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Structure–Function Relationships in TPN-Dependent Isocitrate Dehydrogenase. I. Electron Paramagnetic Resonance Studies of the Interaction of Enzyme-Bound Mn(II) with Substrates, Cofactors, and Substrate Analogues†

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ABSTRACT: Electron paramagnetic resonance (EPR) spectra were obtained for various isocitrate dehydrogenase–Mn(II) complexes. The qualitative effects of the binding of substrates, nucleotides, and substrate analogues on the isotropic character of the electronic environment of enzyme-bound Mn(II) were subsequently investigated. The addition of isocitrate produces a markedly anisotropic spectrum whereas α-ketoglutarate does not alter the spectrum of enzyme–Mn(II) substantially. This suggests direct coordination of isocitrate to the Mn(II) but perhaps a different mode of binding for α-ketoglutarate. Other studies demonstrated mutually exclusive binding relationships between TPN and TPNH, between Mn–isocitrate and TPNH, and between HCO_3^- (CO_2) and formate or thiocyanate. Indirect evidence supporting CO_2 rather than HCO_3^- as the actual reactive species which binds to the enzyme in the reductive carboxylation reaction is presented, on the basis of the results of the formate and thiocyanate studies. From the EPR results recorded for ternary, quaternary, and quinary enzyme–substrate complexes, correlations between the appearance of fine structure signals and the binding of individual substrates and/or nucleotides are found, and tentative assignments of such signals are made on this basis. Additional studies were conducted to determine binding constants for Mg(II), Co(II), and Co–isocitrate, and a comparison was made with kinetically determined binding constants.

TPN-dependent isocitrate dehydrogenase (*threo*-D_S-isocitrate:TPN oxidoreductase (decarboxylating), EC 1.1.1.42) is found in both the mitochondria and the cytoplasm of mammalian tissues. It is composed of only one subunit, and its molecular weight is approximately 60 000 (Plaut et al., 1957).

The enzyme catalyzes the oxidation of the D_S isomer of isocitrate (I) to produce oxalosuccinate (II) which is then decarboxylated to α-ketoglutarate (III). The reaction proceeds

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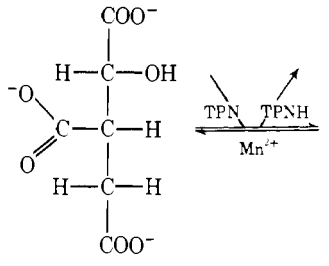
with a net retention of configuration in the replacement of the carboxyl group by a solvent proton (Rose and Lienhard, 1963):



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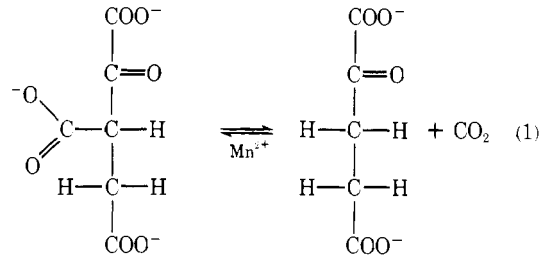

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In order for the reaction to occur, triphosphopyridine nucleotide (TPN) is required as a coenzyme, and a divalent metal ion as a cofactor. Several metal ions have been used to catalyze the reaction with varying degrees of efficiency, but Mn(II) provides the best activation with the highest overall maximum velocity (V_{max}) (Colman, 1972). The metal ion is believed to be coordinated to the C-1 carboxyl and C-2 hydroxyl (-carbonyl) as well as to the enzyme during the reaction, facilitating proton removal from the hydroxyl and stabilizing the enolate ion (Villafranca and Colman, 1974). Additional mechanisms have also recently been proposed (Colman, 1975), implicating methionyl, cysteinyl, and glutamyl residues at the active site with the metal ion binding the C-1 carboxyl and central carboxyl groups of isocitrate. All the data that have been obtained, however, implicate the metal ion as stabilizing the transiently formed enolate of α-ketoglutarate during catalysis.

To date, much of the work done involving this enzyme concerned the determination of substrate and cofactor binding constants (Colman, 1969, 1972; Villafranca and Colman, 1972), determination of amino acid residues in the vicinity of the active site (Colman, 1968, 1973; Colman and Chu, 1970), and proton relaxation rate studies in the absence of nucleotides (Villafranca and Colman, 1972, 1974). The spatial orientations of the nucleotide, substrate, and metal ion sites have yet to be determined. The isotope exchange data of Rose (1960) led to the first speculation that TPNH binding might induce a conformational change in the enzyme while it was later suggested that TPNH and isocitrate occupy mutually exclusive binding sites on the basis of a decrease of TPNH fluorescence in the presence of isocitrate (Ehrlich and Colman, 1975).

Electron paramagnetic resonance techniques furnish a unique opportunity to examine the effects of substrate and cofactor binding on the environment of enzyme-bound metal ion, by taking advantage of the paramagnetic properties of the metal ion cofactor, Mn(II). By working with high concentrations of enzyme (0.3–1.0 mM), it is possible to monitor changes in the low intensity spectrum of enzyme-bound manganese upon the progressive addition of various substrates, cofactors, and their structural analogues. Qualitative examination of the gross features of such spectra can provide information concerning ligand substitutions, rearrangements, and structural or conformational changes in the vicinity of the paramagnetic probe. Studies of this type with isocitrate dehydrogenase are reported in this paper and have led to new conclusions about the binding and interactions of substrates and cofactors at the active site of the enzyme.

Experimental Section

Isolation and Purification of TPN-Dependent Isocitrate Dehydrogenase. The enzyme was initially obtained from fresh porcine hearts. The hearts were trimmed, homogenized, and partially purified using the previously described centrifugation and ammonium sulfate fractionation procedures (Cleland et al., 1969). Further purification was achieved by the use of carboxymethylcellulose and Sephadex G-150 chromatography (Colman, 1968). Some of the enzyme used in these studies was supplied in a 50% glycerol solution by Boehringer and was also purified to homogeneity via the column chromatographic procedures of Colman (1968).

A single band was observed in polyacrylamide gel electrophoresis in Tris buffer, pH 8.5. The specific activity of homogeneous enzyme samples was ~29 μmol min^{-1} mg^{-1} in a buffer containing 0.1 M NaCl–0.1 M triethanolamine–10% glycerol, pH 7.7. This standard buffer system was used throughout the course of this study.

Determination of Enzyme Activity. The specific activity of isocitrate dehydrogenase was measured at 25 °C, using the standard assay mixture (Colman, 1968), with the exception that $MnCl_2 \cdot 4H_2O$ was substituted for $MnSO_4$. Total activity was determined spectrophotometrically by monitoring the appearance of TPNH at 340 nm (the millimolar ϵ value = 6.22). The concentration of homogeneous enzyme was determined by measuring the optical density at 280 nm, where 1 mg/mL = 0.91 OD unit.

EPR Experimental Techniques and Theory. EPR measurements were recorded on a Varian E-12 spectrometer at 9.1 GHz (X band). Samples were placed in 1-mm i.d. quartz capillary tubes. Solutions were prepared in a small test tube and drawn into the capillary, and the capillary was plugged with a Teflon cap. A temperature of 1 ± 1 °C was maintained, in order to maximize enzyme stability and to minimize the amplitude of the free Mn(II) signal. This was achieved by passing nitrogen through a Dewar containing dry ice–ethanol and then heating it to the desired temperature. Samples were temperature equilibrated for several minutes prior to recording spectra.

For most crystal field strengths, the ^6S ground state of Mn(II) is by far the most stable energy state; hence spin–lattice relaxation times are long, and EPR signals should be easily observable for most crystal field symmetries and temperature ranges. However, spectra of Mn(II) macromolecular complexes have always been difficult to observe, despite the fact that there is little ground state–excited state interaction ("homogeneous" relaxation). This phenomenon has been attributed to the inability of the slow macromolecular rotation to isotropically average static distortions prevalent in large molecule complexes, resulting in an extremely asymmetric electronic environment (Reed and Cohn, 1970; Reed and Ray, 1971). A much larger zero-field splitting (ZFS)[1] of the Mn(II) electronic energy levels occurs, with the result that the line shape of the spectrum resembles that of a polycrystalline powder spectrum. The concentration of free hexa-aquo-Mn(II)

[1] Abbreviations used: ZFS, zero field splitting; EPR, electron paramagnetic resonance; Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance.

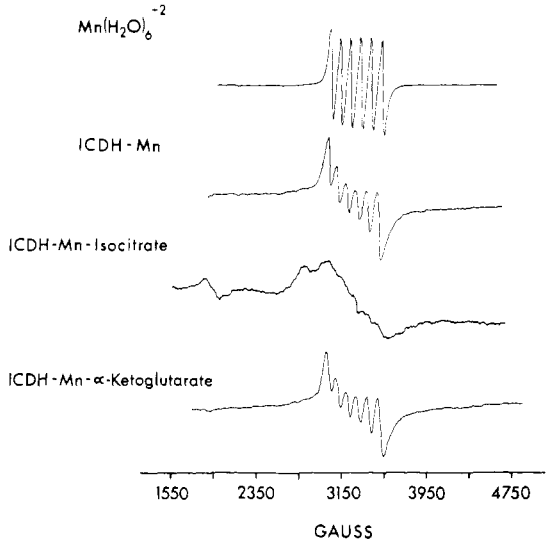


FIGURE 1: EPR spectra of binary and ternary complexes of divalent manganese and isocitrate dehydrogenase (ICDH) in the standard buffer. Temp = ±1 °C, modulation amp. = 20.0 G, power = 150 mW. Spectrum 1, 0.2 mM, $MnCl_2$, GAIN = 1×10^2; spectrum 2, ICDH = 1.01 mM, Mn = 0.20 mM, GAIN = 1.6×10^3; spectrum 3, ICDH = 0.94 mM, Mn = 0.19 mM, isocitrate = 2.34 mM, GAIN = 5.0×10^3; spectrum 4, ICDH = 0.92 mM, Mn = 0.19 mM, α-ketoglutarate = 1.81 mM, GAIN = 2.0×10^3.

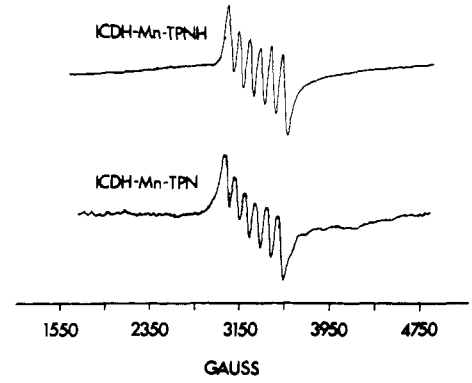


FIGURE 2: EPR spectra of ternary complexes of isocitrate dehydrogenase (ICDH) and nucleotides. Temp = ±1 °C, Mod. amp. = 20.0 G, power = 100 mW. Top: ICDH = 0.90 mM, Mn = 0.20 mM, TPNH = 1.0 mM, GAIN = 1.0×10^3. Bottom: ICDH = 0.90 mM, Mn = 0.20 mM, TPN = 0.95 mM, GAIN = 5×10^3.

in the sample must be kept to a minimum since its high intensity signal can easily mask the much weaker enzyme-bound manganese signal.

Results

EPR Spectra of Enzyme Solutions Containing Substrates. The EPR spectra of binary and ternary complexes of isocitrate dehydrogenase are displayed in Figure 1.

The spectrum of $Mn(H_2O)_6^{2+}$ consists of six hyperfine lines (Figure 1) due to the interaction of the electron spin moment with that of the nucleus ($I = 5/2$). For aquo-Mn(II) the cation is in a perfectly symmetrical, isotropically averaged environment, and each of the six lines is a superposition of the five electron-electron fine structure transitions that occur between the six electronic energy levels resulting from the d^5 configuration. The coordination sphere around Mn(II) is cubically symmetric, and the tumbling time for this small complex is sufficiently fast to average any environmental anisotropies.

At the concentration ratios specified for the binary enzyme-Mn(II) complex, it can be determined that approximately 95% of the added manganese is bound. The spectrum (Figure 1) suggests that the electronic environment of the metal ion is still quite isotropic—the absence of displaced fine structure transitions due to zero field splitting, and the smooth, regular line shape support this conclusion. When the spectrum was run at 15 °C, there was only a very slight increase in signal amplitude and sharpness, indicating that the contribution from free Mn(II) is negligibly small, a fact suggested by the pronounced downward sloping of the sextet. A spectrum of apoenzyme shows no Mn(II) signal.

Addition of *threo*-D_S-isocitrate in a 5:2 ratio with respect to the enzyme produces an extremely anisotropic "powder" spectrum (Figure 1). The ZFS is pronounced and several broad low-field fine structure transitions are observed. The pronounced spectral change upon addition of isocitrate strongly suggests binding of the substrate directly to or extremely near the manganese. Recently obtained proton relaxation rate data (Villafranca and Colman, 1974) support this hypothesis, as do earlier kinetics studies (Colman, 1972).

The anisotropy present in the isocitrate spectrum is in direct contrast to that obtained for a ternary enzyme–metal–substrate complex containing α-ketoglutarate (Figure 1). Enzyme complexes containing α-ketoglutarate bind Mn(II) much more weakly than their isocitrate counterparts (Villafranca and Colman, 1974). It follows that the EPR spectra of such complexes would exhibit much less anisotropy if the primary coordination sphere of Mn(II) was not greatly perturbed and the metal ion was more accessible to bulk solvent. The isotropic character of the spectrum is almost identical with respect to both line shape and amplitude to that of the binary enzyme–Mn(II) complex. Spectra of solutions containing a higher α-ketoglutarate to enzyme ratio are quite similar, with the exception of the appearance of weak ZFS signals below 2750 G on the low-field side of the sextet.

Quaternary Enzyme Complexes Containing Nucleotides. Addition of TPN to enzyme–Mn(II) complexes (TPN/enzyme ~1) results in a slight decrease in the sextet amplitude of the EPR spectrum, with the individual signals becoming more asymmetric (Figure 2). Addition of the reduced nucleotide to a similar solution in a similar ratio produces completely different results. The sextet amplitude sharply increases and becomes more well-defined, clearly indicating that the presence of TPNH leads to the release of a significant amount of Mn(II) from the enzyme surface (Figure 2). Since the concentration of the binary enzyme–Mn(II) complex is small with respect to the ternary nucleotide complex because of the high affinity of TPNH (K_D = 1.45 μM) for isocitrate dehydrogenase (Ehrlich and Colman, 1975), a calculation of the dissociation constant of manganese in the presence of a saturating level of TPNH (10 μM) could be performed. This value is 280 μM, and it represents an approximate sixfold increase over the K_D value of 45 μM found for the enzyme–manganese complex alone (Villafranca and Colman, 1972). Experiments were not conducted at subsaturating levels of TPNH.

As isocitrate is gradually added to a solution containing enzyme–Mn(III), the binary complex sextet progressively decreases in intensity as expected. At 1:1 isocitrate to Mn(II) ratios, six discrete transitions are no longer detectable. However, an intense broad signal appears at ~2870 G. In Figure 3A, the spectrum for an isocitrate to Mn(II) ratio of 3:1 is presented and is identical with the spectrum obtained for a 1:1 ratio. As shown in Figure 1, the intensity of this signal de-

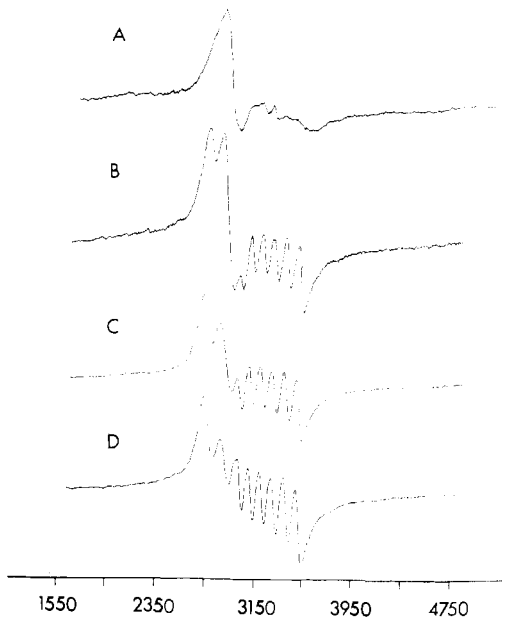


FIGURE 3: Effects of nucleotide binding to ternary isocitrate dehydrogenase (ICDH) complexes containing isocitrate. Temp = ±1 °C, mod. amp. = 20.0 G, power = 100 mW. Spectrum A, ICDH = 0.91 mM, Mn = 0.25 mM, isocitrate = 0.72 mM, GAIN = 4.0×10^3; spectrum B, ICDH = 0.90 mM, Mn = 0.24 mM, ISO = 0.81 mM, TPNH = 0.74 mM, GAIN = 4.0×10^3; spectrum C, ICDH = 0.87 mM, Mn = 0.35 mM, ISO = 1.33 mM, TPNH = 0.72 mM, GAIN = 2.0×10^3; spectrum D, ICDH = 0.84 mM, Mn = 0.34 mM, ISO = 1.28 mM, TPNH = 0.69 mM, TPN = 0.63 mM, GAIN = 2.0×10^3. Spectrometer frequency equals 9.128 GHz throughout.

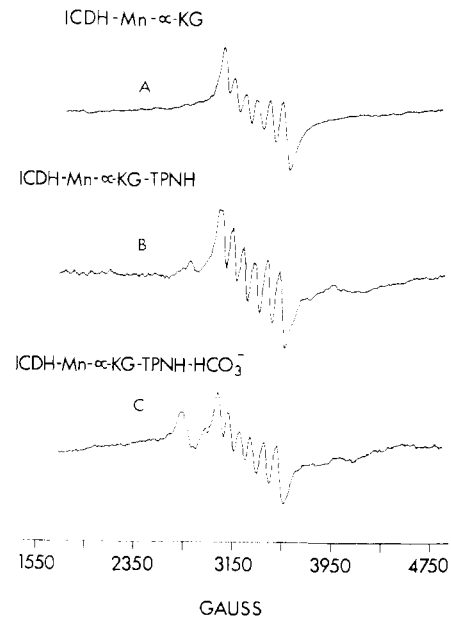


FIGURE 4: EPR spectra of ternary, quaternary, and quinary isocitrate dehydrogenase (ICDH) complexes of α-ketoglutarate. Temp = ±1 °C, mod. amp. = 20.0 G, power = 150 mW. Spectrum A, ICDH = 0.92 mM, Mn = 0.19 mM, α-ketoglutarate = 1.00 mM, GAIN = 2×10^3; spectrum B, ICDH = 0.93 mM, Mn = 0.19 mM, α-ketoglutarate = 0.99 mM, TPNH = 0.96 mM, GAIN = 4×10^3; spectrum C, ICDH = 0.77 mM, Mn = 0.15 mM, α-ketoglutarate = 0.89 mM, TPNH = 0.80 mM, HCO_3^- = 17.86 mM, GAIN = 4×10^3.

creases markedly when the isocitrate:enzyme ratio exceeds unity, and it is possible that this signal may be due to an enzyme-bound ternary species different from the one observed in Figure 1.

Addition of TPNH (Figure 3B) to the ternary enzyme-Mn-isocitrate complex produced a spectrum which contained features peculiar to both the enzyme-Mn-isocitrate and enzyme-Mn-TPNH species.

The gross details of the spectrum are similar to those of the solution prior to TPNH addition; i.e., both display a relatively small sextet amplitude compared with the large amplitude of the two low-field signals. Yet the sextet signal intensity has dramatically increased, indicating that more free Mn(II) exists in solution, a phenomenon associated with TPNH binding. The splitting of the 2870-G signal into an apparent doublet may be indicative of an enzyme-Mn(II)-TPNH-isocitrate complex, as will be discussed later.

Additional Mn(II) and isocitrate were added to see if a "powder" spectrum could be achieved, which would represent a complete replacement of the bound nucleotide by the added metal-carboxylic acid species. The spectrum thus obtained is shown as Figure 3C. The manner of the interaction of Mn-isocitrate with the enzyme is obviously altered by the unusually stable binding of TPNH ($K_M \sim 10^{-6}$ M). The K_M for Mn-isocitrate is of the order of 10^{-8} M, and it is thought to be the actual reactive substrate for the enzyme (Colman, 1972); yet the expected broadening due to the presence of an enzyme-Mn(II)-isocitrate species either does not appear or is perhaps masked by the presence of free Mn(II) in the solution.

The oxidized form of the nucleotide, TPN, was subsequently added to the solution that contained enzyme, Mn(II), isocitrate, and TPNH (Figure 3D). This was done to test the hypothesis that the two nucleotides bind in competitive fashion. If this is true, TPN should replace TPNH and therefore promote substrate turnover since all of the substrates and cofactors required to establish an equilibrium between isocitrate and α-ketoglutarate would then be present. This equilibrium lies predominantly in the direction of α-ketoglutarate (Cleland et al., 1974).

As shown in Figure 1, the presence of α-ketoglutarate produces a more isotropic electronic environment around Mn(II), with a weakening of the metal-substrate interaction. This subsequently produces a smoother, less distorted EPR line shape of increased amplitude. The spectrum shown in Figure 3D displays characteristics of the enzyme-Mn(II)-α-ketoglutarate spectrum (Figure 4A) as well as the low-field signals characteristic of isocitrate-TPNH enzyme species. Thus, the resulting spectrum has components which demonstrate the formation of α-ketoglutarate from TPN and isocitrate. Addition of larger amounts of TPN produced spectral changes which more closely resembled the spectrum in Figure 4C, although the line shape of the latter cannot be completely reproduced.

Figure 4 displays spectra recorded for the ternary enzyme-Mn-α-ketoglutarate complex, the quarternary complex with TPNH, and the quinary complex with bicarbonate (an equilibrium mixture). All solutions contain approximately 1:1 α-ketoglutarate to enzyme ratio. Spectra of solutions containing higher substrate concentrations display very weak ZFS patterns below 2750 G, but the higher field regions are nearly identical with those shown here.

The addition of TPNH to the ternary complex (Figure 4B) produces a more distorted line shape and a slight decrease in the amplitude of the sextet. A broad shoulder on the immediate low-field side of the sextet (~2900 G) and the distinct 2760-G signal are noteworthy. No precautions were taken to eliminate the HCO_3^- present in the buffers due to equilibration with

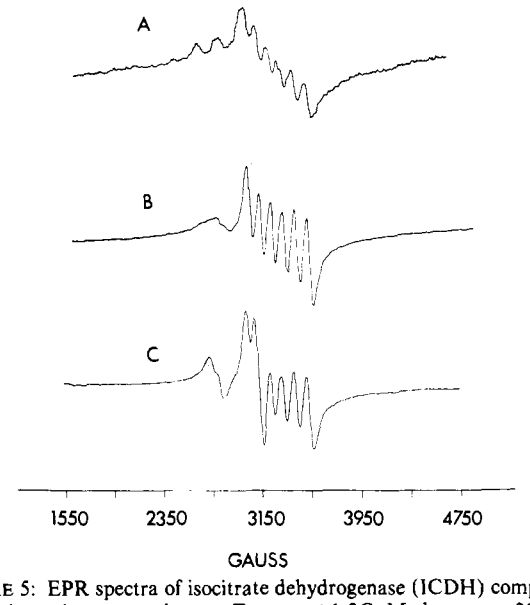


FIGURE 5: EPR spectra of isocitrate dehydrogenase (ICDH) complexes containing substrate analogues, Temp = ±1 °C, Mod. amp. = 20.0 G, power = 150 mW, GAIN = 4.0×10^3. Spectrum A, ICDH = 0.83 mM, Mn = 0.15 mM, isocitrate = 2.09 mM, TPN = 0.83 mM, HCO_3^- = 4.80 mM, formate = 5.29 mM; spectrum B, ICDH = 0.95 mM, Mn = 0.19 mM, L-α-hydroxyglutarate = 0.99 mM, TPNH = 0.89 mM; spectrum C, ICDH = 0.85 mM, Mn = 0.17 mM, L-α-hydroxyglutarate = 6.85 mM, TPN = 0.85 mM.

atmospheric CO_2. Therefore this spectrum represents a solution with a low bicarbonate concentration (~1 mM).

Introduction of saturating quantities of bicarbonate produces an increase in the resolution and spectral amplitude of the low-field transitions (Figure 4C). At this pH and temperature, the large majority of added bicarbonate remains dissolved in solution, and the loss of bicarbonate to the atmosphere via CO_2 escape is insignificant (Umbreit et al., 1964).

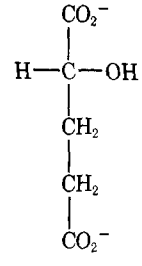
EPR Studies with Substrate Analogues. The question as to whether CO_2 or bicarbonate is the actual reactive species that binds to the enzyme in the reverse reaction (isocitrate formation) is still unsettled (Plaut et al., 1957). One approach to this problem is the substitution of structural analogues of each of the two species in ternary and higher order enzymatic complexes. Thiocyanate (SCN^-) has been chosen as a CO_2 analogue because of its linearity, size, and solubility in polar solvents. The main drawback is its negative charge. Formate (CHO_2^-) is a good analogue of bicarbonate by virtue of its similar charge, structure (planar), and atomic composition.

In general, in all equilibrium or nonequilibrium experiments in which these analogues were tested (enzyme–Mn–substrate–nucleotide plus HCO_3^- or analogue), it was found that they restored the appearance of low-field fine structure transitions which the previous addition of bicarbonate/CO_2 had eliminated. Bicarbonate/CO_2 binding appeared to induce a conformational change which decreased the amount of anisotropy in the Mn(II) electronic environment by effectively eliminating ZFS effects, while the sextet amplitude was essentially unaltered.

In contrast, the addition of either formate or thiocyanate reproducibly increased the amount of fine structure in a similar fashion when added subsequent to HCO_3^-/CO_2. The appearance of distinct ZFS signals at 2670 and 2840 G always attended the addition of either analogue (without modifying the sextet signal), although the relative intensities and line widths of the two signals sometimes differed slightly between different enzyme complexes. For a given complex, the two ZFS signals produced by each analogue possessed a characteristic relationship. The concentration of either analogue was routinely between 5:1 and 8:1 vs. enzyme, and similar effects were observed even when HCO_3^-/CO_2 concentrations were significantly higher than these values.

Replacement of thiocyanate by formate resulted in the slight modification of the line shape of the two signals that was characteristic of the latter analogue. Hence, binding of either analogue appeared to be competitive with HCO_3^-/CO_2 as well as with each other since alternating additions reproduced the spectral changes peculiar to the added species, which the previous addition had eliminated. Preliminary kinetic data also substantiate the competitive nature of this interaction (Villafranca, unpublished observations). A spectrum of a solution containing an equilibrium mixture of enzyme components, to which first bicarbonate then formate was introduced, appears in Figure 5A.

L-α-Hydroxyglutarate is a substrate analogue that lacks the C-3 carboxylate group present in isocitrate and the C-2 carbonyl moiety present in α-ketoglutarate. However, quaternary complexes containing nucleotides and L-α-hydroxyglutarate display spectral characteristics which are reminiscent of those which appear in the spectra of similar complexes of the natural enzyme–substrates.



L-α-hydroxyglutarate

In solutions containing the reduced nucleotide, enzyme–L-α-hydroxyglutarate complexes produce spectra such as that shown in Figure 5B. The solution contains an analogue to enzyme ratio of 1:1, and the line shape is essentially invariant when larger amounts of the analogue are introduced. The spectrum contains a broadened, yet distinctive, fine structure component centered at ~2790 G. Bicarbonate addition to this solution eliminated nearly all of the ZFS intensity on the low-field side of the sextet, unlike the situation that exists in the quinary α-ketoglutarate spectrum (Figure 4C). Since even high concentrations of the analogue failed to produce the anisotropic "powder"-type spectra observed for the corresponding isocitrate complexes, it appears that the interaction of the C-3 carboxylate with the enzyme–metal binding site is an important factor in binding and catalysis.

The addition of TPN to a ternary complex containing enzyme–Mn(II) and L-α-hydroxyglutarate produces the spectrum shown in Figure 5C. The complex, which is not catalytically active, displayed a strong doublet line shape reminiscent of that observed in isocitrate complexes containing TPNH (Figure 3B). This may be the result of an extremely slow conformational change which affects the immediate environment of the enzyme-bound Mn(II).

Determination of Metal Ion Cofactor Binding Constants. In addition to Mn(II), Mg(II) and Co(II) can serve as cofactors in the overall enzymatic oxidative decarboxylation of isocitrate. Activation constants (K_M values) have been determined for these metal ions (Northrop and Cleland, 1970). These are kinetic, not thermodynamic constants, and therefore

TABLE I: Displacement of Mn(II) by Co(II) in Binary Complexes of Isocitrate Dehydrogenase (ICDH).

Solution Added	Final vol (μL)	Total ICDH (μM)	Total Mn(II) (μM)	Total Co(II) (mM)	% free Mn(II)	Co/Mn	App K_D (μM)
Mn-buffer standard	36		60.0		100.0		
Mn-ICDH	36	249	60.0		33.0		
20 mM Co(II)	37	242	58.4	0.54	44.7	9.26	140
20 mM Co(II)	38	236	56.8	1.05	50.5	18.5	240
40 mM Co(II)	39	230	55.4	2.05	61.7	37.0	289
40 mM Co(II)	40	224	54.0	3.00	69.6	55.6	296
100 mM Co(II)	41	219	52.7	5.37	79.7	102.0	301

TABLE II: Affinity of Isocitrate Complexes of Cobalt and Manganese for Isocitrate Dehydrogenase (ICDH).

Experiment	Solution added	Total ICDH (μM)	Total Mn(II) (μM)	Total Co(II) (mM)	Total ISO (mM)	% free Mn(II)	Co/Mn
1	Mn-buffer standard		60.0			100.0	
2	Mn-ICDH	249	60.0			33.5	
3	2 plus Co(II)	242	58.4	0.54		44.4	9.26
4	3 plus isocitrate	236	56.8	0.53	0.24	30.0	9.26
5	4 plus isocitrate	224	54.0	0.50	0.70	16.8	9.26
6	5 plus Co(II)	213	51.4	5.24	0.66	72.9	102.0
7	6 plus isocitrate	204	49.1	5.00	1.08	64.8	102.0

contain, in addition to the enzyme-metal dissociation constants, additional constants which may reflect the affinities of other substrates for metal ion and enzyme. The K_M values obtained in this manner were ~2.0 mM for Mg(II) and ~11 μM for Co(II). The extremely tight value for Co(II) may reflect the affinity of the cobalt-isocitrate species for the enzyme. The cobalt complex with isocitrate has the highest stability constant of the three cations; Mn-isocitrate is lower, while Mg-isocitrate is clearly the weakest (Bolard and Chottard, 1974; Grzybowski et al., 1970).

Apparent dissociation constants for each of the metal ions were determined at ~15 °C, by taking advantage of the fact that displacement of enzyme-bound manganese by either metal ion will give the usual isotropic EPR signal for Mn(II), and the amount of free manganese can be determined by comparing the amplitude of the resulting signal with that of a previously measured standard.

The K_D value for each metal ion was determined using the following equations (using Co(II) as an example)

$$(\text{ICDH})_{\text{free}} = \frac{K_D^{\text{Mn}}[(\text{Mn})_{\text{total}} - (\text{Mn})_{\text{free}}]}{(\text{Mn})_{\text{free}}} \quad (2)$$

$$[\text{ICDH-Mn}] = (\text{Mn})_{\text{total}} - (\text{Mn})_{\text{free}} \quad (3)$$

$$[\text{ICDH-Co}] = (\text{ICDH})_{\text{total}} - (\text{ICDH})_{\text{free}} - (\text{ICDH-Mn}) \quad (4)$$

$$K_D^{\text{Co}} = \frac{[(\text{Co})_{\text{total}} - (\text{ICDH-Co})][(\text{ICDH})_{\text{free}}]}{[\text{ICDH-Co}]} \quad (5)$$

where ICDH = isocitrate dehydrogenase.

The dissociation constants determined in this manner represent an upper limit. The calculated K_D values for cobalt initially increase at low Co(II) concentrations and then asymptotically approach a constant value at the point where essentially all of the enzyme-bound Mn(II) has been displaced by the added metal ion. This final value is taken as the apparent dissociation constant.

$MnCl_2 \cdot 4H_2O$ dissolved in standard buffer was used as the "control" with which all other solutions were calibrated. Mn(II)-enzyme solutions were prepared in which approximately two-thirds of the total Mn(II) was bound to the enzyme. Buffer solutions of $CoCl_2 \cdot 6H_2O$ or $MgCl_2$ were then added to one of the enzyme solutions. A summary of the cobalt data appears in Table I.

The apparent K_D value determined for Co(II) was approximately 300 μM. For Mg(II), the addition of much larger amounts of metal ion is required to displace the Mn(II). When 85% of the total manganese is free, the apparent K_D value for Mg(II) is ~2.0 mM. This final K_D value for Mg(II) agrees well with the kinetically determined K_M value noted above.

In order to compare the binding of Co(II)-isocitrate and Mn(II)-isocitrate to the enzyme, varying proportions of Co(II) and isocitrate solutions were added to a solution containing Mn(II) and enzyme. The resulting binding patterns are presented in Table II.

It can be seen that the percentage of free Mn(II) decreased when isocitrate was added. This suggests that, although Co(II)-isocitrate has a larger stability constant than Mn(II)-isocitrate, the affinity of the latter for the enzyme is greater. However, subsequent introduction of additional Co(II) results in an increase in the concentration of free manganese. This observation seems to indicate that cobalt may successfully compete with the enzyme for bound isocitrate, thus facilitating dissociation of bound Mn(II), since the dissociation constant for Mn(II) for the binary enzyme complex is approximately three orders of magnitude greater than that for Mn(II) dissociation from the ternary isocitrate complex.

Using the CRAMS (Chemical Reaction Analysis and Modeling System) computer program (Butler and deMaine, 1975), the equilibrium concentrations of individual species were computed using the simultaneous multiple equilibria for the various complexes. The dissociation constant for the cobalt-isocitrate complex was ~50 μM, from the published range of values (Bolard and Chottard, 1974); the cobalt-enzyme dissociation constant was 300 μM, as determined above. The K_M value of 11 μM was taken as the binding constant of the cobalt-isocitrate complex to the enzyme and was used as an initial input for the K_D value for this complex. This K_D value was then adjusted until the free manganese concentration predicted by the computer program matched the value deter-

mined by EPR. The K_D value thus determined was 110 μM, approximately ten times weaker than the cobalt K_M value. The K_M value may therefore indicate that the presence of TPN tightens the binding of Co-isocitrate to the enzyme. Using the K_D value of 110 μM for the ternary cobalt complex, computed values for free manganese for solutions 5–7 were then determined. The observed and computed data are compared in Table III.

TABLE III: Comparison of Calculated and Observed Free Manganese Concentrations in Solutions of Cobalt(II), Isocitrate, and Isocitrate Dehydrogenase.

Solution No.	Obsd free Mn(II) (μM)	Calcd free Mn(II) (μM)
4	17.0	16.9
5	9.0	6.6
6	37.5	40.9
7	31.8	38.7

Discussion

In a continued effort to understand the nature of the metal ion participation in the reactions catalyzed by TPN-dependent isocitrate dehydrogenase, this paper reports changes in the environment of enzyme-bound Mn(II) upon addition of substrates and substrate analogues. Importantly, the EPR spectrum of Mn(II) in the various complexes was obtained in the solution state and distortions in the metal ion environment can be readily observed for this paramagnetic $S = 5/2$ ion.

The predictable occurrence of certain fine structure transitions provides evidence for the tentative assignment of such signals to their respective isocitrate- or α-ketoglutarate-containing species. There exists repeated suggestion that the signal in the 2870–2900-G region is indicative of the presence of isocitrate-Mn(II)-enzyme complexes (Figure 3A). The signal is strongest in ternary enzyme-Mn-isocitrate complexes containing a range of isocitrate concentrations, varying from isocitrate-Mn(II) ratios of 1:1, to isocitrate-enzyme ratios of 1:1. It drastically decreases in intensity at higher isocitrate concentrations as does the sextet itself. The signal appears weakly in the severely anisotropic spectrum of the 5:2 isocitrate-enzyme ternary complex (Figure 1). Perhaps at higher isocitrate to enzyme ratios isocitrate can bind at additional sites on the enzyme; this alternative explanation for the anisotropic spectrum in Figure 1 is not substantiated by the binding studies of Ehrlich and Colman (1975) that demonstrate only one isocitrate binding site. In any case, the signal at ~2890 G is clearly associated with ternary isocitrate complexes.

The oxidized and reduced forms of the nucleotide are thought to bind at a site other than the Mn(II) site and to maintain a specific protein structural conformation conducive to the facile interconversion of the carboxylic acid substrate (Villafranca and Colman, 1972; Rose, 1960). If this is so, the enzyme conformations dictated by their binding may be different from those in complexes which lack TPN or TPNH. Once again the signals at ~2890 G attributed to the ternary isocitrate-enzyme-Mn complex are present and even more distinct in all the isocitrate solutions containing the nucleotides (Figure 3), as well as in the two α-ketoglutarate equilibrium mixture spectra (Figures 4B and 4C).

More importantly, the behavior of the signal intensity and line shape correlates with predicted changes in isocitrate concentrations relative to concentrations of other components in the equilibrium mixture. The signal at 2870 G decreases from Figure 3C to 3D due to TPN addition. This is understandable in terms of the progressive disappearance of the isocitrate complex, since the turnover to form α-ketoglutarate would diminish the concentration of isocitrate-enzyme species. It is also observed as a weak shoulder on the sextet in Figure 4B, where a small amount of isocitrate exists due to the presence of dissolved CO_2 (HCO_3^-), which completes the list of components needed for isocitrate formation. Successive additions of bicarbonate also enhance the intensity of this signal, as would be expected, due to the increased concentration of bound isocitrate produced by catalysis. It should be pointed out that the spectra of solutions that contain the components of the equilibrium mixture may consist of the superposition of two or three different Mn(II) spectra. It is still clear, however, that the low-field line(s) are characteristic of an enzyme-Mn(II)-isocitrate complex. If Mn(II)-isocitrate binds to the enzyme surface but the nature of the enzyme-substrate interaction is modified due to a TPNH-induced conformational change then the spectrum in Figure 3C may be due to the enzyme-Mn(II)-isocitrate-TPNH complex, although this conclusion is not substantiated by the data of Ehrlich and Colman (1975).

Introduction of TPNH into solutions containing ternary α-ketoglutarate-enzyme complexes (Figure 4B) results in the appearance of a distinct signal at ~2750 G. Fine structure signals in this immediate vicinity are also present in all spectra containing enzyme-bound TPNH. In the spectra of Figures 3B and 3D with TPNH and isocitrate complexes, it is the strongest recorded signal. Also, in Figure 4C (which represents a large addition of bicarbonate to the quaternary α-ketoglutarate complex), the signal intensity again increases as expected. Bicarbonate addition appears to stabilize the formation of enzyme complexes of α-ketoacid substrates, i.e., oxalosuccinate and/or α-ketoglutarate (Levy and Villafranca, unpublished observations), here increasing the concentration of the TPNH-α-ketoglutarate species. Thus the 2750-G signal appears to be representative of the presence of enzyme-bound TPNH and α-ketoglutarate (Figure 4). When all the components necessary to establish the substrate interconversion equilibrium are present, the signal at 2750 G is most prominent (Figure 4C).

By determining the respective areas under the 2750- and 2870-G signals in Figure 3C, which represents an equilibrium mixture, an approximation of the isocitrate to α-ketoglutarate ratio can be determined. The value of 0.33 suggests, as does the kinetically determined equilibrium constant of 1.1 M (Cleland et al., 1974), that the equilibrium established among enzyme-bound species clearly lies in the same direction as the overall thermodynamic equilibrium for reactants and products.

All the spectra displayed in Figure 1, as well as those in Figures 3B and 3C, represent solutions which are predominantly composed of a single major enzyme species. As such, it was possible to completely simulate the essential features of these spectra by using a computer program to generate solutions to the simplified spin-Hamiltonian (Reed and Ray, 1971):

$$\mathcal{H}_S = g\beta\mathcal{H} \cdot S + D[S_z^2 - \tfrac{1}{3}S(S+1)] + E[S_x^2 - S_y^2] + A \cdot IS$$

This was accomplished by introducing varying amounts of axial and rhombic distortion (the D and E parameters, respectively), and subsequently generating the individual lines for the various transitions by solving the above Hamiltonian using perturbation theory. The ternary isocitrate spectrum in

Figure 1 was found to possess the largest amount of axial distortion in the Mn(II) coordination sphere, ~450 G, while the two α-ketoglutarate equilibrium spectra displayed the maximum amount of rhombic distortion.

It seems that neither of the bicarbonate/CO_2 analogues (thiocyanate or formate) are ideal replacements for whichever of the two species is the actual reactive molecule that binds to the enzyme. The analogues introduce more anisotropy into the electronic environment of Mn(II) than does the actual substrate. Initially, it seems that HCO_3^- or CO_2 causes an enzyme conformational change that induces a structural alteration near the Mn(II) binding site. The analogues seem to have a similar effect with respect to each other, but their relationship to HCO_3^-/CO_2 binding cannot be unambiguously determined from these studies. An interesting observation is that the two analogues produce similar effects despite their very different properties. One property they do have in common, however, is their charge. This is also a characteristic that they have in common with bicarbonate, but not with CO_2. If charge is the determining factor in their behavior, which it seems to be since the two analogues differ so much in every other way, then the question arises as to why HCO_3^- has a different effect on the EPR spectrum. If this were the only consideration, it would suggest that CO_2 is the species which binds to the enzyme.

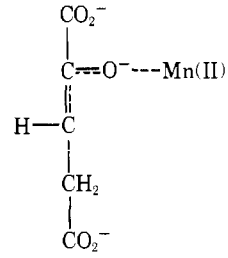
Since the enzyme probably binds isocitrate through all three carboxyl groups at the beginning of the reaction, the enzyme may have a positively charged group that binds the central carboxyl group. At the decarboxylation step, CO_2 is released and a solvent proton is added to the enol form of α-ketoglutarate. The reverse of this step undoubtedly involves attack of CO_2 by the π electrons of the double bond of the enolate of α-ketoglutarate. During these reactions, a positively charged group (perhaps Arg) may protonate and deprotonate depending upon which reactive species are present. The analogues thiocyanate and formate may bind to the positively charged group on the enzyme and thus stabilize this form of the enzyme. The spectra of enzyme-bound Mn(II) in the presence of formate and thiocyanate may reflect this enzyme conformer, whereas the presence of HCO_3^-/CO_2 may stabilize the conformer that binds CO_2.

The spectra in Figure 5 for L-α-hydroxyglutarate have features of spectra for both ternary and quaternary enzyme complexes with isocitrate and α-ketoglutarate. The strong signal at 2750 G, as well as the amplitude of the sextet signals centered at 3200 G, underlines this analogy. This analogue may bind at carbons 1 and 2 similarly to isocitrate but not allow hydride abstraction by TPN due to other enzyme–substrate–metal ion interactions too subtle to be observed by the techniques employed in this study.

Our studies of the binding constants of Mg(II) and Co(II) to isocitrate dehydrogenase have led to a better understanding of the variation in activity with these different metal ion cofactors. The K_D and K_M values for Mg(II) are similar, while those for cobalt differ by a factor of nearly 30. The K_D value for magnesium is expected to agree more closely with the K_M since the stability constant for the Mg(II)–isocitrate complex, as noted before, is relatively weak. Thus, formation of the binary Mg–enzyme complex may be the important binding step prior to catalysis when Mg(II) is the metal ion cofactor used.

By contrast, Co(II) forms a strong 1:2 complex with isocitrate (Bolard and Chottard, 1974). The stability constant for this complex is at least an order of magnitude larger than that for manganese–isocitrate. The catalytically important complex when Mn(II) is the cofactor is probably the binary Mn–isocitrate complex binding to the enzyme (Colman, 1975), and it is probable that the situation is analogous for cobalt. If this is so, one would expect the K_D and K_M values to be more disparate, and the values clearly reflect this expectation. The high K_D/K_M ratio for Co(II) suggests that metal binding to the enzyme is facilitated by the presence of isocitrate. A large K_D/K_M ratio is also observed for Mn(II) (Villafranca and Colman, 1974; Colman, 1975).

In conclusion, previous data with isocitrate dehydrogenase have suggested that the metal ion is implicated in catalysis (Villafranca and Colman, 1972, 1974). The data presented herein demonstrate that the metal ion environment of enzyme-bound Mn(II) is distinctively altered when isocitrate is simultaneously bound. The Mn(II) environment is not altered greatly by α-ketoglutarate which is not surprising since isocitrate and α-ketoglutarate have different structures. When TPN or TPNH are present, the metal ion environment is altered depending upon whether the complex formed with substrates is a catalytically competent complex or a dead-end complex. Overall these data provide direct evidence that the metal ion is involved in substrate binding and perhaps catalysis. The role of the metal ion in catalysis is most likely to stabilize the enolate of α-ketoglutarate subsequent to decarboxylation of oxalosuccinate. The following paper presents NMR data that further substantiate these conclusions.



Note Added in Proof

A recent paper by O'Leary and Limburg (1977) presents data on carbon isotope effects that are consistent with the view that the carbonyl oxygen of oxalosuccinate is coordinated to the metal ion during catalysis. These conclusions are precisely what we would predict based on the EPR data in this paper.

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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†

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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.