_D values for the two nucleotide complexes may signify that the bound nucleotides impose different conformational restrictions upon the enzyme which markedly influence the enzyme affinity for α-ketoglutarate.

Since the enzyme turnover rate (30 s^{-1}) is faster than the time it takes to obtain the $1/T_1$ data, the titration curve for the TPNH complex will represent an average of the contributions from both the α-ketoglutarate and isocitrate complexes, as their concentrations vary during the titration. Hence, it is not surprising that the resulting pattern for the overall $1/T_{1p}$ titration with TPNH is complex. In these titrations, the $1/T_{1p}$ values plateau at approximately 1.7 s^{-1} and then increase when the α-ketoglutarate to enzyme ratio approaches unity (~.3 mM α-ketoglutarate, Figure 2). The $1/T_{1p}$ value rises to ~2.2 s^{-1} at ~1.0 mM α-ketoglutarate.

The reverse of the previous titration was performed by using the solution containing enzyme, Mn(II), and TPNH as the titrant. In this experiment the α-ketoglutarate concentration gradually decreases. The relaxation rate remained constant at 0.8 s^{-1} until the α-ketoglutarate concentration was approximately 2.2 mM. The $1/T_{1p}$ value then increased without any further additions, over a 90-min time interval, until it reached a stable value of 1.7 s^{-1}. The value then remained at 1.60–1.65 s^{-1}, although additional titrant was added. The $1/T_{1p}$ values at the end of the forward and reverse titrations were nearly identical. The increase in $1/T_{1p}$ at a 1:1 α-ketoglutarate to enzyme ratio in the forward titration is not reproduced when the titration procedure is reversed. The change in $1/T_{1p}$ that occurred in the reverse titration at the α-ketoglutarate concentration of 2.2 mM is much too slow to be attributed to enzyme turnover (the amount of enzyme present in solution can turnover ~30 μmol of α-ketoglutarate per min, whereas only 0.13 μmol of α-ketoglutarate has been introduced). This change could be the result of second site binding by the substrate, a phenomenon described by Cleland et al. (1974). The second site binding produces a dead-end type of inhibition in the kinetic experiments, and our observations may reflect this alternate site binding.

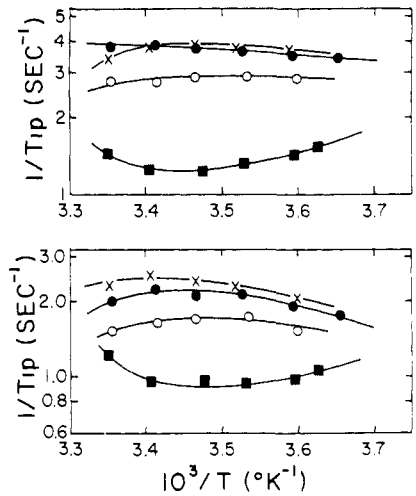

FIGURE 4: (Top) The longitudinal proton relaxation rates of H_2O due to the isocitrate dehydrogenase (ICDH)-Mn(II) complex as a function of reciprocal absolute temperature. The $1/p$ factor is included in the relaxation rates. [ICDH] = 0.33 mM; [Mn] = 0.05 mM. The various frequencies used are: (○) 6 MHz; (●) 12 MHz; (X) 24 MHz; and (■) 48 MHz. Relaxation rates have been multiplied by 10^{-6}. (Bottom) The longitudinal proton relaxation rates due to the ICDH-Mn(II)-TPNH complex. The $1/p$ factor is also included. Solution composition is the same as described above, except that 0.72 mM TPNH has been added. The frequency symbols are identical with those described above. Relaxation rates have been multiplied by 10^{-6}.

Nucleotide Titration of Binary Enzyme-Mn(II) Complexes. In order to study the effects of nucleotide binding to the enzyme in the absence of substrate, TPN and TPNH solutions were independently titrated into solutions of the binary enzyme-metal ion complex. The results, as shown in Figure 3, indicate that the effects of the two nucleotides on the PRR values for the binary complex are remarkably similar. Both titration curves decrease regularly and reach an end point value

TABLE I: Isocitrate Dehydrogenase (ICDH) Solution Compositions for PRR Temperature-Frequency Dependence Studies.

Solution No.	Mn species of interest	Pred % Mn in complex[a]	Pred free Mn[a] (μM)	Obsd free Mn[b] (μM)
1	ICDH-Mn	86.0	7.25	12.6
2	ICDH-Mn-TPNH	40.6	17.80	19.1
3	ICDH-Mn-isocitrate	98.5	0.40	<0.9
4	ICDH-Mn-TPNH-oxalylglycine	73.7	4.59	7.3
5	ICDH-Mn-TPNH-L-α-hydroxyglutarate	80.3	3.39	3.9
6	ICDH-Mn-TPNH-L-glutamate	65.1	5.25	9.5
7	ICDH-Mn-TPNH-α-ketoglutarate	73.9	4.54	9.0
8	ICDH-Mn-TPNH-α-ketoglutarate-HCO_3^-	75.5	0.94	6.1

[a] Calculated via CRAMS program (constants employed were determined at 20-25 °C). [b] Determined from EPR measurements, 25 °C.

similar to that recorded for the titrant solutions alone. The initial and final relaxation rates for the two titrations are nearly identical, and the "apparent" K_D values are similar.

Although the nucleotides display similar decreases in $1/T_{1p}$ values, the reasons behind the respective phenomena are different. EPR spectra of solutions of composition similar to those used for the titrations show that TPNH addition causes significant displacement of Mn(II) from the enzyme surface which accounts for the observed decrease in $1/T_{1p}$. However, TPN addition slightly decreases the amplitude of the bound Mn(II) EPR signal in the binary enzyme-Mn(II) complex. It appears that TPN binds away from the Mn(II) site and induces a conformational change which slightly occludes exchange of Mn(II)-bound water with the solvent. The K_D value calculated for TPN is ~60 μM, while for the reduced nucleotide it is ~7 μM. These values are upper limits of the dissociation constants.

Temperature and Frequency Dependence of $1/T_{1p}$. The following systems were studied: the binary enzyme-Mn(II) complex, ternary complexes with TPNH and isocitrate, quaternary complexes with TPNH and substrate analogues, and quaternary and quinary complexes with α-ketoglutarate.

Before the relaxation rate data could be evaluated, concentrations of individual Mn(II) species in each sample were determined using the CRAMS computer program along with previously published equilibrium constants[2] (Colman, 1972; Villafranca and Colman, 1972; Ehrlich and Colman, 1975; Colman and Chu, 1970; Sillen and Martell, 1971). The concentration of free Mn(II) predicted by CRAMS was verified experimentally for each sample by EPR measurements. Table I lists these theoretical and observed values, which are in close agreement for each of the eight solutions. The marked discrepancy in solution 8 is undoubtedly due to the larger variety of simultaneous equilibria which must be considered which leads to a progressive accumulation of individual errors.

In all cases, the experimentally determined free manganese

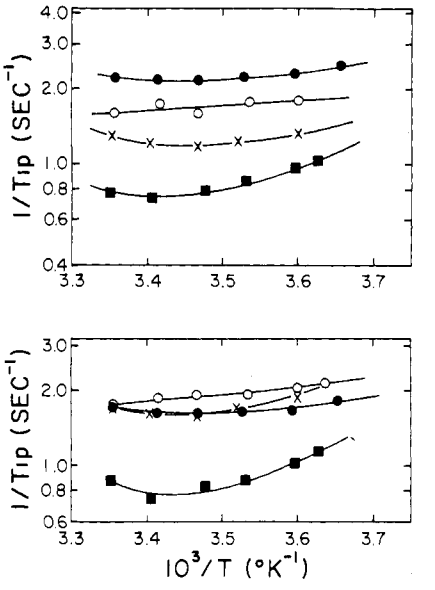

FIGURE 5: (Top) The longitudinal proton relaxation rates of H_2O due to the isocitrate dehydrogenase (ICDH) complex of Mn(II)-α-ketoglutarate-TPNH vs. reciprocal absolute temperature. The $1/p$ factor is included in the relaxation rates. [ICDH] = 0.33 mM; [Mn] = 0.05 mM; [α-ketoglutarate] = 2.10 mM; [TPNH] = 0.72 mM. The frequency symbols used are: (○) 6 MHz; (●) 12 MHz; (×) 24 MHz; and (■) 48 MHz. Relaxation rates have been multiplied by 10^{-6}. (Bottom) The longitudinal proton relaxation rates due to the ICDH-Mn(II)-α-ketoglutarate-TPNH-HCO_3^- complex. The $1/p$ factor is also included. Solution composition is identical with that described above, except that 11.2 mM HCO_3^- has been added. The frequency symbols are the same as those used above. Relaxation rates have been multiplied by 10^{-6}.

values are slightly larger than those predicted by the computer program. This probably occurs because other small-molecule Mn(II) species in addition to the aquo complex are contributing to the EPR signal amplitude. Indeed, combination of the concentrations for all small Mn(II) complexes predicted by CRAMS more closely approaches the total "free" Mn(II) concentration observed by EPR.

The $(1/T_{1p})_b$ data for each solution were plotted as a function of the reciprocal temperature (Figures 4-6). These values represent the paramagnetic contribution to $1/T_{1p}$ from the enzyme-bound Mn(II) species of interest in each solution. In Figure 4 the plots for solutions 1 and 2 (enzyme-Mn and enzyme-Mn-TPNH) are shown. They are similar in overall appearance, the most obvious characteristic for both solutions

[2] K_D values that were used in the computer fit of the PRR data were as follows: [E][Mn]/[E-Mn] = 45 μM; [E][isocitrate]/[E-isocitrate] = 11.5 μM; [isocitrate][Mn]/[isocitrate-Mn] = 870 μM; [E-Mn][isocitrate]/[E-Mn-isocitrate] = 2 μM; [E][TPN]/[E-TPN] = 49 μM; [E][TPNH]/[E-TPNH] = 1.5 μM; [E-Mn][ketoglutarate]/[E-Mn-ketoglutarate] = 290 μM; [Mn][TPNH]/[Mn-TPNH] = 1.7 mM; [Mn][Glu]/[Mn-Glu] = 0.3 mM; [E][ketoglutarate]/[E-ketoglutarate] = 10 μM. The value for Mn-TPNH was obtained in our laboratory while all the remaining values are from the following literature references: Colman (1968, 1972), Ehrlich and Colman (1975), Sillen and Martell (1971), and Villafranca and Colman (1972, 1974).

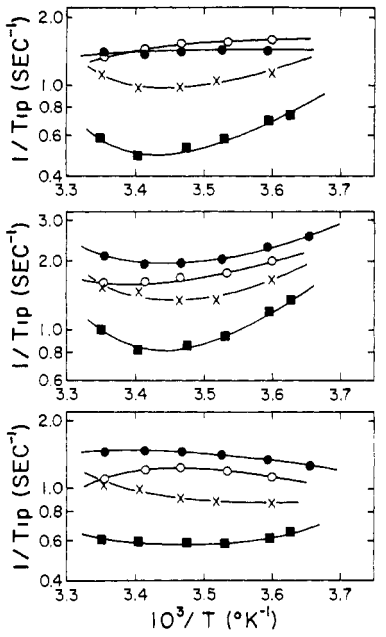

FIGURE 6: (Top) The longitudinal proton relaxation rates of H_2O due to the isocitrate dehydrogenase (ICDH) complex of Mn(II)-α-oxalylglycine-TPNH vs. the reciprocal absolute temperature. The $1/p$ factor is included in the relaxation rates. [ICDH] = 0.33 mM; [Mn] = 0.05 mM; [oxalylglycine] = 2.00 mM; TPNH = 0.72 mM. The frequency symbols used are: (○) 6 MHz; (●) 12 MHz; (×) 24 MHz; and (■) 48 MHz. Relaxation rates have been multiplied by 10^{-6}. (Center) The longitudinal proton relaxation rates due to the ICDH-Mn(II)-L-α-hydroxyglutarate-TPNH complex. The $1/p$ factor is also included. [ICDH] = 0.33 mM; [Mn] = 0.05 mM; [L-α-hydroxyglutarate] = 11.25 mM; [TPNH] = 0.72 mM. The frequency symbols are the same as those used above. Relaxation rates have been multiplied by 10^{-6}. (Bottom) The longitudinal proton relaxation rates due to the ICDH-Mn(II)-Glu-TPNH complex. The $1/p$ factor is also included. [ICDH] = 0.33 mM; [Mn] = 0.05 mM; [Glu] = 11.10 mM; [TPNH] = 0.72 mM. The frequency symbols are unchanged. Relaxation rates have been multiplied by 10^{-6}.

being the displacement to lower $1/T_{1p}$ values of the 6 MHz data, relative to the 12 and 24 MHz relaxation rates.

Apart from small corrections for small molecule Mn(II) complexes, the $1/T_{1p}$ values for solutions 2, 4, 6, 7, and 8 required one further adjustment. Four of the solutions had to be corrected for the presence of a significant amount of Mn-TPNH (>5% of the major solution component), while the equilibrium mixture sample had to be corrected for the quaternary enzyme-Mn-α-ketoglutarate-TPNH complex, in order to obtain the final $(1/T_{1p})_b$ value for the quinary complex.

For each solution, the $1/T_{1p}$ data for the four frequencies were computer fit at each temperature by iterative manipulation of the variables as previously described (Villafranca and Colman, 1974; Levy, 1976). The percentage difference between the predicted $1/T_{1p}$ values (determined by the adjusted parameters) and the experimentally observed relaxation rates for each frequency were computed, and the percentage errors for the four frequencies were summed to give the total error. The data and computer fits at 25 °C are given in Table II for some of the enzyme complexes.

Total errors for all but the glutamate and L-α-hydroxyglutarate solutions were consistently below 10% at 25 °C. Total errors for these substrate analogues were acceptable but clearly larger, varying between 30 and 35%. Small increases in the error percentages for all solutions were observed with decreasing temperature, and the significance of these observations will be discussed later in this paper.

T_2 values were measured at 25 °C as described earlier. Data were determined for each system at 24 and 48 MHz, the two frequencies at which the greatest T_{1p}/T_{2p} ratios are expected for our measurements.

The calculation of the uncorrected rates was performed by a computer program, which divided the height of each echo by the height of the initial echo, plotted the log of each ratio vs. time, and fit the data by a linear least-squares subprogram. The slope of this line equals $-1/T_2$.

Correction of the enzyme-Mn(II) complex data for free Mn(II) was performed by using the $1/T_{2p}$ data for $MnCl_2$ published by Koenig et al. (1971). Contributions from Mn-TPNH were corrected as described earlier for the $1/T_{1p}$ data.

For Mn(II), an equation relating observed T_{2p} values to the τ_M and T_{2M} parameters can be written similar to that relating longitudinal relaxation rates to τ_M and T_{1M} values (eq 2). Since the T_{1p} values were found to have a definite contribution from τ_M, T_{2p} values would be expected to be more strongly dominated by τ_M since $T_1 \geq T_2$, and τ_M is a constant at a given temperature. A positive temperature coefficient for $1/T_{2p}$ values (increasing $1/T_{2p}$ values with increasing temperature) would be expected, typical of a τ_M-dominated process.

The final $1/T_{2p}$ values calculated at 24 and 48 MHz for each system differed slightly and T_{1p}/T_{2p} ratios listed in Table III are presented for all eight solutions.

The fact that the ratios are clearly greater than unity rules out domination of the longitudinal relaxation rates by τ_M, i.e., $\tau_M \gg T_{2M}$. The dramatic increases with increasing frequency suggest τ_c values for the complexes in the range $>5 \times 10^{-9}$ s, as well as the necessary corollary that T_{1M} furnishes the major contribution to the $1/T_{1p}$ rates.

Discussion

This paper reports an extension of previous NMR studies on TPN-dependent isocitrate dehydrogenase from pig heart. Previously, binding constants of isocitrate and α-ketoglutarate to enzyme-Mn(II) were determined by PRR titrations (Villafranca and Colman, 1972). The binding constant for α-ketoglutarate was ~290 μM from this previous study. In this paper, the presence of bicarbonate and TPN increased the K_D value to ~590 μM, demonstrating an antagonistic effect of α-ketoglutarate binding in this abortive complex. When a titration with α-ketoglutarate was performed in the presence of enzyme, Mn(II), TPNH, and bicarbonate, an approximate K_D value of ~90 μM was determined. While this experiment is complicated by enzymatic turnover of substrates, the K_D value is definitely tighter consistent with a synergistic effect of TPNH and bicarbonate on α-ketoglutarate binding.

The substrate analogue oxalylglycine produced a decrease in the PRR of water molecules when titrated into a solution containing the binary enzyme-Mn(II) complex. The decrease in the relaxation rates could indicate the occurrence of one of four possible phenomena: (1) oxalylglycine binding could occur directly to Mn(II), displacing a coordinated water molecule; (2) the analogue could bind elsewhere on the enzyme and induce a conformational change in the vicinity of the manganese which serves to restrict exchange of the Mn(II)-bound water with solvent water; (3) the inhibitor may have a much stronger affinity for Mn(II) than does the enzyme, and compete with the enzyme for Mn(II); or (4) the bonding of the inhibitor to the enzyme may result in the displacement of Mn(II) from the enzyme surface.

EPR spectra recorded for enzyme-Mn-oxalylglycine complexes can be used to distinguish between the first and

TABLE II: Calculated and Observed Relaxation Times for Selected Systems at 25 °C.[a]

Enzyme complex	ν (MHz)	$(1/\tau_s) \times 10^{-8}$ (s^{-1})	$(1/\tau_c) \times 10^{-8}$ (s^{-1})	$T_{1M} \times 10^6$ (s)	pT_{1p} (obsd) $\times 10^6$ (s)	pT_{1p} (calcd) $\times 10^6$ (s)
Mn (0.26×10^{-6})[b]	6	4.39	4.90	0.282	0.279	0.278
	12	1.96	2.47	0.155	0.202	0.213
	24	0.64	1.14	0.178	0.227	0.226
	48	0.18	0.70	0.784	0.529	0.530
Mn-TPNH (0.71×10^{-7})	6	4.28	5.90	0.373	0.222	0.222
	12	3.95	4.55	0.296	0.176	0.182
	24	2.07	2.65	0.223	0.154	0.147
	48	0.76	1.39	0.503	0.287	0.287
Mn-isocitrate (0.12×10^{-6})	6	3.47	3.96	0.209	0.322	0.321
	12	2.28	2.79	0.157	0.256	0.265
	24	1.03	1.55	0.155	0.278	0.266
	48	0.44	0.95	0.560	0.668	0.672
Mn-TPNH-oxalylglycine (0.40×10^{-6})	6	0.70	1.20	0.080	0.496	0.482
	12	0.62	1.13	0.101	0.474	0.485
	24	0.49	1.00	0.200	0.600	0.600
	48	0.43	0.93	0.735	1.138	1.140
Equilib mixture (0.19×10^{-6})	6	2.52	3.06	0.196	0.387	0.388
	12	2.12	2.66	0.181	0.396	0.377
	24	1.33	1.88	0.195	0.387	0.387
	48	0.58	1.12	0.586	0.779	0.778

[a] $\tau_r = 0.49 \times 10^{-8}$ s at 25 °C for all systems. [b] Numbers in parentheses represent τ_M values (s).

fourth possibilities above. The resultant spectra are typical for those of enzyme-Mn(II) (Levy and Villafranca, 1976), proving that the addition of oxalylglycine does not result in displacement of Mn(II) bound to the enzyme. In addition, although the stability constant for Mn(II)-oxalylglycine is not known with certainty, it can be reasonably assumed to be of the same order of magnitude as that for Mn(II)-α-ketoglutarate (Sillen and Martell, 1971) and, thus, does not compete with the enzyme for Mn(II). Therefore, one of the first two alternatives seems likely.

Of the remaining possibilities, the advocation of direct binding of the inhibitor to manganese seems the least plausible. The largest decrease in the measured paramagnetic relaxation rates during any of the three oxalylglycine titrations is only 18%, and there was no observed change in the EPR spectrum of bound Mn(II) upon addition of saturating levels of this inhibitor. This latter fact is also consistent with the suggestions that the inhibitor does not bind to Mn(II) but that oxalylglycine induces a conformational change at the metal ion site resulting in restricted solvent exchange but little change in coordination geometry.

In oxalylglycine titrations where the enzyme was saturated with TPNH, $1/T_{1p}$ values at first decrease and then increase sharply at inhibitor concentrations which corresponded approximately to a 1:1 oxalylglycine to enzyme ratio. This suggests the possibility that the small amount of free Mn(II) may now be totally bound to the enzyme and that the presence of this analogue is responsible for the additional Mn(II) binding. However, the change may be due to a slight conformational change in the bound Mn(II) environment since the $1/T_{1p}$ value recorded for the titrant solution alone (a situation that duplicates the conditions present at the final stages of the actual titration) is nearly identical with the lowest $1/T_{1p}$ value recorded prior to the marked upturn. This result seems to indicate that this upturn is the result of an initial conformational adjustment of the enzyme which permits Mn(II)-bound H_2O to be more readily exchanged with solvent water.

TABLE III: T_{1p}/t_{2p} Ratios for Various Complexes of Isocitrate Dehydrogenase.

Solution No.	Complex	NMR Frequency	T_{1p}/T_{2p}
1	ICDH-Mn	24	1.39
		48	4.27
2	ICDH-Mn-TPNH	24	1.56
		48	3.33
3	ICDH-Mn-isocitrate	24	1.53
		48	4.28
4	ICDH-Mn-oxalylglycine	24	2.10
		48	4.95
5	ICDH-Mn-L-α-hydroxyglutarate	24	1.95
		48	3.38
6	ICDH-Mn-L-glutamate	24	2.31
		48	4.16
7	ICDH-Mn-α-ketoglutarate	24	2.47
		48	4.44
8	ICDH-Mn-α-ketoglutarate-TPNH-HCO_3^-	24	1.74
		48	4.30

The K_D values determined for TPN (~60 μM) and TPNH (~7 μM) are upper limits for these constants since our experiments were performed under conditions where [enzyme] > [Mn(II)]. During the course of the titration, the nucleotide concentration varied from values lower to values higher than the enzyme concentration and therefore large corrections had to be made for the amount of nucleotide bound to enzyme that contained no metal ion. The agreement between our K_D values and those of Ehrlich and Colman (1975) (K_D for TPNH of ~2 and μM and K_D for TPN of ~50 μM) are satisfactory considering the differences in experimental conditions.

Extensive studies were performed of the frequency and temperature dependence of the PRR of water interacting with enzyme–Mn(II) complexes. These studies were conducted with complexes of substrates or inhibitors, TPN or TPNH, in the absence or presence of bicarbonate. All the data were successfully analyzed in terms of the Solomon–Bloembergen–Morgan scheme as was previously reported for other isocitrate dehydrogenase complexes (Villafranca and Colman, 1974).

The present data demonstrate features that can be used to gain an insight into understanding how substrates or inhibitors bind near or at the enzyme-bound metal ion. For example, the values at every frequency decrease in going from the binary mixture to the ternary nucleotide complex (as demonstrated earlier by the titration data at 24 MHz), but the relative positions of the frequencies and their corresponding temperature dependence patterns remain essentially unchanged. The binary complex data are similar in all respects to results obtained earlier by Villafranca and Colman (1974), with the exception that here the 48-MHz data display a positive slope at lower temperatures.

This latter characteristic is difficult to rationalize when compared with the temperature behavior of the relaxation rates predicted by the Solomon–Bloembergen–Morgan equations (Villafranca and Colman, 1974; Villafranca et al., 1976). Examples of systems which display negative slopes at higher temperatures and positive slopes at lower temperatures for a given frequency are rare, and no satisfactory explanation for such behavior has been offered. Attempts to generate such curves by computer variation of the values for individual physical parameters have been unsuccessful.

With decreasing temperatures, the τ_M values determined by the computer fit decrease while the other physical parameters remain essentially unchanged. However, the total percentage error increased with decreasing temperature (not in excess of 20%, however), and could not be reduced despite manipulation of the individual parameters. However, many of the properties of the systems under discussion can be analyzed by consideration of the data in the high-temperature range only.

Since the 6-MHz data in Figure 4 are lower than the 24-MHz data, τ_s must contribute to τ_c since this is the only time constant that is frequency dependent (Bloembergen and Morgan, 1961).[3] Also, the 6-MHz data for the binary complex have a nearly zero slope. This suggests either of two possibilities: first, $\tau_M \simeq T_{1M}$ with τ_M dominating at low temperatures, and T_{1M} dominating at higher temperatures, or $T_{1M} > \tau_M$ and $\omega_I\tau_c \simeq 1$ for the 6-MHz data. A similar situation exists for the TPNH complex; and the computer fit to the data shows that τ_M may be important in the determination of the relaxation rates in both systems but that $T_{1M} \simeq \tau_M$ for frequencies greater than 12 MHz (Table II). Overall, the temperature dependence for the four frequencies suggests a correlation time of the order of 10^{-8} s.

For the data with α-ketoglutarate shown in Figure 5 (the quaternary complex with TPNH and the quinary complex with bicarbonate), the temperature dependence patterns for all frequencies display a positive slope in the 3.4 to 3.7 region, and a zero or negative slope at higher temperatures for all frequencies but 6 MHz. Similar data were obtained by Villafranca and Colman (1974) for ternary enzyme–Mn–α-ketoglutarate complexes, except that the order of the frequencies is slightly different. The decreasing order for the $1/T_{1p}$ values for the ternary complex was 24, 12, 6, and 48 MHz. In the quaternary complex, the 24-MHz values were shifted below the 6-MHz data, and, in the quinary complex, the 6-MHz values were shifted above the 24–12-MHz data.

Again, the data below 3.45 (above 17 °C) are suggestive of a correlation time $\leq 10^{-8}$ s. It is interesting to note that the upward curvature at lower temperatures occurs essentially at all frequencies in the α-ketoglutarate complexes. Villafranca and Colman (1974) ascribed the positive slopes in this region to a decrease in the effective number of water molecules bound to Mn(II) with increasing temperature. The decrease is attributed to a possible alteration in the mode of α-ketoglutarate binding to the enzyme complex in going from low to higher temperatures, involving a more direct interaction between metal and substrate at higher temperatures. However, a decrease in the effective number of bound water molecules could also occur if a small conformational change of the enzyme took place as the temperature increased serving to impede H_2O exchange with bulk solvent.

For the data in Figure 6, the computer fits for oxalylglycine were generally less than 10% total error for the four frequencies, while the L-α-hydroxyglutarate and glutamate data were fit within 33% total error at certain temperatures. Oxalylglycine is a significantly more effective inhibitor for isocitrate dehydrogenase than either glutamate or L-α-hydroxyglutarate. The K_I of the former is ~350–400 μM, whereas the K_I of the latter two analogues is μ2.0 mM (Levy, 1976).

There were no gross discrepancies in the overall computer fits as judged by an inspection of the figures since the solid lines are the computer-generated fits to the experimental data. For oxalyglycine, the data recorded above 18 °C again seems to suggest a correlation time of the order of $\sim 2.8 \times 10^{-8}$ s, with a small τ_M contribution. The high temperature data for L-α-hydroxyglutarate also suggest a τ_c value around 3×10^{-8} s, and a similar value is predicted for glutamate.

The relaxation rates for the isocitrate–enzyme–Mn(II) solution were determined only at 25 °C since this complex was studied in detail earlier by Villafranca and Colman (1974). The total error for the best fit to the isocitrate data was ~5%.

The correlation times for the individual systems all lie in the $(0.3\text{–}2) \times 10^{-8}$ s range (since the correlation times themselves are frequency dependent, the τ_c value for each frequency varies). These τ_c values are modulated predominantly by τ_s, as shown in Table II, with a distinct contribution from τ_r. This is unusual as noted earlier, since τ_r is generally an insignificant factor in the data analysis for Mn(II)-activated enzymes. The reason it is predominant for these data is because of the relatively small size of isocitrate dehydrogenase (60 000 mol wt). τ_r values were determined from a Stokes law calculation (Levy, 1976).

The $1/\tau_s$ values are observed to decrease with increasing frequency as expected. At 6 MHz, $1/\tau_s$ values exceed $1/\tau_r$ values by a ratio of between two and ten to one, depending upon

[3] Three correlation times can contribute to the correlation time which describes the overall processes that modulate the dipolar electron–nuclear interaction between Mn(II) and the water protons:

$$1/\tau_c = 1/\tau_r + 1/\tau_s + 1/\tau_m \quad (3)$$

where τ_r is the time constant for rotation of the complex, τ_s is the longitudinal electron spin relaxation time, and τ_m is the residence time of the nucleus in the primary coordination sphere of the paramagnetic metal ion. τ_c is determined by the fastest rate process, i.e., the shortest τ value. For small molecular weight complexes, $\tau_c \simeq \tau_r$ and the $1/T_{1p}$ values are similar to those for aquo-Mn, for which temperature-frequency dependence measurements have been recorded (Reuben and Cohn, 1970). For larger Mn(II) complexes, $\tau_c \simeq \tau_s$, with some contribution from τ_r for isocitrate dehydrogenase (Villafranca and Colman, 1974). In most cases, the concentrations of small molecular weight complexes were less than 5% of the concentration of the primary macromolecular complexes and therefore did not contribute significantly to the $1/T_{1p}$ values.

the system in question. This ratio decreases progressively as the frequency increases, and the ratio approaches unity at 48 MHz for each solution. In all solutions, and at all frequencies, the τ_M value makes almost no contribution to τ_c. The value of τ_M does contribute somewhat in the determination of the $1/T_{1p}$ values (Table II). At the lowest $(1/T_{1p})b$ values (in all cases recorded at 48 MHz), τ_M had the smallest contribution to the observed rates. The relative magnitude of the τ_M contribution was of course a function of frequency and temperature since the former variable affects the T_{1M} and the latter affects both T_{1M} and τ_M.

An interesting similarity in the τ_v values for certain of the solutions also became apparent. τ_v is the time constant for symmetry distortions of the Mn(II) complex that modulates the electron spin relaxation time τ_s (Bloembergen and Morgan, 1961). Interpretation of τ_v data for enzyme-bound Mn(II) is not completely straightforward but an analogy can be drawn between such impact times and the relative accessibility of the manganese coordination sphere to penetration by solvent water. In this regard it is noteworthy that the three solutions containing TPNH with enzyme substrates displayed τ_v values in a narrow 6.5–8.0 ps range. The τ_v values for complexes containing the substrate analogues were distinctly lower than this (2–3 ps). This observation suggests that TPNH binding to isocitrate dehydrogenase imparts a distinct and characteristic conformation upon the bound Mn(II) which seems to be essentially invariant in the presence of any combination of natural substrates or cofactors.

Neglecting hyperfine contribution to T_{2p} as well as the τ_M contribution to T_{1p} and T_{2p}, estimated τ_c values can be determined from the T_{1p}/T_{2p} ratio for the 24- and 48-MHz data using a rearranged form of the Solomon-Bloembergen equations (Villafranca and Colman, 1974). The results for each system varied only slightly with frequency, and all τ_c values were within the narrow range of 4×10^{-9} to 8×10^{-9} s. Thus, the overall computer fits to the $1/T_{1p}$ data along with the T_{1p}/T_{2p} ratios at two frequencies have defined all of the PRR parameters for the various complexes of isocitrate dehydrogenase.

In summary, the substrates isocitrate or α-ketoglutarate bind close to, if not directly in, the coordination sphere of enzyme-bound Mn(II). The inhibitors oxalylglycine, L-α-hydroxyglutarate, and L-glutamate also bind near the Mn(II) but probably not in the inner coordination sphere. The nucleotides, TPN and TPNH, interact with the enzyme in a different manner. TPNH weakens Mn(II) binding to the enzyme (Levy and Villafranca, 1977) while TPN does not. However, TPNH binding does alter the accessibility of Mn(II) to bulk solvent as demonstrated by a decrease in $1/T_{1p}$ values for water interacting with enzyme-Mn(II). Thus both EPR results presented in the previous paper and the NMR results reported herein provide information about Mn(II), substrate, and cofactor interactions with isocitrate dehydrogenase that are important for an understanding of the catalytic function of this enzyme. It has been previously speculated that Mn(II) functions to aid in the decarboxylation of oxalosuccinate by stabilizing an intermediate enolate of α-ketoglutarate (Villafranca and Colman, 1974). The data reported in the previous paper and in this one demonstrate that the nucleotide is very important in stabilizing certain enzyme conformations and these enzyme "states" may be related to enzyme conformational changes that occur during catalysis.

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