Metal Ion Specificity at the Catalytic Site of Yeast Enolase†

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ABSTRACT: A new, more gentle enzyme purification for yeast enolase was developed. A series of kinetic experiments was performed with yeast enolase where the concentration of Mg(II) is kept constant and at the K_m' level; the addition of Mn(II), Zn(II), or Cu(II) gives a hyperbolic decrease in the enzyme activity. The final velocity of these mixed-metal systems is the same as the velocity obtained only with Mn(II), Zn(II), or Cu(II), respectively. The concentration of the second metal that gives half-maximal effect in the presence of Mg(II) is approximately the same as the apparent K_m (K_m') value measured for that cation alone. Direct binding of Mn(II) to apoenolase in the absence and presence of Mg(II) shows that Mn(II) and Mg(II) compete for the same metal site on enolase. In the presence of D-2-phosphoglycerate (PGA) and Mg(II), only a single cation site per monomer is occupied by Mn(II). Water proton relaxation rate (PRR) studies of enzyme–ligand complexes containing Mn(II) and Mn(II) in the presence of Mg(II) are consistent with Mn(II) binding at site I under both conditions. PRR titrations of ligands such as the substrate PGA or the inhibitors orthophosphate or fluoride to the enolase–Mn(II)–Mg(II) complex are similar to those obtained for the enolase–Mn(II) complex, also indicating that Mn(II) is at site I in the presence of Mg(II). High-resolution ^1H and ^{31}P NMR was used to determine the paramagnetic effect of enolase-bound Mn(II) on the relaxation rates of the nuclei of the competitive inhibitor phosphoglycolate. The distances between the bound Mn(II) and the nuclei were calculated. In the presence of Mg(II) at site II, the distance between Mn(II) and the protons of phosphoglycolate was 5.73 Å and the distance between Mn(II) and the phosphorus of phosphoglycolate was 5.28 Å. In the absence of Mg(II), these distances were 6.00 and 6.59 Å, respectively. These results indicate that the presence of Mg(II) at site II causes an effect on the structure of the ligand at the active site. The frequency dependence of the PRR of various enolase–metal complexes was measured. The enolase–Mn–PGA, enolase–Mn–PGA–Mg, and the enolase–Mn–PGA–Mn complexes give similar frequency dispersions. The τ_c values for these complexes calculated for 24.3 MHz were 6.65, 4.31, and 6.24 ns, respectively. The number of exchangeable water molecules coordinated to the bound Mn(II) was estimated to be 0.5 for each of these complexes. The binding of Mg(II) or Mn(II) at site II appears not to affect the properties of Mn(II) at site I, suggesting that the metal sites are far apart in the enzyme complex and are magnetically independent. The τ_c and the number of water molecules were also calculated from a measurement of the T_{1p}/T_{2p} ratio of water at 300 MHz. The second metal ion appears to be >12 Å from the metal site I and not at the catalytic site or involved in the catalytic process. The cation at site I influences the catalytic constant of the enzyme.

Yeast enolase [phosphoenolpyruvate hydratase (E.C. 4.2.1.11)] catalyzes the reversible dehydration of PGA[1] to yield PEP. The enzyme is a symmetrical dimer with a molecular weight of 93 000. The primary structure has been determined (Chin et al., 1981a,b; Holland et al., 1981), and the secondary structure for enolase has been predicted (Sawyer et al., 1986). The X-ray structure of the enzyme has recently been reported at 2.25-Å resolution (Lebioda et al., 1989), and the enzyme–metal–substrate complex is partially resolved (Lebioda & Stec, 1991). Enolase requires a divalent metal ion for catalytic activity, and a number of metal ions elicit activation (Brewer, 1981). This enzyme binds 2 mol of activating divalent metal ions per dimer, and these cations facilitate the binding of the substrate as well as activate the enzyme (Faller & Johnson, 1973). It has been reported that the metal at site I causes a conformational change in the tertiary structure of the enzyme (Hanlon & Westhead, 1969). On this basis, the metal at site I has been called "conformational". The enzyme is reported not to be active until another 2 mol of divalent metal ions bind to the enolase dimer (Faller et al., 1977). The site II is thus called "catalytic". Brewer and Collins (1980), using spectrophotometric titration and stopped-flow kinetics, suggested that the first metal ion plays a crucial role in activating the substrate but the catalytic activity is governed by the second metal ion. Certain divalent metal ions can inhibit enolase by binding at sites that are apparently distinct from the two previous sites (Elliott & Brewer, 1980). This metal site has been called "inhibitory" and may be referred to as site III. Nowak et al., using nuclear relaxation rate studies and steady-state kinetics, showed that the Mn(II) at site I coordinates to the hydroxyl oxygen at the C-3 position of PGA (Nowak et al., 1973). This metal was referred to as the catalytically important site. The location of the metal at site II has not been determined and is not observed by X-ray diffraction (Lebioda & Stec, 1991). Brewer and Ellis suggested that the "catalytic" metal ion coordinated directly to the phosphate and the carboxyl group of the substrate (Brewer & Ellis, 1983).

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[1] Abbreviations: PGA, D-(+)-2-phosphoglyceric acid; PEP, phosphoenolpyruvate; PRR, longitudinal water proton relaxation rate; EPR, electron paramagnetic resonance; EDTA, ethylenediaminetetraacetate; SDS, sodium dodecyl sulfate.

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Dickinson et al. (1980), using Cu(II) EPR, reported that enolase sites do not include nitrogenous ligands. Recently, a pH study of the kinetics of activation by Mn(II) and of Mn(II) binding to enolase and enolase in the presence of substrate is consistent with the ligands at sites I and II being carboxylates and/or carbonyl groups but not histidine or lysine (Lee, B. H., & Nowak, 1992). Results from EPR studies showed that Co(II) at the "conformational" site appears to be in a tetragonally distorted octahedral complex while the second pair of cobalt ions appear to be in a more regular tetrahedral symmetry. It was proposed from that study that the "catalytic" metal determines the enzyme activity (Rose et al., 1984). A Cd(II) NMR study of the enzyme–metal complexes was interpreted as Cd(II) binding at the "conformational" site which consists of all oxyligands in a distorted octahedral environment and the "catalytic" site also consists of all oxyligands in a distorted octahedral geometry (Spencer et al., 1985). The involvement of carboxylic groups in metal binding has been suggested (Collins & Brewer, 1982). Recent X-ray data indicates that one of the cation binding sites contains two aspartate and one glutamate residues (Lebioda et al., 1989; Lebioda & Stec, 1991).

In this study, a mixed-metal kinetic analysis was performed to determine the separate roles of the "conformational" and the "catalytic" metal ions. Results show differential binding of several transition metals and of Mg(II) to enolase, and it is possible to occupy the two sites of the monomer with different divalent cations. A physical characterization was used to indicate the locations of the respective metal sites. Separate roles for the cations are determined by examining the kinetic and physical properties of the enzyme complexes.

Materials and Methods

PIPES and PGA (sodium salt) were obtained from Sigma. TSK DEAE-650M was obtained from EM Science, and CM-Sephadex C-50 was from Pharmacia. All other reagents were reagent grade.

Enolase was prepared from Baker's yeast using a revised method developed in our laboratory. In our hands, enolase is sometimes unstable in the acetone and ethanol fractionation steps using Westhead's method (Westhead & McLain, 1964; Westhead, 1966); thus, these steps were replaced with milder procedures. Baker's yeast was obtained from a local bakery. After the toluene autolysis (Yun et al., 1976) and centrifugation, enolase activity was collected in the supernatant obtained from 55% ammonium sulfate fractionation and centrifugation (19000g for 15 min). The enolase activity was collected in the pellet obtained from 70% ammonium sulfate fractionation. The 70% pellet was back-washed with decreasing concentrations of ammonium sulfate solution to 63% and 61%. The enolase activity that is then in the supernatant was collected, and the activity was concentrated into an 80% ammonium sulfate pellet. The pellet was resuspended in 20 mM Tris-HCl/2 mM $MgCl_2$/1 mM EDTA, pH 8.2, and desalted on a G-50 column (5 × 60 cm) using the same buffer. The protein was eluted into a TSK DEAE-650M column (5 × 24 cm) equilibrated in the same buffer. Enolase does not bind to the resin. The activity was collected, and the protein was concentrated by ammonium sulfate (80%) precipitation. After the buffer was changed to 20 mM PIPES/2 mM $MgCl_2$/1 mM EDTA, pH 6.0, by desalting on G-50 (5 × 60 cm), the protein was adsorbed on a CM-Sephadex C-50 column (5 × 18 cm). The column was washed with the same buffer, and enolase was collected by gradient elution varying the ionic strength from 0 to 1.5 M KCl (3-L total volume). The activity was recovered as a single peak between 0.38 and 0.42 M KCl. The enolase fraction was desalted, lyophilized, and stored as a dry powder at −20 °C. The purification factor and the yield are given in the Results section. The enzyme was demetalized by treating with Chelex-100 followed by gel filtration on G-50 (1.5 × 30 cm).

Enzyme activity was assayed as described by Westhead (1966) with a slight modification. The assay consisted of 50 mM Tris-HCl, pH 7.5, 2 mM PGA, 1 mM $MgCl_2$, 50 mM KCl, and 0.01 mM EDTA in 1 mL and was performed at room temperature. The increase in absorbance was measured at either 230 or 240 nm and was measured by using a Gilford 250 spectrophotometer. The enzyme concentration was determined by using the extinction coefficient at 280 nm (0.89 mL mg^{-1} cm^{-1}) and the molecular weight of 93 000 per dimer (Chin et al., 1981b). The specific activity was initially determined as the change in absorbance at 240 nm/min or at 230 nm/min divided by enzyme concentration expressed as absorbance at 280 nm (Westhead & McLain, 1964). The kinetic data is reported as specific activity in standard activity units (micromoles of product per minute per milligram = units per milligram). One unit of activity measured at 230 nm [$(\Delta A_{230}/A_{280})$ min^{-1}] corresponds to 0.32 unit/mg, and one unit of activity measured at 240 nm [$(\Delta A_{240}/A_{280})$ min^{-1}] corresponds to 0.576 unit/mg. The specific activity of pure yeast enolase at pH 7.5 was 330 $(\Delta A_{230}/A_{280})$ min^{-1} at 30 °C or 124 units/mg at 22 °C (Westhead, 1966).

Kinetic Studies. Kinetic studies were performed spectrophotometrically at room temperature. Initial velocities were measured at 230 nm on a Gilford 240 spectrophotometer as a function of various metal ion concentrations and at various pH values. For buffers, 50 mM Tris-HCl or 50 mM PIPES was used in the experiments. Specific details for each experiment are given in the figure legends.

Binding Studies. The binding of Mn(II) to the apoenzyme and the enzyme–metal–substrate complexes was measured by EPR spectroscopy. The concentration of free Mn(II) was measured on an X-band Varian E-9 EPR spectrometer. The number of binding sites and the binding constant were obtained from analysis of a Scatchard plot (Scatchard, 1949) of the data. The principal data that were treated were that obtained over the range of 20–80% occupancy (Deranleau, 1969) when technically feasible. The Mn(II) binding to the apoenzyme and the enzyme–metal–substrate complex was also determined in the presence of varying levels of Mg(II). Specific experimental details are given in the figure legend for the experiments described.

The binding of ligands to the various enzyme–metal complexes was determined by measuring the PRR with a Seimco-pulsed NMR spectrometer operating at 24.3 MHz. The treatment of the data has been well described elsewhere (Nowak, 1981). The enhancement value was calculated from $1/pT_{1p}$ values and plotted against the substrate concentration. The binding of the inhibitory ligands inorganic phosphate and fluoride to enolase–Mn was studied in a similar fashion (Maurer & Nowak, 1981). The binding constant of the ligand to the enolase–Mn complex (K_3) was obtained by the best fit of the data using the approach initially described by Reed et al. (1970) and modified for use on the VAX 750.

High-Resolution NMR Studies of Phosphoglycolate. The ^1H and ^{31}P NMR experiments were performed at 300 MHz and 121.5 MHz, respectively, using a Nicolet NTC-300 NMR spectrometer. The $1/T_1$ values were measured by the inversion recovery method (Vold et al., 1968) and $1/T_2$ values were measured by the Hahn spin–echo method (Hahn, 1950). The $1/T_1$ and $1/T_2$ values were measured as a function of added

Mn(II) concentration, and the $1/pT_{1p}$ and $1/pT_{2p}$ were determined from the slopes of the plots. Each experiment contained 100 μM enolase sites, 20 mM phosphoglycolate, 20 mM cacodylate, pH 6.8, and 50 mM KCl. The relaxation rates were used to calculate the distance between Mn(II) and the nuclei measured (Solomon & Bloembergen, 1956). Since the methodolgy has been well described elsewhere (Nowak, 1981), only a brief outline will be described. The paramagnetic effect of the enzyme-bound Mn(II) ($1/T_{1p}$) on the relaxation rates of the nuclei being studied is obtained from the experimental $1/T_1$ ($1/T_{1,obs}$) corrected for the relaxation in the absence of Mn(II) ($1/T_{1,0}$). This value is related to the ratio of bound Mn(II) to free ligand, p ($p = [Mn_b]/[L_f]$), the number of ligands at the Mn(II) site, q, normally unity, T_{1M}, the relaxation time of the nucleus at the Mn(II) site, and τ_m, the lifetime of the ligand at the Mn(II) site (eq 1). When $\tau_m \ll$

$$\frac{1}{T_{1p}} = \frac{1}{T_{1,obs}} - \frac{1}{T_{1,o}} = \frac{pq}{T_{1M} + \tau_m} \quad (1)$$

T_{1M}, relaxation is in fast exchange and $1/pT_{1p} = 1/T_{1M}$. Analogous arguments are made for $1/pT_{2p}$. When $1/pT_{1p}$ is in fast exchange, the electron Mn(II)–nuclear dipolar distance, r, can be calculated using the abbreviated form of the Solomon–Bloembergen equation (Solomon, 1955; Bloembergen, 1957) given by

$$r\ (\text{Å}) = C[pT_{1p}f(\tau_c)]^{1/6} \quad (2)$$

where C is a constant and $f(\tau_c)$ is a correlation time function. When the paramagnetic species is Mn(II) and the measured nucleus is ^{1}H, C is 812 Å $s^{-1/3}$. When the nucleus is ^{31}P, C is 601 Å $s^{-1/3}$. The $f(\tau_c)$ is defined as

$$f(\tau_c) = \frac{3\tau_c}{(1 + \omega_I^2\tau_c^2)} + \frac{7\tau_c}{(1 + \omega_s^2\tau_c^2)} \quad (3)$$

where τ_c is the correlation time for the dipolar interaction, ω_I is the nuclear precession frequency, and ω_s is the electron precession frequency. For some of the studies reported, τ_c was calculated from the T_{1p}/T_{2p} ratio for proton relaxation rates at 300 MHz (eq 4).

$$\tau_c = \frac{6(T_{1p}/T_{2p} - 7)}{1.42 \times 10^{19}} \quad (4)$$

Frequency Dependence of Various Enzyme–Metal–PGA Complexes. The frequency dependence of PRR was determined by measuring the values for $(T_{1p})_{bound}$ of various enzyme–metal complexes with a Seimco-pulsed NMR spectrometer. The experiment was performed at pH 6.8 at frequencies of 7.03, 10.3, 14.0, 24.3, 35.0, and 45.0 MHz. The concentration of free Mn(II) was measured by EPR as explained previously. The value for $(T_{1p})_{bound}$ was calculated from the relationship

$$\frac{1}{(T_{1p})_{obsd}} = \frac{[Mn]_{free}}{[Mn]_{total}}\frac{1}{(T_{1p})_{free}} + \frac{[Mn]_{bound}}{[Mn]_{total}}\frac{1}{(T_{1p})_{bound}} \quad (5)$$

The τ_c and q for the complexes studied can be obtained from the frequency dependence of $(T_{1p})_{bound}$ (Navon, 1970):

$$\frac{1}{T_{1p}} = \frac{NqD}{55.6}\frac{\tau_c}{1 + \omega_I^2\tau_c^2} \quad (6)$$

where N is the molar concentration of bound Mn(II), D is a collection of constants, and the correlation time τ_c is given as

$$\frac{1}{\tau_c} = \frac{1}{\tau_r} + \frac{1}{\tau_s} + \frac{1}{\tau_m} \quad (7)$$

Table I: Enolase Purification

purification steps	total units	total protein (mg)	sp act. (units/mg)	% yield	purification factor
toluene autolysis	259 000	69 800	3.85	100	1
$(NH_4)_2SO_4$ fractionation	322 000	55 500	5.81	120	1.51
back-wash	310 000	17 900	17.3	115	4.49
TSK DEAE	118 000	1 200	98.6	43.9	25.6
CM-Sephadex	76 400	616	124.0	28.4	32.2

In this equation, τ_r is the rotational correlation time, τ_s is the Mn(II) electron spin relaxation time, and τ_m is the lifetime of the nucleus in the vicinity of the bound Mn(II). The τ_s, as described by Bloembergen and Morgan (1961) is

$$\frac{1}{\tau_s} = B\left(\frac{\tau_v}{1 + \omega_s^2\tau_v^2} + \frac{4\tau_v}{1 + 4\omega_s^2\tau_v^2}\right) \quad (8)$$

where B is the zero-field splitting constant and the τ_v is the correlation time for the modulation of the zero-field splitting. The values for q and τ_c can be evaluated by estimating values for B, τ_v, τ_r, and τ_m which give the best fit of the data (T_{1p}) as a function of frequency. The data were treated for us by Dr. J. J. Villafranca to obtain a best fit.

The values for the correlation time (τ_c) and the number of water molecules (q) in the complex were also obtained by measuring the T_{1p}/T_{2p} ratio for water protons at a fixed frequency (Navon, 1970). The $1/T_{1p}$ and $1/T_{2p}$ for the various enzyme–metal–substrate complexes were obtained at pH 6.8 at 300 MHz with a Nicolet NT-300 NMR spectrometer.

The $1/T_1$ and $1/T_2$ values were measured for control solutions which had an identical composition except that it did not contain Mn(II). The $1/T_{1p}$ and $1/T_{2p}$ values were the differences between the relaxation rates of the enzyme solution which contained Mn(II) and those of a control solution as described by Navon (1970). The τ_c and q were calculated as

$$\tau_c = \frac{T_{1p}/T_{2p} - 1.1887}{0.6887\omega_I^2} \quad (9)$$

$$q = (3.26 \times 10^{-14})\frac{[(T_{1p}/T_{2p}) - 0.5]}{(T_{1p}/T_{2p}) - 1.19}\frac{\omega_I}{NT_{1p}} \quad (10)$$

where ω_I is the nuclear resonance frequency and N is the molar concentration of the bound Mn(II). The principal assumption made in these calculations is that scalar effects on T_{1p} and T_{2p} values are negligible at higher fields.

Results

Enzyme Purification. Enolase was purified starting from two pounds of Baker's yeast. A new purification procedure was developed to eliminate the solvent fractionation steps used in the earlier procedures (Westhead & McLain, 1964; Westhead, 1966). The solvent steps in the previous method were not consistently reliable in our hands. Following toluene autolysis of the yeast, an ammonium sulfate fractionation and back-wash were performed followed by chromatography on a TSK DEAE column and then on CM-Sephadex. This procedure yields enolase with specific activity of 124 units/mg in 29% yield. The purification scheme is summarized in Table I. The protein gives a single band by SDS–polyacrylamide gel electrophoresis and a single peak that elutes at pH 7.75 by chromatofocusing. There is no indication of more than one isozyme or of multiple forms sometimes observed in earlier preparations (Westhead & McLain, 1964; Westhead, 1966).

Table II: Kinetic Constants for Metal Activation of Enolase in Single and in Mixed-Metal Systems

pH	M(II)	$K_{m,1}$[a] (μM)	$V_{m,1}$[b]	$K_{m,2}$[c] (μM)	$V_{m,2}$[d]
7.5	Mg	83 ± 6	133		
	Mn	3 ± 1	46.9	7 ± 2	36.9
	Zn	3 ± 1	30.5	5 ± 2	14.1
6.8	Mg	909 ± 50	51.1		
	Mn	9 ± 3	19.0	40 ± 10	17.9
	Zn	3 ± 1	18.6	2 ± 1	12.2
5.8	Mg	3500 ± 200	35.7		
	Mn	42 ± 4	4.82	50 ± 5	3.85
	Zn	4 ± 2	5.46	9 ± 2	2.89

[a] Concentration of M(II) that gives $V_m/2$. [b] V_m measured with only the cation indicated (units per milligram). Statistical deviations are approximately 5%. [c] The concentration of M(II) that gives half-maximal velocity change in the presence of K_m values of Mg(II). [d] Final v obtained by the addition of the second cation to Mg(II)–enolase (units per milligram). Statistical deviations are approximately 5–8%.

These multiple forms are apparently due to partial deamidation of the protein (Westhead & McLain, 1964).

Mixed Metal Kinetics. Activation of enolase by Mg(II) and by Mn(II) was studied at several pH values from pH 5.8 to pH 7.5. Data were fit to obtain the values for V_m and K_m' where K_m' is the concentration of cation giving half-maximal activation. The Mg(II)-activated enzyme has a higher turnover number (higher V_m) than Mn(II) at each pH measured and has a greater K_m' (Table II). In the presence of a high but subsaturating Mg(II) concentration, the addition of Mn(II) gives a hyperbolic decrease in velocity to yield a final value that is the same maximal velocity as that measured for Mn(II) alone. This same observation was made at each pH value studied (Figure 1). No apparent synergism was observed in these mixed-metal experiments. Activation of enolase by Mg(II) and by Zn(II) was also studied and compared at several pH values. A greater V_{max} was elicited by Mg(II) than by Zn(II), but a higher value for K_m' for Mg(II) is also measured. As with Mn(II), addition of Zn(II) to enzyme containing subsaturating Mg(II) yields a hyperbolic decrease in activity to give a final velocity similar to that measured with Zn(II) alone (Figure 2). In Figure 2C, data is shown for Zn(II) addition to apoenzyme or to enzyme with 3.5 mM Mg(II).

The concentrations of Mn(II) at which a half-maximal effect in the presence of Mg(II) was obtained were 7, 40, and 50 μM at pH 7.5, 6.8, and 5.8, respectively, as tabulated in Table II. The concentrations of Zn(II) which gave a half-maximal effect were 5, 2, and 9 μM at pH 7.5, 6.8, and 5.8, respectively. These apparent activator constants were of the same order of magnitude as the K_m' values for Mn(II) and for Zn(II), respectively, at the same pH values, suggesting that Mn(II) or Zn(II) replaced Mg(II) at the catalytically effective metal binding site.

Activation of enolase by Cu(II) was also studied in 50 mM HEPES, pH 7.5. The effect of Cu(II) addition on the activity of enolase at subsaturating Mg(II) was similar to the effects observed by Zn(II) and Mn(II). The added Cu(II) showed a hyperbolic decrease in activity and the final velocity was the same as V_m measured for Cu(II) alone. The K_m' measured for Cu(II) was 3 μM, and the concentration of Cu(II) which gives a half maximal effect in the presence of the K_m' value of Mg(II) was 5 μM (data not shown).

Determination of Mn(II) Binding to Enolase. A single binding site for Mn(II) per monomer of enolase was measured in the absence of the substrate. Mn(II) binding to apoenolase was also determined in the presence of Mg(II). There was only a single binding site per monomer, and the K_d values measured were 0.9 μM, 6.5 μM, and 17.5 μM at 0 μM, 200 μM, and 500 μM Mg(II), respectively. Assuming that the two cations are mutually competitive, these results allow an estimate of a K_d value for Mg(II) = 30 ± 3 μM. Measurements made at higher concentrations of Mn(II) give increased scatter but no evidence for more than one binding site per monomer.

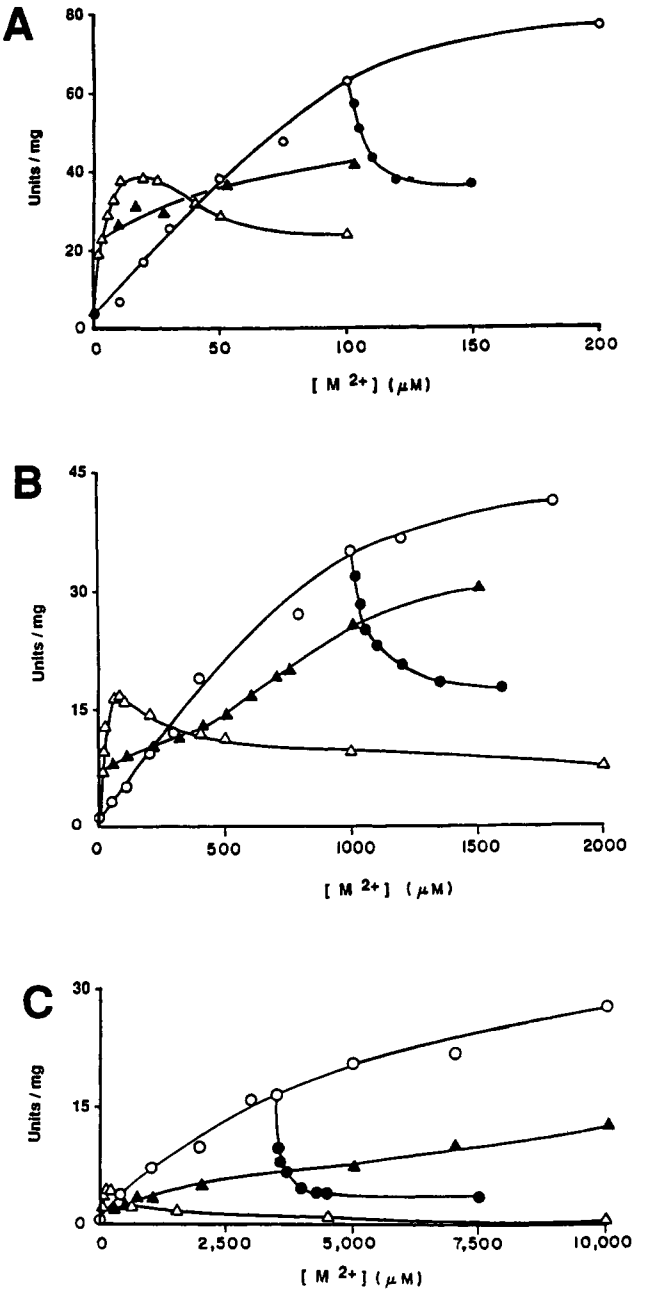

FIGURE 1: Activation of enolase by Mg(II) and Mn(II) at various pH values. (A) At pH 7.5, the assay mixtures contained 50 mM Tris-HCl, pH 7.5, 50 mM KCl, and 2 mM PGA. Mg(II) was added up to 200 μM (○) and Mn(II) up to 100 μM (△). To observe the mixed-metal effect, Mn(II) was added (1–50 μM) to the assay in the presence of 100 μM Mg(II) (●). Similarly, 0–100 μM Mg(II) was added in the presence of a fixed (3 μM) concentration of Mn(II) (▲). The concentration values on the abscissa are for the total concentration of metals in the assay. (B) At pH 6.8, the assay mixtures contained 50 mM PIPES, pH 6.8, 50 mM KCl, and 2 mM PGA. Velocity was measured with Mg(II) alone (○), with Mn(II) alone (△), with Mn(II) in the presence of 1 mM Mg(II) (●), and with Mg(II) in the presence of 10 μM Mn(II) (▲). (C) At pH 5.8, the assay mixtures contained 50 mM PIPES, pH 5.8, 50 mM KCl, and 2 mM PGA. Velocity was measured as a function of Mg(II) alone (○), Mn(II) alone (△), Mn(II) in the presence of 3.5 mM Mg(II) (●), and Mg(II) in the presence of 30 μM Mn(II) (▲).

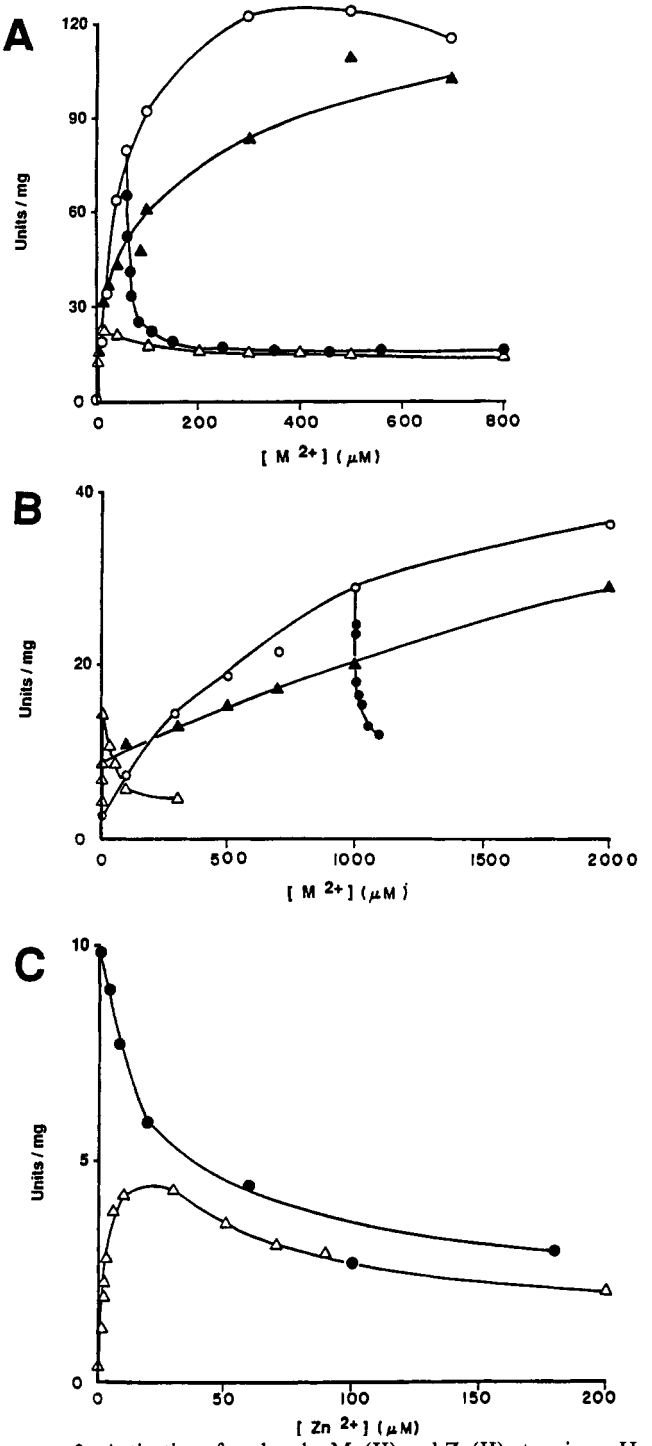

FIGURE 2: Activation of enolase by Mg(II) and Zn(II) at various pH values. (A) At pH 7.5, the assay mixtures contained 50 mM Tris-HCl, pH 7.5, 50 mM KCl, and 2 mM PGA. Velocity increased as a function of Mg(II) alone (○), Zn(II) alone (△), Zn(II) in the presence of 60 μM Mg(II) (●), and Mg(II) in the presence of 2 μM Zn(II) (▲). (B) At pH 6.8, the assay was the same as in (A) except that 50 mM PIPES, pH 6.8, was used instead of Tris-HCl. Velocity was measured as a function of Mg(II) alone (○), Zn(II) alone (△), Zn(II) in the presence of 1 mM Mg(II) (●), and Mg(II) in the presence of 3 μM Zn(III) (▲). (C) At pH 5.8, the assay was the same as in (B) except that the PIPES buffer pH is 5.8. For simplicity, only the velocity effects as a function of Zn(II) are shown. The velocity measured at 3.5 mM Mg(II) and pH 5.8 is 17 units/mg. Velocity was measured as a function of Zn(II) (△) and Zn(II) in the presence of 3.5 mM Mg(II) (●).

Two Mn(II) sites per monomer are observed in the presence of the substrate (Lee, B. H., & Nowak, 1992). In the presence of 800 μM PGA and in the presence of 0.5 mM Mg(II) and 1 mM Mg(II), only one Mn(II) site per monomer was found with K_d values of 5.4 μM and 16.1 μM, respectively. An estimate of K_d for Mg(II) at site I under these conditions is 70 ± 17 μM. Binding experiments performed at higher concentrations of Mn(II) gave increased scatter but gave no significant evidence for more than one Mn(II) site per monomer under these experimental conditions.

Table III: Parameters Obtained from Fits to PRR Titration Data[a]

complex	titrant	K_3 (μM)	ϵ_T
E–Mn	PGA	36.0 ± 11.7	1.46 ± 0.47
E–Mn_2	PGA	50.0 ± 5.5	1.34 ± 0.15
E–Mn–Mg	PGA	50.0 ± 6.7	1.07 ± 0.14
E–Mn–F	P_i	0.50 ± 0.17	1.79 ± 0.25
E–Mn_2–F	P_i	0.15 ± 0.04	0.79 ± 0.21
E–Mn–Mg–F	P_i	0.10 ● 0.02	0.41 ± 0.09
E–Mn–P_i	F^-	1000 ± 164	0.81 ± 0.13
E–Mn_2–P_i	F^-	935 ± 210	1.33 ± 0.30
E–Mn–Mg–P_i	F^-	951 ± 69	0.35 ± 0.03

[a] The experiments were performed as described in the legend to Figure 3. The parameters K_3 and ϵ_T are obtained from best fits to the data. The deviations are obtained from replicate experiments, and the fits to the data vary from 0.8 to 12% standard deviation.

The binding of Mn(II) to apoenzyme appears competitive with Mg(II) binding. In the presence of substrate and Mg(II), Mn(II) appears to bind selectively to only one of the two cation sites on the monomer even at high concentrations of Mg(II). This appears to be the cation site that is important in modulating catalytic activity. Conversely, Mg(II) appears to bind quite selectively at the second site and less tightly to site I. Under experimental conditions, we have no evidence that Mn(II) does significantly displace Mg(II) from the second site.

PRR Binding Studies of Ligands to the Enolase–Mn(II) and the Enolase–Mn(II)–Mg(II) Complexes. To determine the binding ability of PGA to the enolase–Mn(II), enolase–Mn(II)–Mn(II), and enolase–Mn(II)–Mg(II) complexes and to further characterize the Mn(II) site in the mixed-cation complex, titration of PGA into the various enolase–cation complexes was studied by PRR at 24.3 MHz and pH 6.8. The $1/T_1$ values were measured as a function of added PGA concentration. The enhancement values were calculated and treated as described earlier (Nowak, 1981) and were plotted versus the PGA concentration (Figure 3A). The titration data was fit to obtain K_3 values for PGA to the respective complexes and the final enhancements (ϵ_T) for the respective complexes (Table III). The complexes containing only Mn(II) were titrated with PGA at Mn(II) levels where site I is predominantly occupied (enzyme–Mn(II)) and also where both cation sites are occupied (enzyme–$Mn(II)_2$) in the respective complexes that contain PGA. The similarity in the results of the titrations between formation of the enzyme–Mn–substrate and enzyme–Mn–substrate–Mg complexes suggests that the Mn(II) in the enolase–Mn(II)–substrate–Mg(II) complex occupies the same site as does Mn(II) in the ternary enolase–Mn(II)–substrate complex (site I metal).

The inhibitory ligands fluoride and P_i bind synergistically to enolase–Mn(II) at different regions of the catalytic site (Maurer & Nowak, 1981; Nowak & Maurer, 1981). The titrations of P_i into the enolase–fluoride complexes containing Mn(II), $Mn(II)_2$, and Mn(II) and Mg(II) were also performed (Figure 3B). The binding parameters obtained from the best fit to the data are tabulated in Table III. The results also suggest that Mn(II) occupies site I but in this case the binding of a cation at site II increases the affinity of the complex for P_i.

Fluoride ion was titrated into the enolase–P_i complexes containing Mn(II), $Mn(II)_2$, and Mg(II) and Mn(II) (Figure

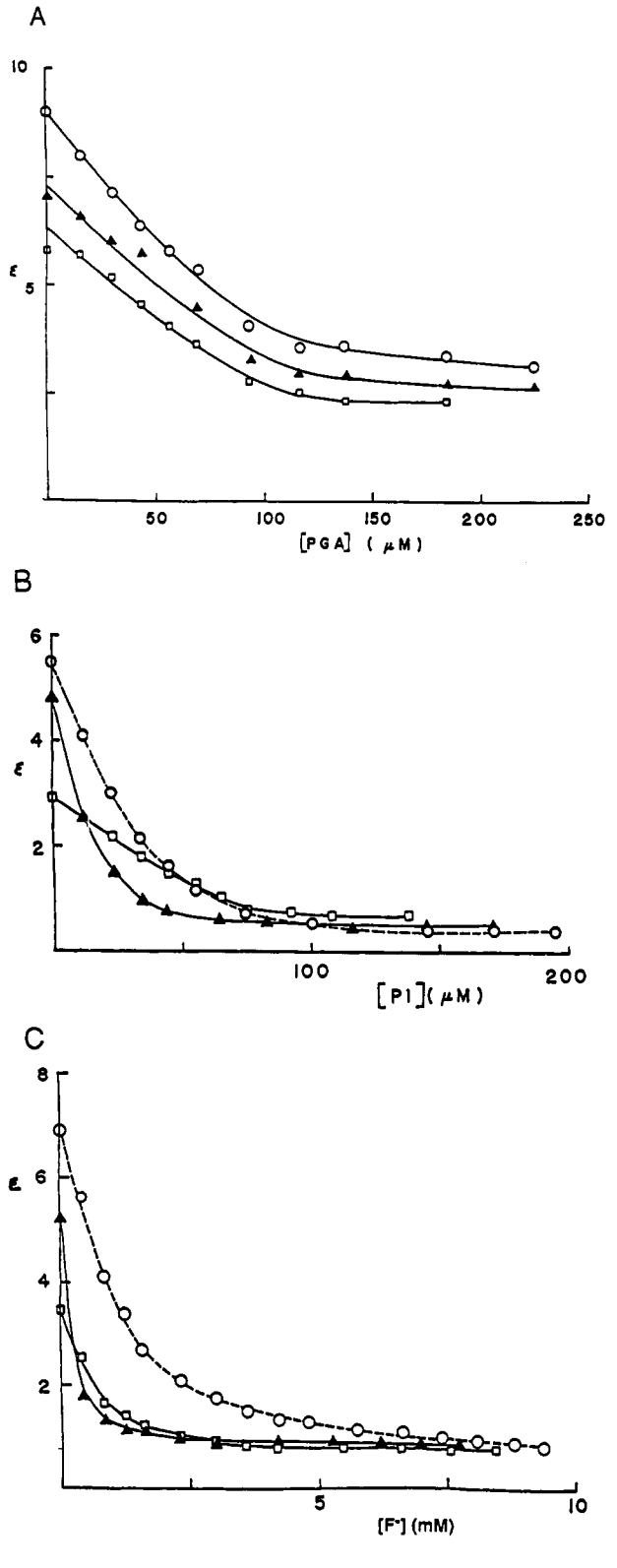

FIGURE 3: PRR titration of ligands to the enolase–Mn(II) and the enolase–Mn(II)–Mg(II) complexes. (A) A titration of PGA into the enolase complex was performed in the presence of 50 mM PIPES, pH 6.8, 50 mM KCl, and 76.8 μM enolase sites. The mixtures also contained 50 μM Mn(II) (○), 50 μM Mn(II) and 1 mM Mg(II) (▲), or 100 μM Mn(II) (□). (B) Phosphate titration of enolase in the presence of 50 mM PIPES, pH 6.8, 50 mM KCl, 100 μM enolase sites, and 200 mM KF. The titration mixtures also contained 50 μM Mn(II) (○), 50 μM Mn(II) and 1 mM Mg(II), (▲), or 200 μM Mn(II) (□). (C) Fluoride titration of enolase in the presence of 50 mM PIPES, 50 mM KCl, 123 μM enolase sites, and 1.8 mM KH_2PO_4. The titration mixtures also contained 50 μM Mn(II) (○), 50 μM Mn(II) and 1 mM Mg(II) (▲), or 200 μM Mn(II) (□).

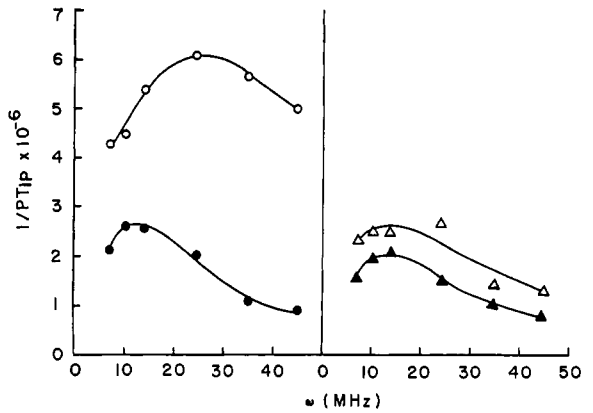

FIGURE 4: The frequency dependence of PRR of various enolase–metal–PGA complexes. The water proton relaxation rates were measured for various enolase complexes at six different frequencies (7.03, 10.3, 14.0, 24.3, 35.0, and 45.0 MHz). Each sample contained 50 mM PIPES, pH 6.8, 50 mM KCl, and 120 μM enolase sites. The samples also contained 50 μM Mn(II) (○); 50 μM Mn(II) and 800 μM PGA (●); 50 μM Mn(II), 1 mM Mg(II), and 800 μM PGA (△); or 200 μM Mn(II) and 800 μM PGA (▲).

Table IV: Parameters Used To Obtain Best Fits for the Frequency Dependence of PRR[a]

	complex			
parameters	E–Mn	E–Mn–PGA	E–Mn–PGA–Mn	E–Mn–PGA–Mg
$B \times 10^{-18}$ ($(s)^{-2}$)	8.64	4.33	3.48	4.50
$\tau_v \times 10^{11}$ (s)	1.22	6.88	4.03	9.43
$\tau_c^b \times 10^9$ (s)	2.40	6.65	6.24	4.31
$\tau_s^b \times 10^9$ (s)	9.77	82.2	60.6	107
$\tau_m \times 10^9$ (s)	3.18	7.24	6.96	4.49
q	1.62	0.34	0.28	0.43
% error	2.8	4.8	3.7	11.7

[a] q is the hydration number for bound Mn(II), and B is a zero-field splitting parameter. Other parameters are defined in Materials and Methods. The experimental conditions used for each complex are the same as that described in Figure 4. [b] τ_c and τ_s are calculated for 24.3 MHz.

3C). The parameters obtained from the calculation of a best fit to the data are presented in Table III. In all of the PRR studies, there is no substantial difference in ligand binding to the enolase–Mn(II) or the enolase–$Mn(II)_2$ complexes.

Frequency Dependence of the Water Relaxation Rates of Various Enolase–Metal Complexes. The PRR for the enolase–Mn(II), enolase–Mn(II)–PGA, enolase–Mn(II)–PGA–Mg(II), and enolase–Mn(II)–PGA–Mn(II) complexes were measured at six different frequencies from 7.03 MHz to 45 MHz (Figure 4). The curves used to fit the data are calculated using the Bloembergen–Morgan theory (Bloembergen & Morgan, 1961) and agree well with the experimental data (percent error is 2.8–11.7%). The parameters used to generate the best fit to the data and the percentage of error of the fit are tabulated in Table IV.

The enolase–Mn–PGA and enolase–Mn–PGA–Mg complexes give a similar frequency dispersion and yield τ_c values of 6.65 and 4.31 ns, respectively. The enolase–$(Mn)_2$–PGA complex also appears similar and gives a τ_c of 6.24 ns.

The electronic properties of Mn(II) in the quaternary enolase–Mn_2–PGA complex are similar to those for the ternary enolase–Mn–PGA complex, indicating that neither Mg(II) nor Mn(II) at site II affects the electronic properties of Mn(II) at site I. The distance between the two metal sites appears to be far apart (>12 Å) in the enzyme complex since the electronic properties of Mn(II) at site I were not affected by the presence of the paramagnetic Mn(II) at site II. The

Table V: Solvent Relaxation Rates for Various Enzyme-Mn(II)-Substrate Complexes at 300 MHz[a]

	complex			
parameters	E-Mn[b]	E-Mn-PGA[c]	E-Mn-PGA-Mn[d]	E-Mn-PGA-Mg[e]
T_{1p}/T_{2p}	31.2	37.0	20.9	68.3
τ_c (at 300 MHz) (ns)	3.50	3.82	2.84	5.24
q	2.91	0.81	1.03	0.47

[a] Each experiment contained 50 mM PIPES, pH 6.8, 50 mM KCl and 120 μM enolase sites. Further additions are indicated by the rest of the footnotes: (*b*) 50 μM Mn(II); (*c*) 50 μM Mn(II) and 800 μM PGA; (*d*) 200 μM Mn(II) and 800 μM PGA; (*e*) 50 μM Mn(II), 800 μM PGA, and 50 μM Mg(II).

properties of Mn(II) at site II appear similar to those for Mn(II) at site I in the presence of PGA.

The effect of enolase-bound Mn(II) on relaxation rates ($1/T_{1p}$ and $1/T_{2p}$) of water protons were measured at pH 6.8 and at 300 MHz. The τ_c and q values were calculated according to Navon (1970), as described in Materials and Methods. In these studies, fewer parameters are fit and the primary assumption is that there is an insignificant scalar effect on $1/T_{2p}$. Table V shows the values measured for the different enolase–metal–substrate complexes. The τ_c values for the enolase–Mn(II), enolase–Mn(II)–PGA, enolase–$Mn(II)_2$–PGA, and the enolase–Mn(II)–PGA–Mg(II) are similar. These results indicate that the properties of the enolase–Mn(II)–PGA, enolase–$Mn(II)_2$–PGA, and the enolase–Mn(II)–PGA–Mg(II) are similar to each other and that the Mn(II) or Mg(II) at site II does not significantly affect the electronic properties of Mn(II) at site I.

High-Resolution Nuclear Relaxation Rate Studies of Phosphoglycolate. The effect of enolase–Mn(II) on the relaxation rates ($1/pT_{1p}$ and $1/pT_{2p}$) of the inhibitor 2-phosphoglycolate were measured. The experiments were performed as described in Materials and Methods but also in the presence of 800 μM Mg(II). The $1/pT_{1p}$ and $1/pT_{2p}$ values for the protons in the presence of Mg(II) were 4710 s^{-1} and 18 200 s^{-1}, respectively. The values measured for phosphorus under identical conditions are 2380 s^{-1} and 145 000 s^{-1}, respectively. The τ_c for the Mn(II)–nuclear interaction was calculated from the T_{1p}/T_{2p} ratio for the proton relaxation and gave a value of 1.07 ns. From the relaxation rate measurements and the τ_c value calculated, the distance between Mn(II) and the protons was calculated to be 5.73 Å. The distance between Mn(II) and the phosphorus was calculated to be 5.28 Å using eq 2. In the absence of Mg(II), where Mn(II) binds primarily to site I, the $1/pT_{1p}$ and $1/pT_{2p}$ values for the protons were 3650 s^{-1} and 13 540 s^{-1} and for the phosphorus were 373 s^{-1} and 31 100 s^{-1}, respectively. The τ_c value was also calculated from the T_{1p}/T_{2p} ratio for the protons of that complex and was 1.04 ns. The Mn(II)–proton distance was calculated to give 6.00 Å, and the Mn(II)–phosphorus distance was 6.59 Å.

Discussion

Our nomenclature for the cation binding sites on enolase in the presence of substrate is based on Mn(II) binding where the site of highest affinity is defined as site I, the next site is site II, and the weakest binding site, the inhibitor site, is site III (Lee, B. H., & Nowak, 1992). The primary conclusion drawn from the results presented in this work is that the cation at site I determines the enzyme activity in contrast to the previous model (Faller et al., 1988; Brewer & Collins, 1980) that suggests the metal at site II determines the enzyme activity.

Three experimental results support the conclusion. The mixed-metal kinetic data show that the enzyme activity in the presence of a high concentration of Mg(II) yields a hyperbolic response to the addition of the activating transition metal ion (Mn(II), Zn(II), Cu(II)) to give catalytic activity that is obtained by the activation of enolase only by the transition metal. These results suggest that the transition metal that is added selectively displaces Mg(II) at one site. Binding experiments with Mn(II) show that there is only one tight Mn(II) binding site per monomer in the case where Mg(II) and substrate are present. The Mn(II) binds only at site I. The assignment of this binding site as site I is made because the Mn(II) in the enolase–Mn–PGA, enolase–Mn–PGA–Mn, and the enolase–Mn–PGA–Mg complexes sees a similar solvent environment on the basis of PRR measurements. The substrate binds to the Mn(II) in each respective complex, and the electronic properties of the Mn(II) at the catalytic site are similar in each such complex.

Synergism of cation activation of enzymatic activity using mixed-metal systems has been observed in cases of metal-requiring enzymes where there is more than one cation site in the enzyme–substrate(s) complex and each cation plays a specific role in the catalytic process (Lee et al., 1981; Baek & Nowak, 1982). Synergism is defined as resultant activity from two activators that differs from the effect of a single activator and different from the sum of two activators. In the several cases previously studied (Lee et al., 1981; Baek & Nowak, 1982), the kinetic results with two activators were greater than the sum of the independent effects of two activators. Synergism occurs because each cation site has a differential affinity for the cations and each cation has a different effect on catalysis. The lack of synergism observed with enolase (Figures 1 and 2) suggests that only the site I cation plays an active role in the catalytic process. The cations Mn(II), Zn(II), and Cu(II) bind selectively to site I in contrast to Mg(II). The cation that binds at site II, Mg(II) in these studies, plays no active role in catalysis. This conclusion is consistent with the observation that labeling of site II with the substitution-inert Co(III) yields fully active enzyme.[2] Site II metal may provide activation of the enzyme by eliciting a conformational change (Lee, B. H., & Nowak, 1992).

The binding constant for Mg(II) to site I was estimated assuming competition between Mn(II) and Mg(II) for the same site. When K_d and K_d' for Mn(II) are the dissociation constants for Mn(II) in the absence and in the presence of Mg(II), respectively, K_d for Mg(II) can be obtained from the following equation:

$$K_{d',Mn} = K_{d,Mn}(1 + [Mg]/K_{d,Mg})$$

In the binding studies of Mn(II) to the enolase complex that contains Mg(II) and PGA, the Mg(II) is easily displaced from site I, and by assuming simple competition, the K_d for Mg(II) is estimated to be 70 ± 17 μM at site I. We are unable to measure any significant binding of Mn(II) to site II under these conditions. It is estimated that an observed K_d for Mn(II) of 0.2 mM could be measured for site II under these conditions. If this is correct, K_d for Mg(II) at site II must be <10 μM. The $K_{d,Mg}$ in the absence of PGA is 30 ± 3 μM. These values were approximately the values previously reported for Mg(II) (40 μM; Wold, 1971), and much greater than the values that have been reported by Faller (1973, 1977). These results are consistent with the previous suggestion of reciprocal affinity for Mg(II) and for Mn(II) at site I and II (Hanlon

[2] M. E. Lee and T. Nowak, unpublished observations.

& Westhead, 1969). This reciprocal affinity can explain the confusion between the "conformational" and "catalytic" cation sites (Brewer, 1981) and sites I and II. With the nomenclature used in this paper (based on Mn(II) binding), the site I cation is at the "catalytic" site. In the kinetic mechanism of Mn(II) activation, site I is occupied first. In the kinetic mechanism of Mg(II) activation, site II is occupied first. Thus, the kinetic mechanisms of cation activation differ for Mg(II) and Mn(II). This is consistent with differences in inhibition observed with lanthanide ions. With most of the lanthanides studied, noncompetitive inhibition is observed against Mg(II) but competitive inhibition is observed against Mn(II).[3]

The Mn(II) at site I is at the catalytic site and binds to the substrate via the hydroxyl group at C-3 (Nowak et al., 1973). The PRR titration studies with the ligands PGA, P_i, and F^- that bind to the catalytic site, for enolase that contains only one Mn(II) per subunit or Mn(II) and an excess of Mg(II), show identical behavior. The same is true for the PRR frequency dispersion studies. These results indicate that, in all of these complexes, the Mn(II) resides at the same site (site I). The frequency dependence of the PRR suggests that the two cation sites are far apart in the enolase–Mn–PGA–Mn complex. This conclusion is drawn from the lack of observation of any spin–spin coupling that would occur if the two paramagnetic species are in close proximity. This spin coupling would be reflected in a decrease in the Mn(II) electron relaxation rate estimated from the PRR measurements (Tables IV and V). This would be expected to result in a smaller value for τ_c. This conclusion that the cations are far apart and not spin-coupled is contrary to that reported by Chien and Westhead (1971) on the basis of Mn(II) EPR spectra of the enolase complexes taken at 77 K. The long distance between cation centers is consistent with a lack of Mn(II)–Mg(II) interaction observed by ^{25}Mg NMR (Lee, M. E., & Nowak, 1992).

The values for τ_c and q for various enzyme–Mn–PGA complexes were also determined by a measure of the T_{1p}/T_{2p} ratio of water protons at 300 MHz according to Navon (1970). The results were comparable to those determined by the frequency dependence of PRR at lower frequencies in that the τ_c values for each complex calculated (for 300 MHz in these studies) were the same. The values for q for the E–Mn–PGA–Mn complex were similar to the values for the E–Mn–PGA–Mg or the E–Mn–PGA complex. The differences in the absolute values for q are because of the differences in the assumptions of the two methods, experimental deviations, and the different limitations of each measurement. The number of rapidly exchanging water molecules on the Mn(II) in each case is the same. In the case of the E–Mn–PGA–Mn complex, the number of water molecules calculated is the average of the two different water sites that are located on Mn(II) at site I and at site II. Since the value for q is estimated to be 0.5 for this complex, it suggests that there is 0.5 water molecule or a single exchangeable proton per Mn(II) at both sites I and II.

High-resolution NMR relaxation studies indicate that the presence of Mg(II) at site II affects the structure of the competitive inhibitor phosphoglycolate at the active site of enolase. In contrasting the structure of phosphoglycolate in the ternary enolase–Mn–ligand and enolase–Mn–ligand–Mg complexes, the Mn(II)–proton distance decreased by 0.27 Å while the Mn(II)–phosphorus distance decreased by 1.31 Å when Mg(II) was added at site II. If one can extrapolate structural studies at the catalytic site with an inhibitor to changes with substrate, the metal at site II may affect the conformation of the substrate in the enzyme complex. Thus, if there is a specific function of the cation at site II, it may be to elicit a conformational change of the substrate as well as the enzyme to give an optimal structure for catalytic activity. The large distance between the cations at sites I and II shown here (>12 Å) and by ^{25}Mg NMR (Lee, M. E., and Nowak, 1992) argues against a functional role for cation II as previously proposed (Brewer, 1985). The cation does not appear to play a catalytically active role in the chemical process of hydration/dehydration. This is further confirmed by the observation that occupancy of site II of enolase with the kinetically inert Co(III) gives active enzyme.[2]

Dickinson et al. (1980) reported that when both Cu(II) and Mg(II) are in the presence of enolase, Cu(II) competes very strongly for Mg(II) at site I and Mg(II) competes more effectively at site II as measured by Cu(II) EPR. They did not further investigate the catalytic properties of this system, as it was believed at the time that Cu(II) was a nonactivating cation. It has subsequently been found that Cu(II) can activate yeast enolase in MES and HEPES buffer (Sinha & Brewer, 1984). Kinetic data in HEPES, pH 7.5, as reported here, showed that Cu(II) also gives a hyperbolic decrease in activity in the presence of a subsaturating amount of Mg(II) to give finite activity. The K_m' for Cu(II) in the absence of Mg(II) is 3 μM, and the concentration which gives a half-maximal effect in the presence of Mg(II) is 5 μM in 50 mM HEPES, pH 7.5. It appears that activating transition metals have higher affinities for site I than does Mg(II); however, Mg(II) appears to have a higher affinity for site II. Our definition of metal sites (site I and site II) based on Mn(II) binding suggests that we call site I the "catalytic" metal site while site II is called the "conformational" site.

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Registry No. PGA, 3443-57-0; Mn, 7439-96-5; Zn, 7440-66-6; Cu, 7440-50-8; Mg, 7439-95-4; enolase, 9014-08-8; phosphoglycolic acid, 13147-57-4.

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