Magnetic Resonance Studies of the Role of the Divalent Cation in the Mechanism of Yeast Aldolase*

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ABSTRACT: The interaction of Mn^{2+} and substrates with the Zn^{2+}-free apoaldolase from yeast was studied by electron paramagnetic resonance, and by nuclear relaxation rate measurements of the protons of water and of the substrates. By electron paramagnetic resonance, proton relaxation rate of water, and kinetic studies, two tight binding catalytically active sites and additional weak binding sites for Mn^{2+} on the apo enzyme were detected. The substrates, acetol phosphate, dihydroxyacetone phosphate, fructose diphosphate, and glyceraldehyde 3-phosphate, and the competitive inhibitor, arabinitol diphosphate, interact with the tightly bound Mn^{2+} as indicated by 3–16% changes in the enhancement of the proton relaxation rates of water from the value of 5.9 for the binary Mn–aldolase complex. Dissociation constants obtained by proton relaxation rate titrations are in reasonable agreement with K_M and K_I values from kinetic studies on the Mn–enzyme. The temperature dependence of the proton relaxation rates of water in solutions of the binary enzyme–Mn^{2+} complex and the ternary complexes of dihydroxyacetone phosphate, glyceraldehyde 3-phosphate, and fructose diphosphate can be fit by a large outer sphere contribution and a water exchange contribution. Direct coordination of acetol phosphate, dihydroxyacetone phosphate, dihydroxyacetone phosphate hydrate, and fructose diphosphate by the enzyme-bound Mn^{2+} is demonstrated by the enhanced paramagnetic effects on their carbon-bound protons. Distance calculations from $1/T_1$ indicate that in the binary Mn–acetol phosphate complex, the Mn^{2+} is 38% closer to the C-1 protons than to the C-3 protons, indicating phosphoryl coordination only. However, in the ternary aldolase–Mn–acetol phosphate complex, the Mn is approximately equidistant between the C-1 and C-3 protons, suggesting carbonyl and phosphoryl coordination. Estimation of hyperfine coupling constants from $1/T_2 - 1/T_1$ confirms these findings. The high dissociation constant of the binary Mn–fructose diphosphate complex suggests independent binding of Mn^{2+} to the phosphoryl groups. In the ternary aldolase–Mn–fructose diphosphate complex the enzyme-bound Mn is 15% closer to the C-1 than to the C-6 protons, again consistent with carbonyl and C-1 phosphoryl coordination.

The exchange rates of the substrates into the ternary metal bridge complexes appear to be limited by the rate of dissociation of water from the bound Mn^{2+} and are several orders of magnitude greater than the rates of enzyme catalysis. The competitive inhibitor, arabinitol diphosphate, displaces the substrates, acetol phosphate and fructose diphosphate, from their metal bridge complexes at concentrations consistent with its K_I value. Hence, the kinetic and thermodynamic properties of the bridge complexes are consistent with their role in catalysis. The role of the Mn^{2+} in the yeast aldolase reaction thus appears to be polarization of the C-2 carbonyl group of the substrate, as previously suggested for the class II aldolases, and orientation of the C-1 phosphoryl group of the substrates. An analogous dual role is suggested for the protonated Schiff base in class I aldolases.

Two classes of enzymes, which catalyze the reversible aldol cleavage of fructose diphosphate (FDP)[1] to give dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (G-3-P), have been found in biological systems (Warburg and Christian, 1943; Rutter, 1964). Most of the class I aldolases have molecular weights of 160,000, tetrameric structures, broad pH profiles, and functional carboxyl-terminal tyrosine residues, while the class II enzymes have molecular weights of about 80,000, sharp pH optima, and are activated by monovalent K^+ ion. The distinguishing feature between these two classes is that all of the class I aldolases are inactivated by sodium borohydride in the presence of DHAP, while the class II enzymes are inhibited by chelating agents. From this evidence, the class I enzymes are postulated to have a mechanism like rabbit muscle aldolase, which involves a Schiff base formation between DHAP and a lysyl residue on the protein molecule (Grazi *et al.*, 1962; Horecker *et al.*, 1963). In contrast, the class II enzymes probably require a divalent metal ion for catalytic activity. In fact, the yeast enzyme, the prototype of the class II enzymes, was found to contain zinc (Rutter and Ling, 1958). On the basis of this circumstantial evidence, a mechanism for these was proposed in which the metal ion serves as an electrophile in the class II enzymes analogous to the role of lysine in the class I aldolases (Rutter, 1964).

Recently, it has been demonstrated that the metal ion (Zn^{2+}) in yeast aldolase is required for catalytic activity (Kobes *et al.*, 1969b). Removal of zinc from the native aldolase abolishes activity, while the readdition of zinc to apo enzyme restores activity fully. The addition of other

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[1] Abbreviations used are: FDP, fructose 1,6-diphosphate; DHAP, dihydroxyacetone phosphate; G-3-P, glyceraldehyde 3-phosphate; Ar-DP, arabinitol diphosphate; Me_4Si, tetramethylsilane.

divalent metals such as Co^{2+}, Ni^{2+}, Mn^{2+}, and Fe^{2+} also restores activity to the inactive apo enzyme. It is therefore possible to specifically substitute transition metals with formation of new active holo enzymes with properties that enable investigation of the environment of the metal ion at the active site.

The results that have been obtained thus far have demonstrated that the metal ion is necessary for catalytic activity of this enzyme, but have not given definitive evidence whether the metal ion has a structural or mechanistic role.

The present work was undertaken to elucidate the role of the divalent cation in the yeast aldolase catalyzed reaction.

A direct approach in determining the role of metal ion involves study of the interaction of substrates with Mn^{2+} yeast aldolase by nuclear magnetic resonance techniques (Mildvan *et al.*, 1967). The binding of Mn^{2+} to apoaldolase, as well as the binding of substrates and inhibitors to the Mn^{2+}–enzyme, was studied by magnetic resonance methods.

A preliminary report of some of these findings has appeared (Kobes *et al.*, 1969a).

Materials and Methods

Enzyme Preparation. Yeast aldolase was prepared according to the procedure of Rutter *et al.* (1966) with the following modifications. Fleishmann's baker's yeast was extracted by homogenization with glass beads in a colloid mill at 5°, the extract was adjusted to pH 5.0 and centrifuged, and then the pH of the supernatant was readjusted to 7.5 subsequent to the addition of ammonium sulfate. Phenylmethylsulfonyl fluoride (2×10^{-4} M), a potent inhibitor of serine active-site proteases, was included in all steps of the purification procedure. The enzyme was recrystallized three or four times and yielded preparations which were essentially homogeneous on cellulose polyacetate electrophoresis at a number of pH values and on polyacrylamide disc gel electrophoresis at pH 8.3. The enzymatic activities of these preparations, as assayed by the method of Richards and Rutter (1961a), were 85–105 μmoles of FDP cleaved per min per mg of protein. The molecular weight of yeast aldolase was assumed to be 80,000 and the extinction coefficient at 280 mμ was taken as 1.02 $(mg/ml)^{-1}$ cm^{-1} (Harris *et al.*, 1969).

Yeast apoaldolase was prepared by incubation of 10–60 mg of native aldolase in 0.5 ml of 0.05 M EDTA (pH 7.5) for 1 hr at 4°. This mixture was then applied to a Sephadex G-25 column (0.9 × 15 cm), which had been freed of metals by washing with 0.01 M EDTA (pH 7.5) and then equilibrated with 0.05 M Tris-HCl buffer (pH 7.5). The protein was eluted with the latter buffer and diluted to the proper protein concentrations. This procedure has been shown to result in the removal of chelating agent and metals from the protein (Kobes *et al.*, 1969b). Apo enzyme prepared in this manner contained less than 1% of the original zinc content as determined by atomic absorption spectrometry.

Substrates. Acetol phosphate was synthesized as previously described as the dimethyl ketal cyclohexylammonium salt (Richards and Rutter, 1961b).

DHAP and G-3-P were purchased from Sigma Chemical Co. as their dimethyl ketal cyclohexylammonium salt and diethyl acetal barium salt, respectively. The free trioses were prepared by acidification of 50–100 mM solutions in H_2O or D_2O with 0.3 g/ml of Dowex 50 (acid form), filtration, and hydrolysis of the filtrate at 40° for 4 hr, except in the case of G-3-P where the acetal was hydrolyzed in a boiling-water bath for 3 min.

The resulting acetol phosphate was determined by nuclear magnetic resonance by comparing the integrated area under its methyl resonance to that of an internal methanol standard. The resulting DHAP and G-3-P were assayed by measuring the disappearance of DPNH at 340 mμ when these trioses are used as substrates for the triose isomerase and glycerol 3-phosphate dehydrogenase reactions. The sodium salt of FDP, purchased from Wessex Co., England, was used for the titrations. Ar-DP was a generous gift from Dr. Robert Barker of the University of Iowa. All solutions were made metal-free by procedures described above and then brought to pH 7.3 with solid anhydrous potassium carbonate.

All substrates and buffers were treated to remove trace metal contaminants by either extraction with dithizone in carbon tetrachloride or treatment with Chelex-100 (Bio-Rad Corp.) (Himmelhoch *et al.*, 1966). Other precautions to prevent contamination with adventitious metal ions were taken as previously described (Mildvan and Cohn, 1963). The manganese used for reconstitution of the apo enzyme was a solution of the spectrographically pure chloride or sulfate salt.

Enzymatic Assays. Assays of apo enzyme and manganese-reconstituted apo enzyme were performed in the absence of potassium ion and at 15°. A suitable aliquot of aldolase was added to a mixture containing 75 μmoles of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (pH 7.0), 3 μmoles of FDP, 0.9 μmole of DPNH, and 100 μg of a mixture of crystalline α-glycerol phosphate dehydrogenase and triose phosphate isomerase in a total volume of 3.0 ml. The decrease in absorbancy at 340 mμ was recorded.

In the assay for the condensation reaction, the FDP formed from DHAP and G-3-P in the presence of the enzyme was detected by the Roe colorimetric test for fructose (Roe *et al.*, 1949). The assay system contained 25 μmoles of glycylglycine (pH 7.5), 2 μmoles of DHAP, 0.7 μmole of D-G-3-P, 50 μmoles of potassium acetate, and 0.02–0.2 unit of Mn^{2+} yeast aldolase in a total volume of 0.5 ml. The reaction was begun by the addition of aldolase; after 5-min incubation at 25°, it was stopped by the addition of 1.75 ml of 30% HCl. Then, 0.25 ml of resorcinol–thiourea reagent (0.1% resorcinol plus 0.25% thiourea in glacial acetic acid) was added to give a total volume of 2.5 ml. The solutions were then heated at 80° for 10 min and air cooled to room temperature. The optical density at 520 mμ was measured with a nonincubated control as a blank, and related to FDP standards.

The K_M for FDP, the K_I's for Ar-DP and acetol phosphate, and the K_M values for DHAP and G-3-P were determined using the previously described assays for cleavage and condensation, respectively (Richards and Rutter, 1961a).

The K_I for FDP as a product inhibitor of the condensation reaction was measured by quenching the reaction mixture with 6 N HCl to pH 1, neutralizing with KOH to pH 7.5, and assaying the residual triose phosphates. The triose phosphates were assayed using α-glycerol phosphate dehydrogenase and triose phosphate isomerase and measuring the disappearance of DPNH at 340 mμ. All of these assays were performed using Mn–aldolase under metal-free conditions as described above.

Magnetic Resonance Studies. Two methods were used to study the interaction of the paramagnetic ion, Mn^{2+}, with enzyme, and with the substrates and inhibitor. The first was a measurement of the amplitude of the electron paramagnetic resonance spectrum of the ion in aqueous solution to determine the concentration of free manganese (Cohn and

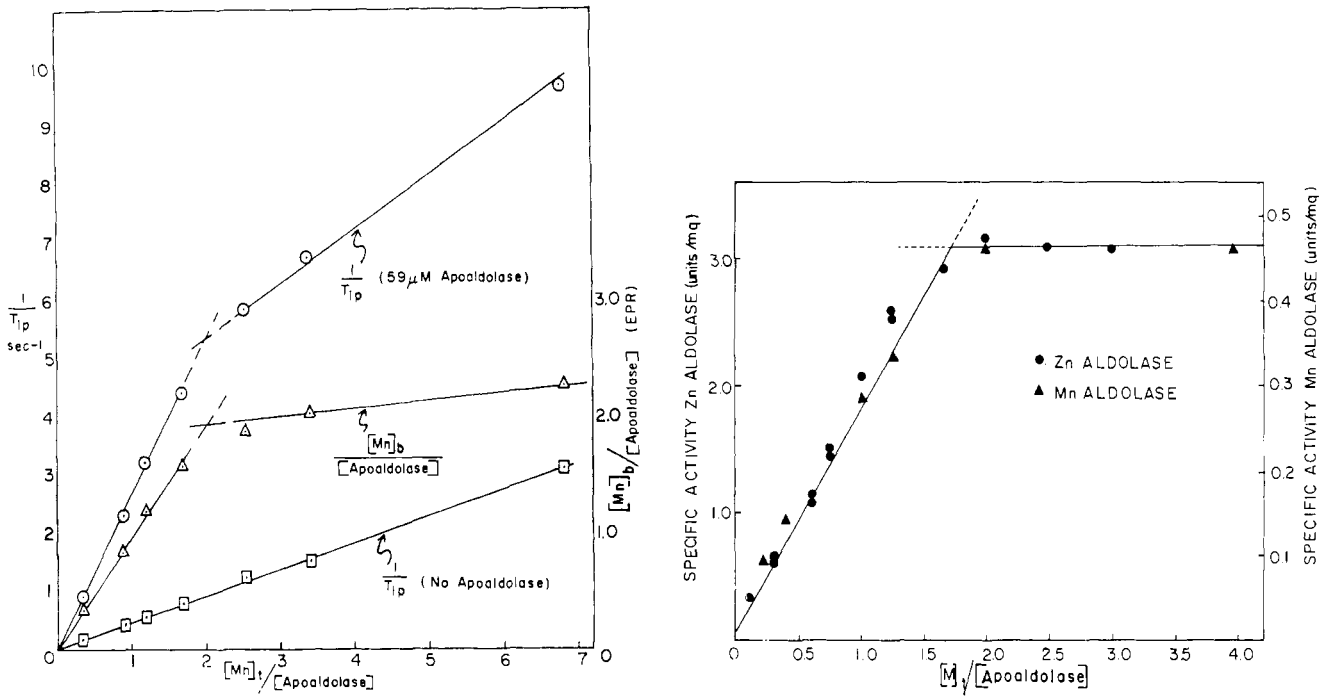


FIGURE 1: Manganese binding to aldolase. (A, left) The relative concentration of bound Mn^{2+} and apoaldolase, determined by electron paramagnetic resonance, and the paramagnetic effect of Mn^{2+} on the longitudinal relaxation rate of water protons ($1/T_{1p}$) in the presence and absence of apoaldolase, determined by pulsed nuclear magnetic resonance, is plotted against the total (Mn^{2+}). The molecular weight of the apoenzyme is 80,000 (Harris *et al.*, 1969). The solutions contained 25 mM Tris-HCl (pH 7.5) and 100 mM NaCl, T = 21°. The same range of Mn^{2+} concentrations was used in the absence of apoenzyme and these concentrations may be obtained by multiplying the abscissa by 59 μM. (B, right) Dependency of enzymatic activity on the total concentration of zinc or manganese $[M]_t$ added to the apoenzyme. Enzyme assays were done as described in Materials and Methods. (●) Zn-aldolase activity; (▲) Mn-aldolase activity.

Townsend, 1954). Measurements were made with Varian E-3 and E-4 electron paramagnetic resonance spectrometers at 9145 MHz as previously described (Mildvan and Cohn, 1963).

The second magnetic resonance method used for binding studies was the measurement of the proton relaxation rate of water by the pulsed nuclear magnetic resonance method of Carr and Purcell at 24.3 MHz as previously described (Mildvan and Cohn, 1963). This method makes use of the observation that a paramagnetic ion such as Mn^{2+} increases the longitudinal nuclear magnetic relaxation rate of water protons (proton relaxation rate). When a macromolecule is present which binds Mn^{2+}, the effectiveness of the ion in increasing the relaxation rate is enhanced. The enhancement factor is defined as the ratio of the paramagnetic contribution to the PRR of water in the presence of the aldolase–Mn complex to that in the presence of an equal concentration of free Mn^{2+} (Mildvan and Cohn, 1970). The solutions were the same as those used for measurement of the electron paramagnetic resonance of Mn^{2+}.

The affinity of the apo enzyme for Mn was high, permitting direct analysis of the electron paramagnetic resonance and proton relaxation rate data to give the number of Mn^{2+} binding sites (n) and the enhancement factor (ϵ_b) of the binary enzyme–Mn^{2+} complex. Only a crude estimate of the dissociation constant was possible.

In order to determine the dissociation constants (K_3) and the enhancement values (ϵ_t) of the ternary complexes of enzyme, Mn^{2+}, and substrate, the proton relaxation rate was measured at a constant concentration of the Mn–aldolase complex and variable concentrations of the substrates. The results were analyzed to yield K_3 from the concentration of free substrate which produced half-maximal change in enhancement (Mildvan *et al.*, 1966).

The relaxation rates of the carbon-bound protons of acetol phosphate, DHAP, and FDP were studied in D_2O by continuous-wave nuclear magnetic resonance as previously described (Mildvan *et al.*, 1967; Mildvan and Scrutton, 1967) using the Varian HA-60 and HA-100-15 nuclear magnetic resonance spectrometers. For the nuclear magnetic resonance studies with DHAP, the triose phosphate isomerase, which contaminates most preparations of yeast aldolase by ~1%, was inactivated by treatment with the specific inactivator, glycidol phosphate (Rose and O'Connell, 1969a), generously provided by I. A. Rose. Apoaldolase (27 mg/ml) was treated with 10 mM glycidol phosphate for 12 hr in 0.05 M Tris-HCl (pH 7.5) at 22°. This compound had no effect on the aldolase activity, but completely inactivated the triose phosphate isomerase, as reported by Rose and O'Connell (1969a).

For the continuous-wave nuclear magnetic resonance experiments on fructose 1,6-diphosphate, the isomerase contaminant was not inactivated but utilized with aldolase to enzymatically prepare partially deuterated FDP from DHAP in the nuclear magnetic resonance tube. The resulting FDP had exchanged all but the C_1 and C_6 protons for deuterons in D_2O, as would have been expected from earlier studies (Rose, 1958), resulting in a simplified nuclear magnetic resonance spectrum for FDP.

Results

Magnetic Resonance Studies of the Binary Mn^{2+}–Enzyme Complex. Figure 1A shows the results of titration of yeast

TABLE I: Stoichiometry (n), Dissociation Constants (K_D), and Enhancement Factors (ϵ_b) of Binary Mn^{2+}–Aldolase Complexes.[a]

Method	Type of Binding Site	n	K_D (μM)	ϵ_b
Electron paramagnetic resonance	Tight	2.0 ± 0.1	2.4 ± 0.2	
	Weak	2.0[b]	1600 ± 400	
Proton relaxation rate	Tight	2.0 ± 0.1		5.9 ± 0.6
	Weak	2.0[b]		19.5 ± 2.0

[a] Conditions are given in Figure 1 and in the text. [b] Stoichiometry of weak binding site is assumed to be 2 per 80,000 g.

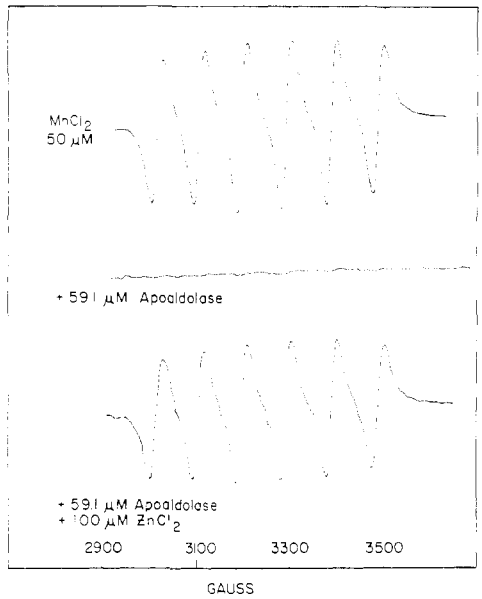


FIGURE 2: The displacement of manganese on aldolase with zinc as determined by electron paramagnetic resonance of free Mn^{2+}. Other components present were 50 mM Tris-HCl (pH 7.5) and 15% D_2O–85% H_2O. The electron paramagnetic resonance spectra were run at 9145 MHz, 10-gauss modulation amplitude, 100-mW microwave power, sweep time 4 min, time constant 0.3 sec, T = 24°.

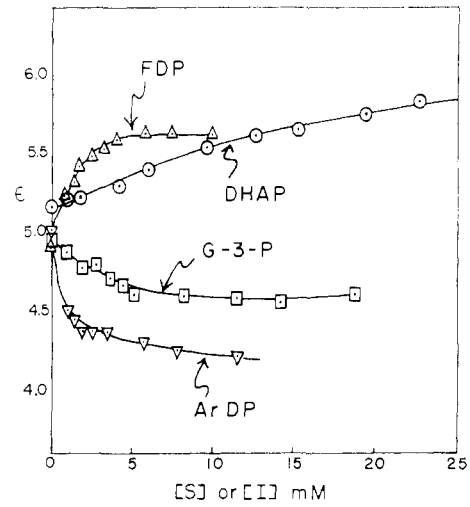


FIGURE 3: Effect of substrates on the enhancement of the longitudinal relaxation rate of water protons in the presence of the aldolase–Mn^{2+} complex. Solutions containing 114–214 μM aldolase sites, 50–100 μM $MnCl_2$, 100 mM KCl, and 25–60 mM Tris-HCl (pH 7.5) were titrated with identical solutions (to avoid dilution), which also contained the substrate. The isomerase activity in this preparation of aldolase was negligible. T = 21°.

aldolase with Mn^{2+}. The paramagnetic contribution to the longitudinal relaxation rate ($1/T_{1p}$) is plotted against the concentration of Mn in the presence and absence of 59 μM aldolase. In the absence of aldolase the relaxivity ($1/T_{1p}$) of water increases linearly with $[Mn]_t$. In the presence of aldolase a linear increase in relaxivity of 5.9-fold greater slope is obtained with a break in the curve at $[Mn]_t/[\text{aldolase}] = 2$, indicating that yeast aldolase binds two Mn^{2+} ions tightly. This stoichiometry is confirmed by calculation of the enzyme-bound Mn^{2+} (Figure 1A, triangles) from electron paramagnetic resonance measurements of the concentration of free Mn^{2+}. A similar stoichiometry (1.7 ± 0.1) is obtained by a titration of the effect of Mn^{2+} or Zn^{2+} on the activity of the apo enzyme (Figure 1B). From the electron paramagnetic resonance data (Figure 1A), the dissociation constant of the two tight binding sites is 2.4 μM. These results are in agreement with the observation that the apo enzyme binds two Zn^{2+} ions or two Co^{2+} ions, as detected by activity and optical spectroscopy (R. T. Simpson, R. D. Kobes, R. W. Erbe, W. J. Rutter, and B. L. Vallee, to be published), respectively. Additional weak binding sites for Mn^{2+} on aldolase are indicated by the failure of the Mn binding curve to level off and by the inequality of the slope of the $1/T_{1p}$ plot in the presence and absence of aldolase beyond a stoichiometry of 2 Mn/enzyme molecule. As calculated from the ratio of slopes in Figure 1, the two tight binding sites cause a 5.9-fold enhancement of the effect of Mn^{2+} on $1/T_{1p}$ of water. By correcting for the tight binding sites as previously described (Mildvan and Cohn, 1963), the enhancement factor of the weak binding sites is calculated as ϵ_b = 19.5. Assuming a stoichiometry of 2, the weak binding site has a dissociation constant of 1.6 mM. The binding data on the binary Mn–aldolase complexes are summarized in Table I.

Conversion of Mn^{2}–Aldolase to Zn^{2}–Aldolase. A solution containing 59 μM apoaldolase and 50 μM $MnCl_2$ revealed no free Mn^{2+} by electron paramagnetic resonance (Figure 2) and an enhancement factor, ϵ_b = 7.5.[2] The addition of 100 μM $ZnCl_2$ displaced 82.8% of the Mn^{2+}, converting it to electron paramagnetic resonance detectable free Mn^{2+} (Figure 2) and reduced the observed enhancement factor

[2] The reason for this atypically high value is not clear, but may be related to the fact that the solution contained 15% D_2O. Different preparations of aldolase–Mn^{2+} typically vary by ±10% in their ϵ_b values for the tight binding sites (Table II).

TABLE II: Binding and Kinetic Constants of Ternary Complexes of Mn^{2+}–Yeast Aldolase.[a]

Substrate Or Inhibitor	ϵ_t/ϵ_b [b]	K_3[c] (mM)	K_M (mM)	K_I (mM)
Acetol phosphate	0.97	1.8 ± 1.0		2.0
Dihydroxyacetone phosphate	1.14	5.5	4.5	
Glyceraldehyde 3-phosphate	0.93	2.6	3.3	
Fructose diphosphate	1.14	1.0	0.2	2.5
Arabinitol diphosphate	0.84	0.6		0.3

[a] Conditions are given in Figure 3. [b] ϵ_t/ϵ_b is the ratio of the enhancement of the ternary complex to the enhancement of the binary complex. The error in ϵ_t/ϵ_b is ±1%. [c] K_3 = [EMn][S]/[EMnS]; the error in K_3 is ±20%.

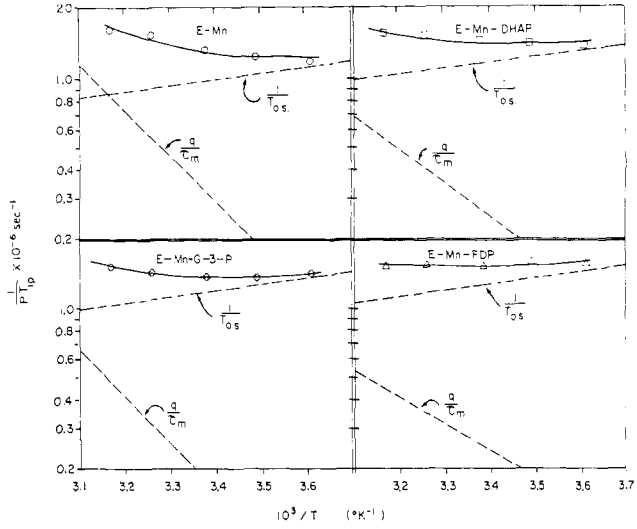


FIGURE 4: Arrhenius plots of the effect of temperature on the paramagnetic contribution to the water relaxation rate in solutions of the binary aldolase–Mn complex and ternary aldolase–Mn–substrate complexes. The solid curves which are fitted to the data were generated by an exchange contribution (q/τ_M) and an outer sphere contribution ($1/T_{os}$) using eq 1, as described in the text. The solution contained 108 μM apoaldolase sites, 50 μM $MnCl_2$, 70 mM Tris-HCl (pH 7.5), and 100 mM KCl. Two concentrations of substrates were used to ensure saturation at all temperatures; DHAP, 25 and 35 mM; G-3-P, 20 and 30 mM; and FDP, 18 and 27 mM.

to 4.2. The enhancement of the 17.2% residual bound Mn^{2+}, ϵ_b = 19.8, was typical of that of the ancillary weak binding sites. Since the two tight binding sites for Mn represent the active sites, subsequent studies were carried out at a Mn^{2+} to enzyme ratio ≤2.

Ternary Aldolase–Mn^{2+}–Substrate Complexes. As exemplified in Figure 3, the addition of substrates or inhibitors to the aldolase–Mn complex causes small changes in the observed enhancement. Detailed titrations were carried out for the substrates FDP, G-3-P, DHAP, and acetol phosphate[3], (not shown), and the competitive inhibitor Ar-DP. From the titration curves the dissociation constants (K_3) and enhancements (ϵ_T) of the respective ternary complexes were calculated and these values are summarized in Table II, together with K_M and K_I values determined kinetically with Mn–aldolase. The dissociation constants obtained from proton relaxation rate titrations are in reasonable agreement with the K_M and K_I values obtained from initial rate studies of the overall reaction with the exception of the K_3 for FDP which, even when corrected for the small amounts of products present at equilibrium, is an order of magnitude greater than the K_M for this substrate, but which agrees with its K_I as a product inhibitor. These findings suggest that the ternary complexes characterized by proton relaxation rate are the enzymatically active complexes and that the Mn enzyme obeys Michaelis–Menten kinetics in the direction of condensation but not in the direction of cleavage.

The ϵ_T values in Table II indicate that FDP and DHAP increase the enhancement while G-3-P, acetol phosphate, and the inhibitor Ar-DP decrease the enhancement, although all of these effects are small (≤16%). As pointed out elsewhere (Mildvan and Cohn, 1970), changes in the enhanced effect of enzyme-bound Mn^{2+} on the relaxation rate of water protons may be due to changes in the coordination number for water on Mn^{2+}, the water exchange rate, or the correlation time for Mn–water interactions. Hence, additional studies such as the temperature and frequency dependencies of $1/T_{1p}$ are required to elucidate the mechanism of the change.

Temperature Dependence of $1/T_{1p}$ in the Presence of Binary and Ternary Aldolase Complexes. The relaxivity of the binary complex is independent of temperature below 13° and increases with temperature above 13° as do the ternary complexes of the triose phosphates (Figure 4). The observed relaxation rates ($1/pT_{1p}$) have been fit (Figure 4) by the equation

$$1/pT_{1p} = q/\tau_M + 1/T_{os} \quad (1)$$

in which p is the ratio of $[Mn^{2+}]/[H_2O]$, q is the coordination number for water on Mn^{2+}, τ_M is the residence time of a water ligand on Mn^{2+}, and $1/T_{os}$ is the outer sphere contribution to the relaxation rate (Luz and Meiboom, 1964; Mildvan and Cohn, 1970). The parameters of τ_M and $1/T_{os}$ used to fit the data (Figure 4) are summarized in Table III.

The relaxivity of the ternary aldolase–Mn–FDP complex shows minimal temperature dependence from 2 to 42° and may in fact be fitted as well by a straight line of zero slope. However, since temperature-dependent outer sphere and exchange contributions are likely, the data for this substrate were also fit by assuming an outer sphere term with an energy of activation of 1.2 kcal/mole (consistent with the value observed for the other complexes) and an exchange contribution. As seen in Table III, the exchange rate of water protons into the coordination sphere of enzyme-bound Mn^{2+} (q/τ_M) is slower in all of the ternary complexes than in the binary E–Mn complex. This effect is most simply explained by a decrease in q, the number of water ligands available for exchange, due to the formation of aldolase–Mn^{2+}–substrate bridge complexes.

The outer sphere contribution to the relaxation rate appears to be greater in the ternary complexes than in the binary complex. This effect contributes significantly to the increase in enhancement of the ternary complexes of DHAP and FDP.

Effect of Mn^{2+} and Aldolase–Mn^{2+} on the Relaxation Rates

[3] Acetol phosphate might be considered a pseudosubstrate since it undergoes rapid proton exchange but slow condensation with the muscle aldolase, yet participates in both reactions with the yeast enzyme (Rose and O'Connell, 1969b).

TABLE III: Outer Sphere ($1/T_{os}$) and Exchange (q/τ_M) Contributions to the Relaxation Rate of Water.[a]

Complex	$1/T_{os}$		q/τ_M			
	Value at 25° ($sec^{-1} \times 10^{-5}$)	E_a (kcal/mole)	Value at 25° ($sec^{-1} \times 10^{-5}$)	E_a (kcal/mole)	$\Delta H^{\ddagger}$ (kcal/mole)	$-T\Delta S^{\ddagger}$ (kcal/mole)
Aldolase–$Mn(H_2O)_3$	9.7	1.2	3.7	9.1	8.5	1.9
Aldolase–Mn–G-3-P(H_2O)	11.4	1.2	2.0	9.4	8.8	1.4
Aldolase–Mn–DHAP(H_2O)	11.4	1.2[b]	3.0	6.7	6.1	3.8
Aldolase–Mn–FDP(H_2O)	12.4	1.2[b]	2.7	5.3	4.7	5.3

[a] These parameters were used to fit the data of Figure 4, with confidence limits ±3% for $1/T_{os}$ and q/τ_M and ±10% for E_a. [b] Assumed for consistency with the binary complex and the ternary complex of G-3-P.

TABLE IV: Paramagnetic Effects of Mn^{2+} and Aldolase–Mn^{2+} on the Relaxation Rates of the Protons of Acetol Phosphate and Dihydroxyacetone Phosphate at 100 MHz.[a]

Complex	CH_2 OP				CH_3 or CH_2OH			
	$sec^{-1} \times 10^{-3}$				$sec^{-1} \times 10^{-3}$			
	$1/pT_{1p}$	$1/pT_{2p}$	ϵ_1	ϵ_2	$1/pT_{1p}$	$1/pT_{2p}$	ϵ_1	ϵ_2
Aldolase–Mn–acetol phosphate	0.90 ± 0.15	4.3 ± 0.7	0.26	0.69	0.90 ± 0.30	5.8 ± 0.7	4.5	5.3
Mn–acetol phosphate	3.5 ± 0.2	6.2 ± 0.1			0.20 ± 0.04	1.1 ± 0.2		
Aldolase–Mn–DHAP	0.20 ± 0.04	3.8 ± 0.6	0.15	1.7				
Mn–DHAP	1.3 ± 0.2	2.3 ± 0.1						
Aldolase–Mn–DHAP hydrate	0.51 ± 0.10	1.6 ± 0.2	0.36	0.54	0.19 ± 0.03	6.6 ± 0.6	0.26	1.9
Mn–DHAP hydrate	1.4 ± 0.30	2.9 ± 0.3			0.72 ± 0.13	3.4 ± 0.3		

[a] Each experiment consisted of at least ten spectra run at different radiofrequency powers, and analyzed as described previously (Mildvan *et al.*, 1967; Mildvan and Scrutton, 1967). The range of concentrations used were acetol phosphate, 21.3–37.6 mM; DHAP, 12.4–43.6 mM; DHAP hydrate, 25 mM; aldolase–Mn, 30–194 μM; $MnCl_2$, 22–106 μM; Tris-HCl, 5 mM, pD = 7.9; D_2O, 99%. In some experiments the relaxation rates for Mn–acetol phosphate and the Mn–DHAP complexes were determined in the presence of the alolase–Zn complex (72.5–139 μM), which did not alter the paramagnetic effect of Mn^{2+}.

of the Protons of Acetol Phosphate.[3,4] The proton nuclear magnetic resonance spectrum of acetol phosphate at 100 MHz consists of a methyl singlet 2.66 ppm downfield from the external standard, Me_4Si, and a methylene phosphate doublet (J = 6.1 cps) which is 4.92 ppm downfield from Me_4Si. The metal-free apoaldolase has no effect on these resonances (Figure 5A), nor does the diamagnetic Zn–aldolase complex (Figure 5B). However, the aldolase–Mn complex broadens both the methyl and methylene resonances to a similar extent due to an increase in the transverse ($1/T_2$) relaxation rates, and increases the radiofrequency power required to saturate these resonances due to an increase in longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates of the methyl and methylene protons (Figure 5C).[4] The addition of excess Zn^{2+}, which displaces Mn^{2+} from the enzyme (Figure 2; eq 2) and converts the paramagnetic ternary complex into a diamagnetic one, causes a narrowing

$$\text{aldolase–}Mn^{2+}\text{–acetol phosphate} \xrightarrow{Zn^{2+}} \text{aldolase–}Zn^{2+}\text{–acetol phosphate} + Mn^{2+} \quad (2)$$

of the methyl resonance and a decrease in the radiofrequency power required to saturate this resonance (Figure 5D), indicating a lengthening of the relaxation times, $1/T_1$ and $1/T_2$, of the methyl protons. The paramagnetic contributions to the longitudinal ($1/pT_{1p}$) and transverse ($1/pT_{2p}$) relaxation rates calculated from such experiments (Table IV) indicate that the enzyme-bound Mn^{2+} is more effective than free Mn^{2+} in relaxing the methyl protons of acetol phosphate. The enhanced effect of the aldolase–Mn^{2+} complex on the relaxation rates of the methyl protons indicates direct coordination of this substrate by the enzyme-bound Mn^{2+}. The release of Mn^{2+} upon the addition of Zn^{2+} to the aldolase–Mn^{2+}–acetol phosphate complex produced the opposite effects on the resonances of the methylene protons of this ligand (Figure 5D), indicating that the enzyme-bound Mn^{2+} is less effective than free Mn^{2+} in relaxing the methylene protons. Spectra similar to that of Figure 5D are obtained by adding Mn^{2+} to acetol phosphate in the absence of apoaldolase but under otherwise identical conditions. These results indicate that the presence of the enzyme alters the structure of the Mn–acetol phosphate complex such that the methyl group is closer to the Mn^{2+} and the methylene group is farther from the Mn on the enzyme. Such reorientations of ligands on enzymes have previously been found with alcohol dehydrogenase (Mildvan and Weiner, 1969) and pyruvate

[4] The enzyme-catalyzed deprotonation of this substrate in D_2O was slow enough under our experimental conditions to permit observations to be made on the same sample for several hours.

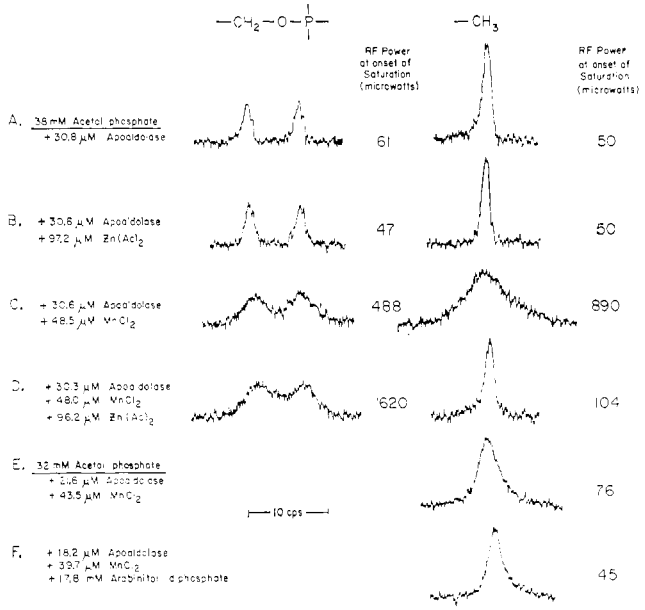


FIGURE 5: Effect of apoaldolase and its metal and inhibitor complexes on the resonances in the 100-MHz proton nuclear magnetic resonance spectrum of acetol phosphate. Other components present were 4 mM Tris-HCl (pD = 7.9), and 99% D_2O. T = 32°.

carboxylase (Mildvan and Scrutton, 1967) by analogous nuclear magnetic resonance methods. The diamagnetic effects of the aldolase–Zn^{2+} complex on the relaxation rates of the protons of acetol phosphate (Figure 5B; Table IV) are at least an order of magnitude smaller than the paramagnetic effects of aldolase–Mn^{2+}, and in most cases are beyond the limits of detectability under our experimental conditions.

Displacement of Acetol Phosphate from Its Ternary Complex by Arabinitol Diphosphate. As shown in Figure 5E,F, the addition of excess Ar-DP to aldolase–Mn–acetol phosphate causes a narrowing of the methyl resonance of acetol phosphate and decreases the radiofrequency power at saturation, indicating that the competitive inhibitor has displaced the pseudosubstrate from the bridge complex. A titration of the effect of Ar-DP on the line width of the methyl resonance of acetol phosphate (Figure 6A) may be fit by assuming simple competition between acetol phosphate (K_3 = 1.8 mM) and Ar-DP (K_3 = 0.45 mM). The latter value is in good agreement with the K_I of Ar-DP (0.3 mM) and its K_3 as determined by proton relaxation rate titrations (0.6 mM) (Table II). Hence, in the metal–bridge complex of aldolase–Mn–acetol phosphate the substrate binds at the active site with a dissociation constant consistent with its role in catalysis.

Manganese to Proton Distances in the Binary Mn^{2+}–Acetol Phosphate and Ternary Aldolase–Mn^{2+}–Acetol Phosphate Complexes. The longitudinal relaxation rate, $1/pT_{1p}$, may be used to calculate intramolecular distances between Mn^{2+} and the nucleus of a coordinated ligand, provided $1/pT_{1p}$ measures the relaxation rate of the ligand nucleus ($1/T_{1M}$) and is not limited by chemical exchange (Mildvan and Cohn, 1970). For the binary Mn–acetol phosphate complex and the ternary aldolase–Mn–acetol phosphate complex, $1/pT_{1p}$ cannot be exchange-limited since it is less than $1/pT_{2p}$. Hence, $1/pT_{1p} = 1/T_{1M}$ and may be used to calculate distances. For Mn^{2+}–proton interactions, the dipolar term of the Solomon–Bloembergen equation may be rearranged to give the following equation which relates the distance,

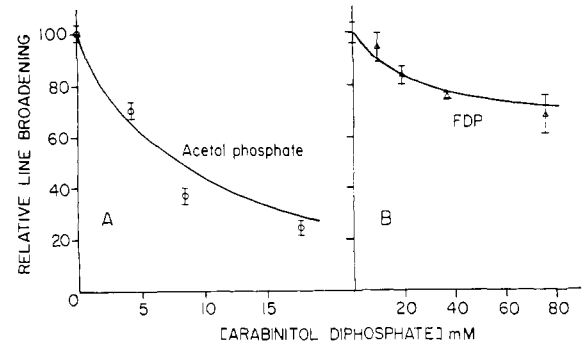


FIGURE 6: Effect of Ar-DP on the paramagnetic line broadening of (A) the C-3 protons of acetol phosphate at 100 MHz and (B) on the C-1 and C-6 protons of FDP at 60 MHz. Conditions are as described in Figures 5, 8, and 10. The solid curves were derived by assuming competition as described in the text.

r, to T_{1M} and to a function of τ_c, the correlation time for dipolar interaction (Mildvan and Scrutton, 1967) (eq 3).

$$r \text{ (in Å)} = 815(T_{1M}f(\tau_c))^{1/6} \cong 979(T_{1M}\cdot\tau_c)^{1/6} \qquad (3)$$

If τ_c is $<10^{-9}$ sec, as is the case in the binary complex and in many ternary complexes, $f(\tau_c) = 3\tau_c$ and the equation simplifies as shown. The correlation time in the binary complex is its tumbling time, which is approximately 3.5×10^{-11} sec, as determined by τ_c for the Mn–water interaction in various small Mn–phosphate complexes (Mildvan *et al.*, 1967) from eq 4, where $\tau_c(Mn(H_2O)_6) = 2.9 \times 10^{-11}$ sec,

$$\tau_c(Mn(H_2O)_q{*}L) = \tau_c(Mn(H_2O)_6)\cdot q/q^*\cdot\epsilon_a \qquad (4)$$

q/q^* is the relative coordination number for water in $Mn(H_2O)_6^{2+}$ and in $Mn(H_2O)_q{*}L$ ($^6/_5$ or $^6/_4$), and ϵ_a is the enhancement of the binary Mn–ligand complex, which is approximately 1 (Mildvan *et al.*, 1967). The correlation times in ternary complexes are in general unknown but, as discussed elsewhere (Mildvan and Cohn, 1970), may be the electron spin relaxation time of Mn^{2+} or an intramolecular rotational time constant of the ligand or of a segment of the protein. Hence, a lower limit is a typical intramolecular rotation time (1.8×10^{-12} sec for CH_3CH_2Br) and an upper limit is the value of τ_c for the Mn–H_2O interaction in the same ternary complex. The latter value is obtained from the ϵ_T value for water in the complex using eq 4. The calculated distances

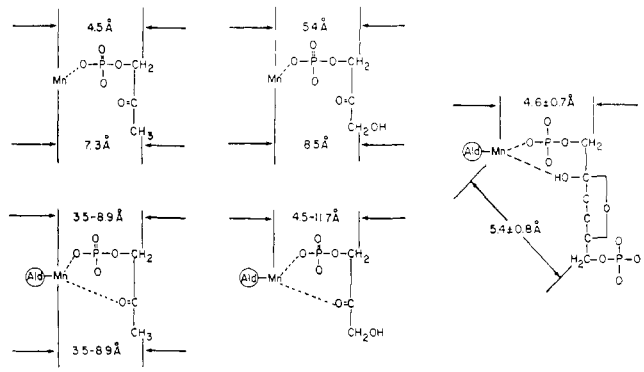


FIGURE 7: Manganese to proton distances in solutions of binary Mn^{2+}–substrate and ternary aldolase–Mn^{2+}–substrate complexes from $1/T_{1p}$ (Table V).

TABLE V: Manganese to Proton Distances in Solutions of Binary Mn^{2+} Complexes and Ternary Aldolase–Mn^{2+} Complexes, from $1/T_1$.[a]

Complex	Distance	$1/pT_{1p}$ ($sec^{-1} \times 10^{-3}$)	τ_c ($sec \times 10^{11}$)	r (Å)	Distance Ratio (Mn–HC-3):(Mn–HC-1)
Mn–acetol phosphate	Mn–HC-1	3.5	3.4[b]	4.53	
					1.61
	Mn–HC-3	0.20	3.4[b]	7.30	
Mn–DHAP	Mn–HC-1	1.3	3.4[b]	5.39	
Mn–DHAP hydrate	Mn–HC-1	1.4	3.4[b]	5.29	
					1.12
	Mn–HC-3	0.72	3.4[b]	5.90	
Aldolase–Mn–acetol phosphate	Mn–HC-1	0.90	0.18[c]	3.47	
			50.5[b]	8.89	
					1.00
	Mn–HC-3	0.90	0.18[c]	3.47	
			50.5[b]	8.89	
Aldolase–Mn–DHAP	Mn–HC-1	0.20	0.18[c]	4.47	
			57.5[b]	11.7	
Aldolase–Mn–FDP	Mn–HC-1	54.0	0.18[c]	1.76	
			19.9[b]	3.86	
			212[d]	5.25	
					1.18[e]
	Mn–HC-6	20.0	0.18[c]	2.08	
			19.9[b]	4.56	
			212[d]	6.20	
Aldolase–Mn–DHAP hydrate	Mn–HC-1	0.51	0.18[c]	3.82	
			57.5[b]	10.0	
					1.18
	Mn–HC-3	0.19	0.18[c]	4.50	
			57.5[b]	11.8	

[a] Calculated from eq 3 as described in the text. [b] Correlation time for Mn–H_2O interaction in the same complex. [c] Intramolecular rotation time for CH_3–CH_2Br at 25° (Higasi *et al.*, 1960). [d] Calculated from T_{1p}/T_{2p} using eq 6, which assumes no hyperfine interaction, *i.e.*, $A/h = 0$. [e] This distance ratio is (Mn–HC-6):(Mn–HC-1).

are rather insensitive to the values of τ_c since this parameter enters eq 3 as a sixth root. Table V and Figure 7 summarize the absolute Mn^{2+} to proton distances and the ratio of the Mn–CH_3 and Mn–CH_2 distances. The relative distances are independent of the value of τ_c chosen, but depend only on the reasonable assumption that τ_c is the same for both types of proton. The distance ratio reveals the Mn^{2+} to be 61% farther from the methyl protons than from the methylene protons in the binary Mn–acetol phosphate complex. The relative and absolute distances calculated from $1/pT_{1p}$ (Table V; Figure 7), when compared with those found in molecular models of the various possible structures of Mn–acetol phosphate (Table VI), indicate that in the binary complex the Mn coordinates only the phosphate, since the Mn to methyl distance is too great for carbonyl coordination. The enzyme alters the structure of the Mn–acetol phosphate complex such that in the ternary metal–bridge complex the Mn^{2+} is equidistant from the methyl and methylene groups (Table V). This change suggests carbonyl coordination in addition to phosphoryl coordination in the ternary complex, but the uncertainties in the absolute values of the distances do not permit the exclusion of alternative structures (Table VI). Additional evidence for carbonyl and phosphoryl coordination in the ternary complex is given in the next section.

Hyperfine Coupling in the Binary Mn^{2+}–Acetol Phosphate and Ternary Aldolase–Mn^{2+}–Acetol Phosphate Complexes. Direct coordination of a ligand by a paramagnetic metal ion such as Mn^{2+} may be demonstrated by the detection of hyperfine coupling between the metal ion and the magnetic nuclei of the ligand (Mildvan, 1971; Mildvan and Scrutton, 1967) since such coupling operates only through chemical bonds (Barfield and Karplus, 1969). The magnitude of the hyperfine coupling is measured by the coupling constant, (A/h), which depends on the number and nature of the chemical bonds connecting the Mn^{2+} and the proton under examination. The values of A/h tend to decrease as the number of chemical bonds intervening between the Mn^{2+} and the proton increase (Mildvan, 1971). The inequality in $1/pT_{2p}$ and $1/pT_{1p}$ may be used to estimate the hyperfine coupling constant if τ_c is less than 10^{-9} sec from eq 5 where τ_e

$$1/pT_{2p} - {}^7/_6(1/pT_{1p}) = 114(A/h)^2\tau_e \qquad (5)$$

is the correlation time for hyperfine interaction which, in Mn complexes, is usually dominated by τ_s, the electron spin relaxation time (10^{-9} sec $< \tau_s < 10^{-8}$ sec). If $1/pT_{2p}$ is limited by chemical exchange, then eq 5 can be used only to set a lower limit on A/h. With acetol phosphate, $1/pT_{2p}$ is

TABLE VI: Manganese to Proton Distances in Molecular Models of Mn^{2+}–Substrate Complexes.

Complex	Structure	Distance (Å)		Distance Ratio (Mn–HC-3): (Mn–HC-1)
		Mn–HC-1	Mn–HC-3	
Mn–acetol phosphate	Monodentate (C-1 phosphate)	2.4–5.3	3.0–8.3	0.6–2.2
Mn–DHAP and hydrate	Monodentate (C-2 carbonyl or hydroxyl)	2.9–4.4	2.9–4.4	0.7–1.4
	Bidentate (C-1 and C-2)	3.0–4.4	3.3–4.5	0.7–1.4
Mn–DHAP and hydrate	Monodentate (C-3 hydroxyl)	2.6–5.8	2.7–3.2	0.5–1.2
	Bidentate (C-3 hydroxyl and C-2 carbonyl or hydroxyl)	4.2–4.4	3.3–3.4	0.8
	Bidentate (C-3 hydroxyl and C-1 phosphate)	2.8–5.3	2.8–3.4	0.6–1.2
	Tridentate	4.4	3.4	0.8
		Mn–HC-1	Mn–HC-6	(Mn–HC-6): (Mn–HC-1)
Mn–FDP[a]	Monodentate (C-1 phosphate)	2.6–5.3	2.6–10.3	0.5–3.1
	Monodentate (C-2 hydroxyl)	2.9–5.3	2.6–7.0	0.5–2.2
	Bidentate (C-1 phosphate and C-2 hydroxyl)	3.6–5.2	3.3–6.8	0.7–1.6

[a] The furanose structure only for FDP was considered (Gray and Barker, 1970).

not limited by chemical exchange since it is 19% greater at 1° than at 33° (Table IV). The absolute values of A/h for the Mn–proton interaction in the binary Mn–acetol phosphate complex (Table VII) are comparable to those of other Mn^{2+} complexes with fewer intervening bonds (Mildvan and Scrutton, 1967; Mildvan, 1971), suggesting a high degree of double-bond character and resulting spin delocalization in the Mn–acetol phosphate complex. Absolute values of A/h of the same order of magnitude are calculated for the ternary aldolase–Mn–acetol phosphate complex, confirming direct coordination of acetol phosphate by the enzyme-bound Mn^{2+}. Since the correlation time, τ_e, is probably the same for all protons of the same ligand, the relative value of the coupling constant for each proton may be calculated independent of any assumed value for τ_e. The ratios of the coupling constants (Table VII) indicate that in the binary complex, Mn^{2+} is more strongly coupled to the methylene than to the methyl protons, as would be expected if the Mn^{2+} coordinated the phosphate. In the ternary complex, the Mn^{2+} is strongly coupled to the methylene protons, suggesting phosphate coordination, but it is even more strongly coupled to the methyl protons than to the methylene protons, suggesting carbonyl coordination as well. However, since coupling constants depend on other factors such as dihedral angles, alternative explanations exist for these differential effects.

TABLE VII: Manganese to Proton Hyperfine Coupling Constants in Solutions of Binary Complexes and Ternary Complexes with Aldolase.[a]

Complex	Interaction	Log (A/h) (cps)	Ratio A(Mn–HC-3): A(Mn–HC-1)
Mn^{2+}–acetol phosphate	Mn–HC-1	≥4.6–5.1	≤0.63
	Mn–HC-3	4.4–4.9	
Aldolase–Mn^{2+}–acetol phosphate	Mn–HC-1	4.7–5.2	≥1.19
	Mn–HC-3	≥4.8–5.3	
Mn–DHAP	Mn–HC-1	≥4.4–4.9	
Aldolase–Mn–DHAP	Mn–HC-1	≥4.8–5.3	
Mn–DHAP hydrate	Mn–HC-1	4.5–5.0	
	Mn–HC-3	≥4.7–5.2	≥1.42
Aldolase–Mn–DHAP hydrate	Mn–HC-1	5.0–5.5	≥2.52
	Mn–HC-3	≥5.4–5.9	

[a] Calculated from $1/pT_{2p}$ and $1/pT_{1p}$ using eq 5, as described in the text.

Rates of Ligand Exchange in the Binary Mn^{2+}–Acetol Phosphate and Ternary Aldolase–Mn^{2+}–Acetol Phosphate Complexes. The maximum transverse relaxation rate may be used to set a lower limit on $1/\tau_M$, the rate of exchange of acetol phosphate into the coordination sphere of Mn^{2+}. Under our experimental conditions, Mn^{2+} and aldolase–Mn^{2+} were saturated with acetol phosphate. Hence, $1/\tau_M$ is limited by and may be equated with k_{off}, the rate constant for dissociation of the Mn–acetol phosphate and aldolase–Mn–acetol phosphate complexes. From the limiting values for k_{off}, and the dissociation constants (K_3),[5] lower limits for the

[5] The dissociation constants of the binary Mn–acetol phosphate and Mn–dihydroxyacetone phosphate complexes were not measured but were assumed to be equal to that of Mn–orthophosphate, 4.7 mM (Mildvan *et al.*, 1967). This value is of the right order of magntiude for binary Mn complexes of monophosphoryl compounds such as fluorophosphate, 1.8 mM (Mildvan *et al.*, 1967); phosphoenolpyruvate, 1.8 mM (Mildvan and Cohn, 1966); and adenosine monophosphate, 4.9 mM (Walaas, 1958).

TABLE VIII: Rate Constants for the Formation and Dissociation of Mn–Substrate and Aldolase–Mn–Substrate Bridge Complexes.[a]

Complex	Temp (°C)	Log (k_{off}) (sec^{-1})	Log (k_{on}) (M^{-1} sec^{-1})	Log ($k_{3,4}$) (sec^{-1})	Log ($1/\tau_M$ H_2O) (sec^{-1})
Mn–acetol phosphate	32	≥ 3.79	≥ 6.12[b]	≥ 4.52	7.63[c]
Mn–DHAP	32	≥ 3.36	≥ 5.69[b]	≥ 4.09	7.63[c]
Mn–DHAP hydrate	32	≥ 3.53			7.63[c]
Aldolase–Mn–acetol phosphate	32	≥ 3.76	≥ 6.54	≥ 4.94	5.23[d]
Aldolase–Mn–DHAP	32	≥ 3.58	≥ 5.83	≥ 4.23	5.23[d]
Aldolase–Mn–FDP	27	4.79	7.79	5.19	5.15[d]
Aldolase–Mn–DHAP hydrate	32	≥ 3.82			5.23[d]

[a] Rate constants are defined as follows:

$$Mn + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} MnL;\ Mn(H_2O) + (H_2O)L \underset{K_0}{\overset{fast}{\rightleftharpoons}} Mn(H_2O)L \underset{k_{off}}{\overset{k_{3,4}}{\rightleftharpoons}} Mn\text{–}L$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad +\ H_2O \qquad\qquad +\ H_2O$$

$k_{3,4} = k_{on}/K_0$, where K_0 is the stability constant of the second sphere complex. For divalent cations interacting with divalent anions such as acetol phosphate and DHAP, the value of $K_0 = 40$ M^{-1} was used (Eigen and Tamm, 1962). For the tetravalent anion, FDP, the value $K_0 = 400$ M^{-1} was used (Hammes and Levison, 1964). [b] Calculated by assuming that the dissociation constant of the binary Mn–acetol phosphate and Mn–DHAP complexes was equal to the dissociation constant of Mn–orthophosphate (4.7 mM) as discussed in footnote 5. [c] For the $Mn(H_2O)_6$ complex (Swift and Connick, 1962), adjusted to 32°. [d] For the aldolase–$Mn(H_2O)_3$ complex (Table IV), adjusted to the temperature of the given experiment.

specific rates of association (k_{on}) of these complexes may be calculated as k_{off}/K_3. The lower limits for k_{off} and k_{on} are summarized in Table VIII. For the ternary aldolase–Mn–acetol phosphate complex, these rate constants exceed by at least four orders of magnitude the maximum velocity of the enzyme-catalyzed deprotonation of the substrate observed by nuclear magnetic resonance[4] and the enzyme-catalyzed detritiation observed with the more active Zn enzyme (Rose and O'Connell, 1969a). Hence, the aldolase–Mn–acetol phosphate complexes detected in the nuclear magnetic resonance experiment form and dissociate rapidly enough to participate in the catalysis of the deprotonation of acetol phosphate. The k_{on} value, when divided by K_0, the stability constant of a second sphere complex, yields $k_{3,4}$, the rate constant for formation of an inner sphere complex from a second sphere complex or ion pair. These rate constants are compared to $1/\tau_M$ or k_{off} of water in Table VIII.

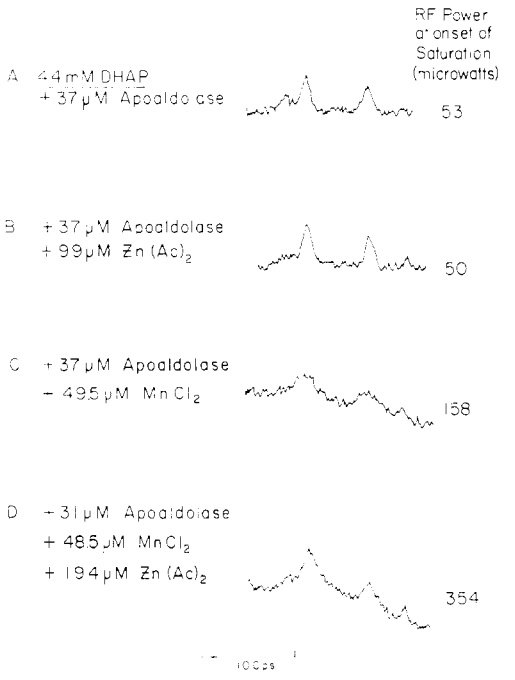
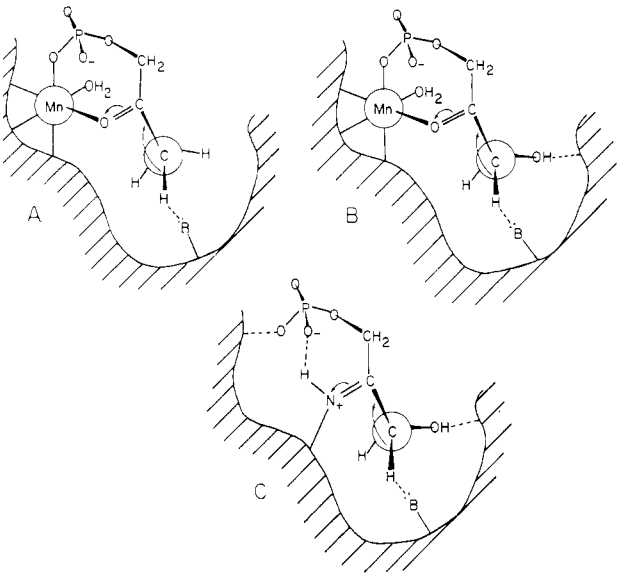


FIGURE 8: Effect of apoaldolase and its metal and inhibitor complexes on the C-1 proton resonances in the 100-MHz proton nuclear magnetic resonance spectrum of DHAP. The small resonances to the left of the doublet are due to the partially deuterated C-3 methylene group and were not studied. The isomerase contaminating this enzyme preparation was inactivated with glycidol phosphate (final concentration, 10 mM). Other components present were as described in Figure 5. $T = 32°$. The small peak upfield from the doublet was not a constant observation and may be due to a small amount of contaminating material in some of our preparations of DHAP.

Effect of Mn^{2+} and Aldolase–Mn^{2+} on the Protons of Dihydroxyacetone Phosphate. The proton nuclear magnetic resonance spectrum of DHAP (Gray and Barker, 1970) consists of a C-1 methylene phosphate doublet ($J = 6.8$ cps), which is 4.96 ppm downfield from Me_4Si, and a C-3 hydroxymethylene singlet 5.01 ppm downfield from Me_4Si. Because of the overlap of the signals in the spectra, only the C-1 methylene phosphate signal of DHAP was suitable for study. As with acetol phosphate, the metal-free aldolase and the diamagnetic aldolase–Zn complex have little or no effect on this resonance (Figure 8A,B), but the aldolase–Mn complex broadens the resonance and increases the radiofrequency power required for saturation (Figure 8C). The addition of Zn^{2+}, which displaces Mn^{2+} from the ternary complex, causes a narrowing of the resonance (Figure 8D). The paramagnetic contributions to the relaxation rates (Table IV) indicate that aldolase enhances the effect of Mn^{2+} on $1/T_2$ of the C-1 protons, consistent with the formation of an aldolase–Mn^{2+}–DHAP bridge complex.

Structural and Kinetic Properties of the Binary Mn–DHAP and Ternary Aldolase–Mn–DHAP Complexes in Solution. The Mn^{2+} to C-1 proton distances in the binary and ternary complexes of DHAP, calculated from $1/pT_{1p}$ using eq 3, are summarized in Table V and Figure 7. Comparisons with the results of molecular model studies (Table VI) indicate



IURE 9: Probable structures and mechanisms of yeast aldolase–bstrate complexes. (A and B) The manganese bridge complexes of etol phosphate and DHAP with yeast aldolase, respectively. eir conformation is depicted to be consistent with the distance ios (Table V), with the relative affinities (Table II), with the values the coupling constants (Table VII), and with the stereochemistry the deprotonation (Rose, 1958; Rose and Rieder, 1958). (C) a mechanism proposed for class I aldolases by analogy with rts A and B. In all cases, B represents a basic group on the enme.

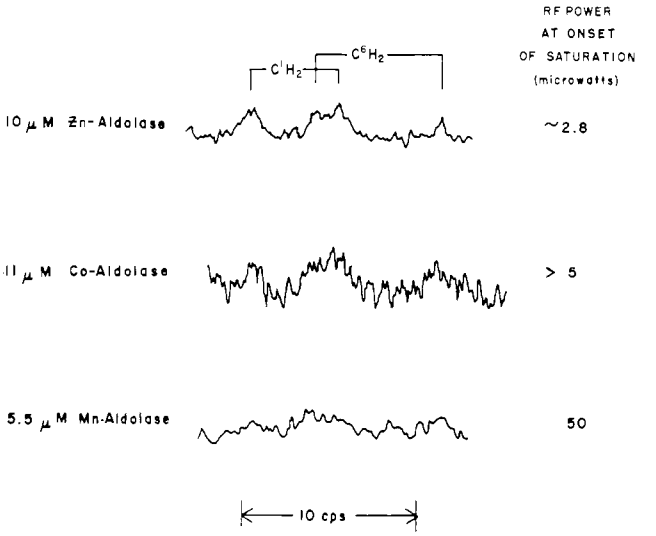


FIGURE 10: Effect of metal–aldolase complexes on the resonances in the 60-MHz proton nuclear magnetic resonance spectrum of partially deuterated FDP. The solutions contained 100 mM FDP, prepared enzymatically from 200 mM DHAP in the nuclear magnetic resonance tube as described in the text. With the Co^{2+} and Mn^{2+} enzymes the concentration of apo enzyme sites was 1.6 times that of the divalent cations. The Zn–aldolase was added as the unmodified enzyme. Other components present were 0.6 mM Tris-HCl (pD 7.9), and 0.6 mM NaCl in 98% D_2O. T = 27°. The radiofrequency powers at onset of saturation at 60 MHz differ from those observed at 100 MHz in Figures 5 and 8 because of a different calibration of the 60-MHz instrument which has no effect on the calculated values of $1/T_1$.

at these distances are consistent with direct coordination the substrate by Mn^{2+} in the binary and ternary complexes, it do not permit a choice to be made among the various mplexes which are possible. The quantitative differences tween the effects of aldolase–Mn^{2+} on the C-1 protons DHAP and the C-1 protons of acetol phosphate may be e to differences in conformation between the two ternary mplexes. As with acetol phosphate, the formation of the nary complex of DHAP increases the hyperfine coupling tween the Mn^{2+} and the C-1 protons, suggesting osphoryl coordination. The similar affinity of aldolase–n for DHAP and acetol phosphate (Table II) and the nimal decrease in q/τ_M of water produced by DHAP able III) argue against coordination of the C-3 hydroxyl oup by the enzyme-bound Mn^{2+}. Structures for the ternary mplexes consistent with these effects are given in Figure A,B.

The limiting values for k_{off}, k_{on}, and $k_{3,4}$ for the binary d ternary complexes of DHAP, calculated from $1/pT_{2p}$ d the dissociation constants as described above for acetol osphate, are given in Table VIII. The rate constants for the lolase–Mn–substrate bridge complex exceed by at least o orders of magnitude the maximal rate of the Mn–enzyme-talyzed reactions (20 sec^{-1}) (Kobes *et al.*, 1969b). Hence, e metal bridge complex of DHAP forms and dissociates idly enough to participate in catalysis.

Interaction of the Hydrate of Dihydroxyacetone Phosphate th Mn^{2+} and Aldolase–Mn^{2+}. As shown by Gray and Barker 970), solutions of DHAP contain appreciable amounts of the drate. Under our conditions, 37 ± 3% of the total DHAP s hydrated. The nuclear magnetic resonance spectrum the hydrate consists of a C-1 methylene phosphate doublet = 8.2 cps) located 4.28 ppm downfield from Me_4Si and C-3 hydroxymethylene singlet 4.03 ppm downfield from Me_4Si. Table IV summarizes the substantial paramagnetic effects of free and enzyme-bound Mn on the C-1 and C-3 protons of the hydrate and reveals an enhanced effect of the enzyme-bound Mn^{2+} on $1/T_2$ of the C-3 protons. The exchange rates of the hydrate into the coordination sphere of free and enzyme-bound Mn^{2+} ($\geq 10^{3.5}$ sec^{-1} and $\geq 10^{3.8}$ sec^{-1}, Table VIII) are at least an order of magnitude more rapid than the rate of hydration of DHAP. The latter rate may be estimated from the difference in chemical shift of free and hydrated DHAP to be less than $10^{2.6}$ sec^{-1}. These findings indicate that the hydrate of DHAP can interact directly with free and enzyme-bound Mn. In the binary Mn–DHAP–hydrate complex the absolute and relative values of the Mn to proton distances (Table V) are consistent with monodentate phosphoryl coordination, but the coupling constants (Table VII) suggest a small contribution by C-2 or C-3 hydroxyl coordination as well. Similarly, the distance ratios and coupling constants in the ternary aldolase–Mn–DHAP hydrate complex are consistent with bidentate coordination (Tables V, VI, and VII), although the precise structure of this complex is undetermined.

Effect of Zn^{2+}–Aldolase, Co^{2+}–Aldolase, and Mn^{2+}–Aldolase on the Protons of Fructose Diphosphate. As shown in Figure 10, when the protons on C-3, C-4, and C-5 of FDP have been replaced by deuterium, the nuclear magnetic resonance spectrum of FDP at 60 MHz consists of four resonance lines, a doublet arising from the protons on C-1 (J = 6.0 cps), which is 4.41 ppm downfield from Me_4Si, and a doublet arising from the protons on C-6 (J = 7.4 cps) at 4.31 ppm. The upper tracing shows the nuclear magnetic resonance spectrum of FDP in the presence of diamagnetic Zn^{2+}–aldolase. In the presence of the paramagnetic Co^{2+}–yeast aldolase, the line width of the resonances and the power at saturation are

TABLE IX: Effect of Metalloaldolases on the Relaxation Rates of the Protons of Partially Deuterated Fructose Diphosphate at 60 MHz.[a]

Interaction	$sec^{-1} \times 10^{-4}$		$sec^{-1} \times 10^{-4}$	
	$\Delta(1/pT_1)$[b]	$1/pT_{1p}$[c]	$\Delta(1/pT_2)$[b]	$1/pT_{2p}$[c]
Aldolase–Zn^{2+}–HC-1	2.1 ± 0.9 (2)		2.0 ± 0.5 (2)	
–HC-6	1.8 ± 1.3 (2)		2.0 ± 0.2 (2)	
Aldolase–Co^{2+}–HC-1	2.2 ± 1.0 (4)	≤1.3	1.8 ± 1.0 (4)	≤1.1
HC-6	2.6 ± 1.6 (4)	<2.1	2.1 ± 1.0 (4)	≤1.0
Aldolase–Mn^{2+}HC-1	7.5 ± 1.2 (2)	5.4 ± 1.5	8.1 ± 1.3 (2)	6.1 ± 1.4
–HC-6	3.8 ± 1.0 (2)	2.0 ± 1.6	5.2 ± 1.9 (2)	3.2 ± 1.9

[a] Conditions are given in Figure 10. [b] $\Delta(1/pT_1)$ and $\Delta(1/pT_2)$ represent the increases in the relaxation rates at a concentration metal = [M] over that found by extrapolation to [M] = 0, normalized by the factor p = [M]/(FDP]. [c] Determined by subtracting the respective value of $\Delta(1/pT_1)$ or $\Delta(1/pT_2)$ for aldolase-Zn-FDP. [d] The number in parentheses indicates the number of experiments as described in Table IV.

altered only slightly. When Mn^{2+}–yeast aldolase is added to FDP, the resonance lines of both sets of protons are markedly broadened, and the radiofrequency required for saturation of these lines is markedly increased. The diamagnetic and paramagnetic contributions to the longitudinal ($1/T_{1p}$) and transverse ($1/T_{2p}$) relaxation rates, calculated for the effect of the three metalloaldolases on the nuclear magnetic resonance of FDP, are summarized in Table IX. The longitudinal relaxation rates and the transverse relaxation rates of the C-1 and C-6 protons of FDP are equally increased by the addition of Zn–aldolase. Unlike the effects on acetol phosphate and DHAP, a significant diamagnetic effect of Zn^{2+}–aldolase on the protons of FDP is observed. Although many processes contribute to diamagnetic relaxation (Jardetsky, 1964), the most likely explanation for this effect is a high degree of immobilization of the substrate on the enzyme. Unlike the triose substrates, the structure of FDP in solution is predominantly a fairly rigid furanose ring (Gray and Barker, 1970). The Co^{2+}–enzyme, due to its low effective magnetic moment, increases $1/T_{1p}$ and $1/T_{2p}$ by an amount which is little greater than the diamagnetic effect of the Zn^{2+}–enzyme. The Mn^{2+}–enzyme with its greater magnetic moment produces large increases in the relaxation rates of the C-1 and C-6 protons of FDP. Since the Zn, Co, and Mn enzymes all bind FDP under the conditions of this experiment, the greater effect of the Mn–enzyme is due to interaction of the protons of FDP, especially those on C-1, with the unpaired electrons of Mn.

The paramagnetic effects of aldolase–Mn^{2+} on $1/T_2$ and $1/T_1$ of the C-1 proton of FDP exceed by one and two orders of magnitude its effects on the C-1 proton of DHAP. Such effects cannot be due entirely to the lower frequency of observation (60 MHz) which, at most, would be responsible for only a 2.8-fold greater relaxivity. The most likely explanation for the greater paramagnetic effect on FDP is a longer correlation time (τ_c) due to a greater degree of immobilization of the larger furanose substrate on the enzyme. The addition of the competitive inhibitor, Ar-DP, diminished the effect of Mn^{2+}–yeast aldolase on the line width and the radiofrequency power required for saturation of the resonance lines of the protons of FDP. A titration of the effect of Ar-DP on the line width of the C-1 proton of FDP (0.1 M) (Figure 6B), assuming competition, yields a dissociation constant for the inhibitor of 0.4 mM, in reasonable agreement with its K_I (0.3 mM) and its K_s from measurements of the proton relaxation rate of water (0.6 mM) (Table II), suggesting that both the substrate and the inhibitor are binding at the active site. At high levels of the inhibitor, 66% of the broadening of the substrate protons remained, which may be due in part to outer sphere relaxation and to residual diamagnetic effects (Mildvan *et al.*, 1967; Mildvan and Scrutton, 1967). A large outer sphere contribution to the relaxivity of FDP is consistent with the large outer sphere effects of ternary complexes on water protons (Table III).

Structural and Kinetic Properties of the Aldolase–Mn^{2+}–Fructose Diphosphate Complex. Because of the high degree of immobilization of enzyme-bound FDP, τ_c may exceed 10^{-9} sec. Hence, it is probable that the simplified Solomon–Bloembergen equation used to calculate distances and hyperfine coupling constants may not be applicable to this system. A more general treatment of the data was used to estimate τ_c in the following manner. The relaxation rates of the C-6 protons are less than those of the C-1 protons and are therefore not exchange limited. Neglecting the hyperfine term for the C-6 protons of FDP, which would be expected to be small, the ratio of $1/pT_{2p}$ and $1/pT_{1p}$ was used to calculate τ_c from the dipolar terms of the Solomon–Bloembergen equations for T_{1M} and T_{2M}, the inner sphere contribution to the longitudinal and transverse relaxation rates

$$T_{1p}/T_{2p} \leq T_{1M}/T_{2M} = {}^7/_6 + {}^2/_3\omega_I{}^2\tau_c{}^2 \qquad (6)$$

The quantity, ω_I, is the nuclear resonance frequency. Equation 6 yields a value of $\tau_c = 2.12 \times 10^{-9}$ sec for the aldolase–Mn–FDP complex, which probably represents the electron spin relaxation time of Mn^{2+}. As discussed above, lower limit values were taken as rates of internal rotation. The Mn^{2+} to proton distances calculated, using these τ_c values given in Table V and Figure 7, are consistent with the direct coordination of FDP by the enzyme-bound Mn^{2+}. The lower limit values for the distances appear to be too small, presumably because internal rotation is slow in the rigid furanose structure. For this reason the upper limit for τ_c is more likely to be correct. The corresponding distances (Table V) are consistent with molecular models of several enzyme–Mn–FDP complexes (Table VI). The hyperfine coupling constant between the Mn^{2+} and the protons at C-1 could not be calculated from the relaxivity data since the difference between $1/pT_{2p}$

and $1/pT_{1p}$ is too small to include a hyperfine contribution. Presumably, $1/pT_{2p}$ of the C-1 protons is limited by the rate of exchange ($1/\tau_M$) of FDP into the ternary complex. Assuming this to be the case, the rate constants for the formation and dissociation of the FDP complex calculated as described are given in Table VIII. As with the other substrates the exchange rate of FDP into the ternary complex is several orders of magnitude faster than the rate of cleavage of FDP catalyzed by the enzyme, indicating that the complex studied forms and dissociates rapidly enough to participate in the catalysis.

The structure of the binary Mn^{2+}–FDP complex was not studied by nuclear magnetic resonance. The dissociation constant of this complex, as determined by electron paramagnetic resonance in the presence of 50 mM Tris-HCl (pH 7.5) and 100 mM tetramethylammonium chloride, was 2.7 ± 0.5 mM at 21°. This value agrees with the dissociation constants of various monophosphate complexes of Mn^{2+} (see footnote 5), suggesting that the Mn binds independently to the two phosphoryl groups of FDP and is not chelated between them. This result is consistent with the predominantly trans orientation of the phosphate groups with respect to the furanose ring found by ^{31}P nuclear magnetic resonance (Gray and Barker, 1970).

The enhancement of the water proton relaxation rate in solutions of the binary Mn–FDP complex ($\epsilon_a = 1.7 \pm 0.2$) is comparable to the ϵ_a values for ADP and ATP (Mildvan and Cohn, 1966) due to slowed tumbling of this rigid complex. Attempts to resolve the nuclear magnetic resonance spectrum of G-3-P at 60 and 100 MHz were unsuccessful since large additional unexplained bands were observed. These additional bands may be due in part to hydration (Trentham *et al.*, 1969) or dimerization of the substrate.[6] Hence, no further studies of this compound were made.

Discussion

Studies of the enhancement of the proton relaxation rate of water have permitted the detection of a binary aldolase–$(Mn)_2$ complex (Table I) and ternary aldolase–Mn–substrate and aldolase–Mn–inhibitor complexes (Table II).

The finding that yeast aldolase binds two atoms of Mn per mole of enzyme is in agreement with the observation that the enzyme binds two atoms of zinc in the native state and two cobalt atoms in the artificially produced cobalt enzyme (R. T. Simpson, R. D. Kobes, R. W. Erbe, W. J. Rutter, and B. L. Vallee, to be published). The fact that the metal-free apo enzyme has a nearly identical molecular weight as the intact metallo enzyme shows that the metal is not involved in the maintenance of the dimeric structure (Harris *et al.*, 1969).

The existence of ternary complexes of G-3-P and DHAP with dissociation constants in reasonable agreement with their Michaelis constants (Table II) suggests that the complexes detected by nuclear magnetic resonance are kinetically active and that the condensation reaction catalyzed by Mn^{2+}–aldolase obeys a rapid equilibrium–random kinetic scheme. However, the potent inhibition by G-3-P and FDP of the detritiation of DHAP catalyzed by the Zn^{2+}–enzyme has previously been interpreted to indicate that the kinetic scheme of the reaction is highly ordered (Rose *et al.*, 1965). Hence, the kinetic scheme of the Mn^{2+}–enzyme may differ from that of the Zn–enzyme, although alternative explanations might exist for the detritiation data.

Direct coordination of acetol phosphate, DHAP, and FDP by the enzyme-bound Mn^{2+} was demonstrated by measurements of the nuclear relaxation rates of the protons of these substrates (Figures 5, 8, and 10; Tables IV and IX). The anomalous increase in the relaxation rate of water upon conversion of the binary aldolase–Mn^{2+} complex into the ternary metal bridge complexes of DHAP and FDP (Figure 3) may be explained by a greater outer sphere contribution (Table III), presumably due to an opening of the site to permit noncoordinated water protons to diffuse closer to the paramagnetic Mn^{2+}.

The metal–bridge complexes of aldolase possess thermodynamic and kinetic properties consistent with their functioning in catalysis. The rates of formation of the ternary complexes of FDP and acetol phosphate from a second sphere complex appear to be limited by the rate of dissociation of a water ligand (Table VIII) suggesting that these complexes form by an SN1–outer sphere mechanism (eq 7) (Eigen and Tamm,

$$EMn(H_2O) + (H_2O)L \underset{K_0}{\overset{\text{fast}}{\rightleftharpoons}} \underset{+\ H_2O}{EMn(H_2O)L} \underset{k_{off}}{\overset{k_{3,4}}{\rightleftharpoons}} \underset{+\ H_2O}{EMnL} \qquad (7)$$

1962). This mechanism is not general for the coordination of phosphoryl ligands since fluorophosphate binds to Mn^{2+} (Mildvan *et al.*, 1967) and methyl phosphate binds to Ni^{2+} (Brintzinger and Hammes, 1966) at rates significantly slower than the respective rates of dissociation of water ligands from the metal ion. The apparent applicability of the SN1–outer sphere mechanism for substrate binding to the Mn^{2+} which is on aldolase may be due to the unusually slow rate of dissociation of water from its inner coordination sphere.

The probable structures, in solution, of the binary Mn^{2+}–ligand and ternary aldolase–Mn^{2+}–ligand complexes of acetol phosphate and DHAP provide insight into the mechanism of catalysis by the enzyme (Figure 9A,B). The Mn^{2+} to proton distances in the binary acetol phosphate complex is consistent only with phosphoryl coordination, but in the ternary complex the distances and coupling constants suggest carbonyl as well as phosphoryl coordination (Tables V and VI; Figure 7). Similar bidentate structures for the ternary complexes of DHAP and FDP are consistent with the more limited data on these bridge complexes. Thus the enzyme-bound Mn^{2+} may be forced to coordinate the carbonyl group of the substrate. Analogous behavior has been found with the enzyme-bound Mn^{2+} of pyruvate carboxylase which appears to coordinate pyruvate and α-ketobutyrate through the carbonyl group, while inorganic Mn^{2+} coordinates these substrates predominantly through their carboxyl groups (Mildvan and Scrutton, 1967).

Coordination of acetol phosphate or DHAP through the carbonyl group by the enzyme-bound Mn^{2+} of aldolase would define the role of the metal as that of an electrophile, as previously predicted (Rutter, 1964). By σ- and π-electron withdrawal, the Mn^{2+} would polarize the carbonyl group, thereby facilitating deprotonation and enolization of the substrate (Figure 9A,B). Simultaneous coordination of the phosphoryl group provides an additional role for the enzyme-bound Mn^{2+}, namely the orientation of the substrate. Since a hydroxyl group at C-3 is necessary for rapid condensation, this group, which is probably not coordinated to the Mn^{2+}, may also influence the orientation of the substrate (Rose and O'Connell, 1969b) (Figure 9B).

[6] J. R. Knowles and A. F. W. Coulson, personal communication (1969).

By analogy with the dual role of the metal in class II or metalloaldolases, it may be suggested that the protonated Schiff base in class I aldolases also binds and orients the phosphoryl group of the substrate, DHAP, by a hydrogen bond (Figure 9C). Such hydrogen bonding would also serve to keep the Schiff base in the protonated form, which is essential for catalysis (Bender and Williams, 1966; Jencks, 1969). Thus, the pK of the Schiff base of DHAP would be expected to be approximately 7 (Koehler *et al.*, 1964; Jencks, 1969). Yet the rate of the tritium exchange on DHAP catalyzed by muscle aldolase (a class I aldolase) is independent of pH from pH 6 to 9.[7] A strong hydrogen bond between the protonated Schiff base and the negatively charged oxygen of the phosphate would raise the pK of the Schiff base by approximately 2 units and permit the enzyme to function at higher pH values as observed. In addition to the electrophilic and orientational effects discussed above, a base on the enzyme is probably required to assist the stereospecific deprotonation of DHAP in both class I and class II aldolases (Rose, 1958, 1965). A base may also operate in the various aldolases to facilitate cleavage of the hexose (Meloche, 1967; Morse and Horecker, 1968).

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