Influence of pH on the Mn^{2+} Activation of and Binding to Yeast Enolase: A Functional Study†

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ABSTRACT: The influence of pH on the activation of yeast enolase by Mn^{2+} was measured by steady-state kinetics. The pH influence on the binding of Mn^{2+} to apoenolase and the enolase–substrate complex was measured by EPR spectroscopy. At pH values above 6.6, activation by Mn^{2+} is fit by Michaelis–Menten kinetics, but at higher concentrations of Mn^{2+}, inhibition is observed. Under conditions analogous to the kinetic studies, the enzyme binds two Mn^{2+} per dimer with a K_d in the micromolar range. In the presence of the substrate 2-phosphoglycerate, three thermodynamically distinct cation binding sites per monomer are detected and the binding constants are determined by a fit to the data. As the pH decreases, the reaction velocity decreases and the cation inhibition becomes minimal. Under these conditions, only two Mn^{2+} binding sites per monomer are observed; the third site must be the inhibitory site. The velocity and kinetic constants are minimally affected by buffer except at pH 5.8 with PIPES. Under these conditions, the velocity is only about 40% that observed with other buffers and only a single binding site for Mn^{2+} per monomer is detected in the presence or absence of substrate. A direct role in the catalytic mechanism by the second cation is called to question. The binding constant for Mn^{2+} at site I is independent of pH over the range from 7.5 to 5.2, and the binding at site II increases only slightly over this same pH range. These results indicate that the cation sites at positions I and II contain ligands that are pH independent over this range. Site III may contain histidine(s) and may also be located at the phosphoryl group of the substrate. Each of these groups has a pK_a value between 6.5 and 7.0 and becomes a poor ligand below 7.0. The results presented support the evidence that the ligands on enolase at cation sites I and II contain oxygen atoms, not nitrogen, and that these two sites are important for catalytic activity.

Enolase catalyzes the reversible trans dehydration of D-2-phosphoglycerate (PGA)[1] to yield PEP and water (Cohn et al., 1970). This reaction is important both in glycolysis and in glucogenesis. The enzyme is a dimer of identical subunits with a molecular weight of 93 000. The primary sequence of the protein has been determined from the protein (Chin et al., 1981a,b) and from the gene (Holland et al., 1981) sequence. Preliminary X-ray structure data have been reported (Lebioda & Brewer, 1984), and from refinements, the partial location of the substrate site and one of the cation binding sites has been determined (Lebioda & Stec, 1991; Lebioda et al., 1991). The enzyme has been shown to require a divalent cation for catalytic activity, and any of eight divalent cations can fulfill this requirement. A review of the cation requirements and their effects has been published (Brewer, 1985). The cation most intensely studied in this regard is Mg^{2+} (Hanlon & Westhead, 1969; Faller & Johnson, 1974b; Faller et al., 1977), although studies with Mn^{2+} (Hanlon & Westhead, 1969; Cohn, 1963; Nowak et al., 1973) and other cations have also been reported (Brewer, 1985). The results demonstrate that the apoenzyme contains two cation sites per dimer with some evidence of anticooperativity or nonequivalence of binding with Mg^{2+} (Faller et al., 1977). The addition of substrate or substrate analogue induces at least two additional cation sites on the enzyme. The roles of each of these cations in the binding of the substrate and the catalytic process of enolase have been a major point of controversy. The binding of cations to the apoenzyme or to the first set of binding sites in the presence of the substrate generates a conformational change on the protein as determined either spectroscopically or calorimetrically (Brewer & Weber, 1966; Faller & Johnson, 1974b). This first set of cation sites has been referred to as "conformational" (Brewer, 1981). The results of Mg^{2+} binding studies indicate very tight, stoichiometric binding per subunit, and quantitative binding and kinetic data have been used to argue that the enzyme is not catalytically active until the second set of cation sites, termed "catalytic", are occupied (Faller et al., 1977). NMR studies using a substrate analogue with a slow turnover showed that the substrate interacts with Mn^{2+} that is bound at the first cation site when the second cation site is unoccupied. The substrate PGA interacts with the cation via the hydroxymethyl group at C-3, and the cation has been postulated to stabilize the leaving hydroxyl group (Nowak et al., 1973) from the intermediate anionic species (Dinovo & Boyer, 1971). Specific roles for both bound cations have been proposed on the basis of, in part, a general review of the current literature regarding the cation binding and activation of yeast enolase. These roles include a function for the cation-bound water at site I and binding of the carboxylate group of PGA to the cation at site II (Brewer, 1985). The second cation has not yet been observed by X-ray diffraction (Lebioda & Stec, 1991). The nature of the cation binding sites and the roles of the cations at each of the binding sites remain to be resolved. In this paper, the activation of enolase by Mn^{2+}

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[1] Abbreviations: PEP, phosphoenolpyruvate; PGA, D-(+)-2-phosphoglyceric acid; EPR, electron paramagnetic resonance; PRR, longitudinal water proton relaxation rates; DMG, dimethylglutaric acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

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as a function of pH and the concomitant binding of Mn^{2+} are reported. The results of this study help to clarify the interactions of the cations with enolase and form the basis for additional detailed studies of the cation activation of this enzyme (Lee & Nowak, 1992).

Materials and Methods

PGA, PIPES, HEPES, dimethylglutaric acid (DMG), Tris, and cacodylic acid were purchased from Sigma (St. Louis, MO). CM-Sephadex was obtained from Pharmacia. All other reagents were reagent grade and were obtained from the best sources available. Solutions were prepared in distilled, deionized water. Commercial yeast enolase was purchased from Sigma. Enolase was prepared from Baker's yeast using a revised procedure that was developed in our laboratory (Lee & Nowak, 1992) that yields enzyme with a specific activity of 125 units/mg measured under standard conditions (see below). This enzyme shows a single band of protein on SDS–polyacrylamide gel electrophoresis and a single peak on a chromatofocusing column. Enolase was stored as a lyophilized powder at −20 °C.

Enolase was prepared prior to experimentation by dissolving the dry powder in a minimal amount of the appropriate buffer and passing the solution through a G-25 column (1.5 × 28 cm) that contained 2 cm of Chelex-100 on the top. The column was equilibrated with 50 mM appropriate buffer. The enzyme was concentrated, if necessary, with a collodion bag concentrator (Schleicher & Schuell, Inc., Keene, NH). The enzyme was normally assayed by a modification of the assay previously described (Westhead, 1966). The assay contained 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM PGA, and 1 mM $MgCl_2$ in a volume of 1.0 mL. Variations of this mixture were designed for specific experiments as described in Results. All changes in buffer were made by simple substitution of the appropriate buffer at the pH indicated. Studies of the activation of the enzyme by Mn^{2+} were performed at saturating PGA (normally 1.5–2 mM), previously determined in an independent assay. A velocity response to PGA concentration was performed at Mn^{2+} concentrations that were optimal either prior to the onset of cation inhibition (higher pH) or at saturating Mn^{2+} concentrations (lower pH), whichever was appropriate. The increase in absorbance due to the formation of the product PEP was measured on either a Gilford 240 or 250 spectrophotometer. The enzyme concentration was determined by using the extinction coefficient at 280 nm of 0.89 mL mg^{-1} cm^{-1} (Warburg & Christian, 1941) and a molecular weight of 93 000 per dimer (Chin et al., 1981). At pH 7.5, the specific activity was initially determined as the change in absorbance at 240 or 230 nm/min divided by the enzyme concentration expressed as absorbance units at 280 nm (Westhead & McLain, 1964). Data are reported as specific activity in standard activity units of micromolar of product per milliliter per minute per milligram. A unit of activity at 230 nm [$(\Delta A_{230}/A_{280})$ min^{-1}] corresponds to 0.32 unit/mg, and a unit of activity at 240 nm [$(\Delta A_{240}/A_{280})$ min^{-1}] corresponds to 0.576 unit/mg. For kinetic studies at pH values other than 7.5, the extinction coefficient of PEP was measured at each pH value and the change in absorbance was corrected accordingly to give concentration units. The extinction coefficient for PEP was measured by first enzymatically determining the concentration of PEP in a stock solution using pyruvate kinase and lactate dehydrogenase. A fixed aliquot of the PEP solution was dissolved in a known volume of appropriate buffer at each pH value, and a spectrum was obtained from 210 to 350 nm. The absorbance at 230 and 240 nm was measured, and the extinction coefficients were calculated. The values were plotted as a function of pH, and each set of studies was appropriately calculated.

Kinetic Studies. Kinetics were measured as described above. The buffers were chosen on the basis of their pK_a values and their weak interactions with Mn^{2+}. Analysis of buffer interactions with Mn^{2+} was performed using EPR prior to these studies. Each buffer chosen had a K_d for $Mn^{2+} > 0.5$ M, and most data indicate no binding. The buffers were overlapped at various pH values as necessary. The enzymatic reactions were initiated by the addition of enzyme or by the metal if necessary, with identical results. Enzyme activity in the absence of added cation was less than 2% of the activity measured at saturating Mn^{2+} concentration. For Mn^{2+} activation studies, calculations were performed to determine concentrations of free Mn^{2+}. The binding of Mn^{2+} to PGA was measured using EPR methods over the pH range studied. Corrections for Mn^{2+} binding to PGA were made, and corrections for binding to buffer were minimal. Kinetic analysis for Mn^{2+} in terms of the concentration of free Mn^{2+}, $[Mn^{2+}]_f$, was performed by a best fit to the Michaelis–Menten equation:

$$v = \frac{V[S]}{K_m + [S]} \quad (1)$$

where [S] is either the concentration of substrate (PGA) or $[Mn^{2+}]_f$. When cation inhibition was observed, the data were fit to the equation for substrate inhibition (Cleland, 1970):

$$v = \frac{V[Mn^{2+}]}{K_m + [Mn^{2+}] + [Mn^{2+}]^2/K_i} \quad (2)$$

Binding Studies. The binding of Mn^{2+} to enolase in the absence and presence of PGA as a function of pH was determined by EPR measurements. $[Mn^{2+}]_f$ was measured on an X-band Varian E-9 EPR spectrometer. Enzyme solutions (20–30 μM) were prepared in 0.1 M KCl and 50 mM appropriate buffer. When substrate was present, the samples also contained 1.5 mM PGA, a concentration found to be saturating for each of the experiments performed under such conditions. The concentration of Mn^{2+} was varied in each experiment, usually over a range of about 12–250 μM. Each sample was prepared in a 50-μL volume, and $[Mn^{2+}]_f$ was measured. The limit of detection for $Mn^{2+}{}_f$ is approximately 2 μM. Corrections for Mn^{2+} binding to PGA were estimated and were usually between 1 and 2%. The binding data were calculated, plotted, and analyzed in the form of a Scatchard plot (Scatchard, 1949) with an attempt to obtain results between 20 and 80% saturation (Deranleau, 1969). The binding data were analyzed for the number of independent binding sites (n) and for the dissociation constants (K_d) for each of the binding sites using the Winlund method (Winlund & Chamberlin, 1970):

$$v = v_1 + v_2 + v_3 = \frac{n_1K_{d1}[Mn]_f}{1 + K_{d1}[Mn]_f} + \frac{n_2K_{d2}[Mn]_f}{1 + K_{d2}[Mn]_f} + \frac{n_3K_{d3}[Mn]_f}{1 + K_{d3}[Mn]_f} \quad (3)$$

This deconvolution of the experimental data treats the information as either a single thermodynamic binding site per subunit if results yield a straight line or two or three binding sites, depending on the results obtained and on the best fits to the data. Specific details are given in the appropriate figure legends. A separate program, written to analyze binding data with multiple binding sites (Dr. Ken Grant, Department of Mathematics, University of Notre Dame), was also used to treat some of the results, and nearly identical results were obtained.

The binding of PGA to the enolase–Mn^{2+} complex was measured by PRR methods using a Seimco pulsed NMR

spectrometer operating at 24.3 MHz. The $1/T_1$ values for the protons of water were measured by the 180°–τ–90° pulse sequence. The treatment of the data has been previously described (Nowak, 1981). Briefly, the paramagnetic contribution to the relaxation rate ($1/T_{1p}$) is the difference in the longitudinal relaxation rate of water protons in the presence of Mn^{2+} ($1/T_{1,obs}$) and in its absence ($1/T_{1,o}$):

$$1/T_{1p} = 1/T_{1,obs} - 1/T_{1,o} \quad (4)$$

The enhancement observed in the presence of enzyme (ϵ^*) is the ratio of the paramagnetic effect in the presence and absence of enzyme. The observed enhancement is related to the mole fraction of Mn^{2+} free and bound:

$$\epsilon^* = \frac{[Mn]_f}{[Mn]_{total}}\epsilon_f + \frac{[Mn]_b}{[Mn]_{total}}\epsilon_b \quad (5)$$

In this equation, ϵ_f is the enhancement of free Mn^{2+}, defined as unity, and ϵ_b is the enhancement of enolase-bound Mn^{2+}. The free Mn^{2+} was determined by EPR. Upon the addition of substrate, the enhancement changes. The enhancement values are plotted as a function of the concentration of added substrate to yield a titration curve. These experiments were performed by preparing an appropriate NMR tube with a fixed concentration of enolase sites (usually between 65 and 100 μM) in the presence of 50 μM $MnCl_2$, 100 mM KCl, and 50 mM appropriate buffer in a final volume of 50 μL. In a second tube, the same sample was prepared with a saturating PGA concentration added. Microliter aliquots of the second sample were titrated into the first tube, and $1/T_1$ values were measured upon each addition. The data were fit using the approach first outlined by Reed et al. to determine the value for the dissociation constant of the substrate from the ternary enolase–Mn–PGA complex (K_3) and the enhancement of the ternary complex (ϵ_r). The program used to fit the data was that of Reed et al. (1970), modified by Joseph Weber (University of Notre Dame) for use on the VAX 750. Best fits were obtained by systematically varying K_3 and K_s (the binding constant for enolase–PGA) until the best fit to the data was obtained.

Deuterium Isotope Effects. Primary deuterium isotope effects on the enolase-catalyzed reaction were performed using [2-^{2}H]PGA in a manner similar to experiments previously described (Dinovo & Boyer, 1971; Shen & Westhead, 1974). Labeled PGA was obtained by the addition of PGA to a buffered solution of D_2O in the presence of 2 mM $MgCl_2$. Enolase (1 mg) was added, and the reaction was allowed to come to equilibrium. The reaction was eluted on a Dowex-1 anion-exchange column and washed with water. The substrates PGA and PEP were differentially eluted with HCl. Under these conditions, the PGA was >98% enriched with ^{2}H at the 2 position, as checked by ^{1}H NMR spectroscopy. The sample was pooled and lyophilized. The [^{2}H]PGA was dissolved in the appropriate buffer prior to use and the concentration determined enzymatically. Primary isotope effects were determined by initial velocity kinetics with [^{2}H]PGA and with [^{1}H]PGA that was prepared in an identical fashion except in H_2O. The results were plotted in a double-reciprocal plot, and the results were fit and compared.

Results

Prior to measuring enolase kinetics, the extinction coefficient of PEP was measured over the pH range 5.0–7.5. Spectra were taken from 210 to 350 nm. Results give extinction coefficients at 240 nm that vary, giving the values 1.55 (mM^{-1} cm^{-1}) at pH 7.5, 1.25 (pH 7.0), 1.12 (pH 6.6), 1.05 (pH 6.2), 0.96 (pH 5.8), 0.81 (pH 5.5), and 0.66 (pH 5.15), and are similar to those previously reported (Wold & Ballou, 1957; Wold, 1971). The extinction coefficients for PEP were used to calculate the activity of enolase at different pH values. The extinction coefficient decreases as the ionizable groups are protonated.

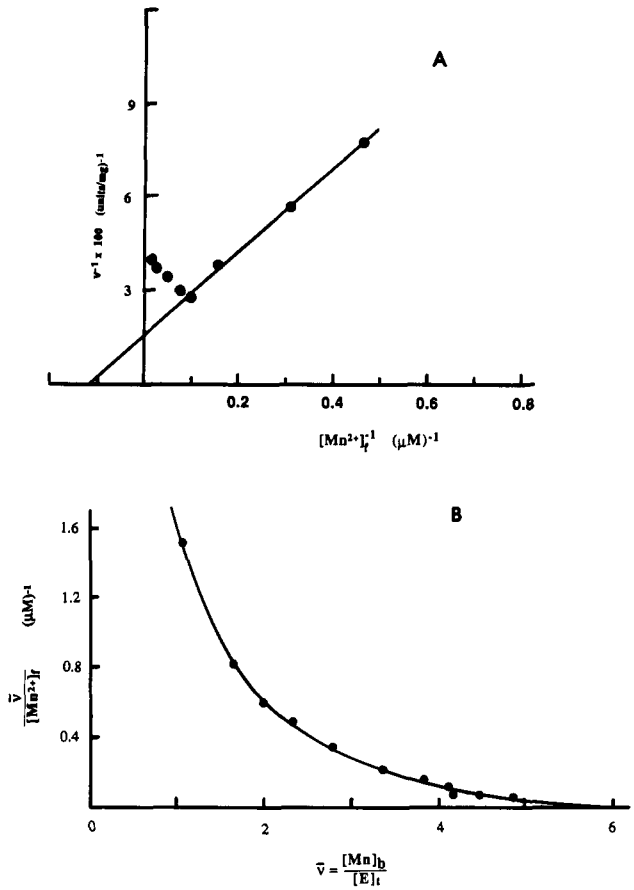

FIGURE 1: Kinetics of activation and binding of Mn^{2+} to yeast enolase at pH 7.0. (A) Lineweaver–Burk plot of the kinetics of Mn^{2+} activation. Enolase kinetics were measured in 50 mM HEPES buffer, pH 7.0, containing 100 mM KCl and 1.5 mM PGA. The concentration of Mn^{2+} was varied and $[Mn^{2+}]_f$ was calculated by accounting for Mn^{2+} binding to PGA (K_i = 3 mM under these conditions). The reaction was initiated by the addition of enolase. The data were fit to the equation for noncompetitive substrate inhibition and the following parameters were obtained: K_a = 7 μM, K_i = 47 μM, and V_m = 68 units/mg. (B) Scatchard plot of Mn^{2+} binding to enolase. The binding of Mn^{2+} to 26 μM enolase was measured at pH 7.0 in the presence of 50 mM HEPES containing 100 mM KCl and 1.5 mM PGA. The concentration of Mn^{2+} was varied from 38.2 to 239 μM, and $[Mn^{2+}]_f$ was measured for each sample. The results are best fit with three K_d values, each representing two binding sites. The K_d values obtained are K_{d1} = 0.8 μM, K_{d2} = 15 μM, and K_{d3} = 42 μM. The curve drawn is the sum of the three sets of binding sites.

Kinetics. Initial kinetic studies were performed to determine the activator constant for Mn^{2+} as a function of pH. At each pH value, the concentration of PGA in the assay was checked for saturation in each experiment. At higher pH values, inhibition is observed as the concentration of Mn^{2+} was increased (Figure 1A). These kinetic data were fit with eq 2, and the values for V_{max}, K_a, and K_i are summarized in Table I. As the pH is decreased, the Mn^{2+} inhibition becomes progressively weaker, and by pH 5.8, Lineweaver–Burk plots are linear. Kinetic data could be obtained to a pH value of 5.15, below which the enzyme was inactive. At pH values > 7.5, the inhibition by Mn^{2+} increases and additional information was judged not to be of sufficient importance for these studies. Several buffers gave differences in K_i values for Mn^{2+}. The error in the calculations of some of the K_i values is high. The

Table I: Kinetic and Binding Parameters for the Activation of Enolase and Binding to Enolase by Mn^{2+}

		Kinetic Parameters					Binding Parameters[e]							
pH	buffer	V_m[a] (units/mg)	K'_a[b] (μM)	K_i[c] (μM)	V_m[d] (units/mg)	K_M (μM)	K_1 (mM)	K_3 (μM)	K_d (μM)	K_{d1} (μM)	K_{d2} (μM)	K_{d3} (μM)	ϵ_T	ϵ_b
7.5	HEPES	103	4	35	92	42	1.7	7.5	2.0	0.7	9.1	45	2.7	12.5
7.0	HEPES	68	7	47	70	66	3.0	17	2.0	0.8	15	42	3.0	12.0
	PIPES	65	13	271	–	–		25	3.8	1.2	35		2.5	12.5
6.6	HEPES	58	4	125	60	68	3.6	13	2.2	0.6	30	111	2.7	13.2
	PIPES	61	28	199	–	–		7	4.1	0.8	40	–	2.5	12.5
	MES	52	4	135	–	–								
	DMG	70	4.7	741	–	–		13	2.1	0.6	30	–	2.7	4.6
6.2	HEPES	65	4	75	–	–	4.1							
	PIPES	77	33	66	–	–								
	MES	71	29	108	–	–								
	BIS-TRIS	68	16	193	–	–								
	cacodylate	61	20	164	–	–			3.5	1.9	36	–	2.5	11.9
	DMG	67	8	138	40	90		20	3.3	2.1	43	–	2.5	4.7
5.8	PIPES	25	42	NI	10.5	91	4.7	3	7.3	1.5	–	–	2.3	12.4
	BIS-TRIS	70	52	101	42.	48								
	cacodylate	60	42	346	30.7	93			5.3	1.7	60	–	2.5	11.7
	DMG	60	29	NI	34.5	111		25	4.0	1.5	50	–	2.5	11.7
5.6	cacodylate	35	10	NI	–	–	8			1.1	3	–		
	PIPES	28	20	NI	–	–				1.1	118	–		
5.5	DMG	38	80	NI	20	180	10	33	3.0	1.1	63	–	2.3	4.3
	MES	34	80	NI	–	–								
5.15	MES	17	80	NI	15	180	35		25	1.4	76	–	2.4	13.1

[a] Calculated by extrapolation to saturating concentrations of Mn^{2+}. [b] Concentration of Mn^{2+} giving $V_m/2$. [c] Calculated by a fit of the data to eq 2; NI = no observed inhibition. [d] Calculated by extrapolation to saturating concentrations of PGA. [e] Symbols: K_1 = [Mn^{2+}][PGA]/[Mn-PGA]; K_3 = [E-Mn][PGA]/[E-Mn-PGA]; K_d = [E][Mn]/[E-Mn]. K_{d1}, K_{d2}, and K_{d3} are Mn^{2+} binding sites I, II, and III, respectively, for the E-PGA complex.

only striking anomaly observed was with PIPES at pH 5.8, where the V_{max} was 40% that observed with the other three buffers used.

The kinetic response of enolase as a function of PGA concentration was also measured over the same pH range and with the same buffers. The Mn^{2+} concentration used in each of the experiments was either a saturating concentration (≥ 10 K_a) or the optimum concentration at the pH values where Mn^{2+} inhibition is observed. The data were fit to a simple Michaelis–Menten model, and the values for K_m and V_m are shown in Table I. The results for the apparent V_m obtained from variations in Mn^{2+} or in PGA concentrations do not always agree, presumably because of the differences in Mn^{2+} concentration. The fits to most of the kinetic data were usually within 10% for values for V_m. Kinetic parameters showed greater deviation, sometimes 15–20%, when Mn^{2+} inhibition was observed. Deviations for K_i values are more difficult to determine, but values of 20–40% are estimated with greater deviations for larger K_i values. Some buffer effects apparently occur, giving variations in K_m and K_i values at a given pH. A fit of the kinetic parameters as a function of pH to obtain pK_a values was not performed since the $V/K_{m,PGA}$ results with various buffers gave too much scatter.

Mn^{2+} Binding. The binding of Mn^{2+} to apoenolase was measured, using EPR, as a function of pH under experimental conditions similar to those used for each of the kinetic studies performed. At each pH value, two thermodynamically equivalent Mn^{2+} binding sites per dimer were measured. There was no evidence for nonequivalence in binding as indicated with Mg^{2+} (Faller et al., 1977). Values for K_d varied from 2.0 to 7.3 μM over this pH range except for pH 5.15, where the value is approximately 25 μM. The results obtained at this pH showed greater scatter, although binding was clearly weaker than observed at higher pH values. The fits to the binding data show approximately 10–15% deviation in the values of K_d and n. The binding of Mn^{2+} at this site appears to be with liganding groups that are insensitive to pH over this pH range. The enhancement of the binary complex was also measured concurrent with the binding constant. For most of the buffers and the pH values used, values of 12.3 ± 0.4 for ϵ_b were obtained. The value for ϵ_b when DMG was used as the buffer was approximately 4.5. Although the ϵ_b is low, there is no apparent effect on catalytic activity with this buffer.

Since the addition of substrate is known to induce additional metal binding sites on enolase, the binding of Mn^{2+} to enolase in the presence of 1.5 mM PGA was also measured over the same pH range under experimental conditions analogous to those used for kinetic studies. Results of two such series of studies are shown and compared to the kinetic studies obtained under comparable experimental conditions (Figures 1 and 2). At pH > 6.6, six Mn^{2+} binding sites per dimer are detected. The data were fit to a model of three independent binding sites per dimer to determine the binding constants for Mn^{2+} at sites I, II, and III. These results are summarized in Table I. Errors in these fits are more difficult to assess, but the errors in the calculations of the three K_d values are reflected in greater deviations for K_{d3} than for K_{d1}. This is due, in part, to the larger errors inherent in the determination of weaker binding cations, in the observation of smaller changes in higher Mn^{2+} concentrations, and in performing multiparameter fits. At pH values below 6.6, the binding of Mn^{2+} to the third site becomes very weak (>150 μM) and is not measurable. The very weak binding to site III is parallel to the decrease in Mn^{2+} inhibition. Site I Mn^{2+} binding is tighter, by a factor of 2–4, than Mn^{2+} binding to apoenzyme. Values for K_{d1}, ranging from 0.7 to 2 μM, are nearly insensitive to pH. This insensitivity to pH is analogous to Mn^{2+} binding to apoenolase. The binding of Mn^{2+} to site II is weaker than the binding to site I by a factor of about 10–50. The value for K_{d2} is also nearly invariant with pH over this range. There is no significant data to indicate the presence of site III at low pH values.

The binding of PGA to the enolase–Mn^{2+} complex was measured over the pH range of 5.15–7.5. To correctly fit the binding data, the binding constant (K_1) for the binary Mn–PGA complex was also necessary. The dissociation constant for this binary complex increases from 1.7 to 35 mM with a

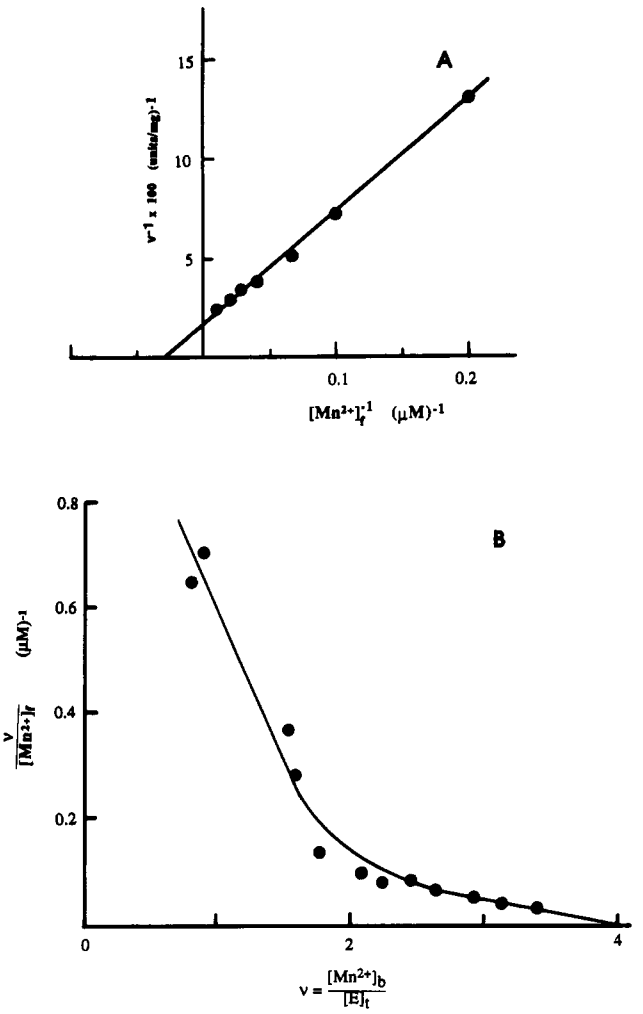

FIGURE 2: Kinetics of activation and binding of Mn^{2+} to yeast enolase at pH 5.8. (A) Lineweaver–Burk plot of the kinetics of Mn^{2+} activation. The kinetics were performed in the presence of 50 mM DMG buffer, pH 5.8, containing 100 mM KCl and 1.5 mM PGA. The concentration of Mn^{2+} was varied, and $[Mn^{2+}]_f$ was calculated to account for any Mn^{2+} binding to PGA (K_1 = 4.7 mM). The reaction was initiated by the addition of enolase. The data were fit to the Michaelis–Menten equation, and the values of K_a = 29 μM and V_m = 60 units/mg were calculated. (B) Scatchard plot of Mn^{2+} binding to enolase. The binding of Mn^{2+} to 25.8 μM enolase was measured at pH 5.8 in the presence of 50 mM DMG buffer containing 100 mM KCl and 1.5 mM PGA. The concentration of Mn^{2+} was varied from 23.9 to 239 μM, and $[Mn^{2+}]_f$ was measured by EPR for each sample. The results are best fit with two K_d values each representing two binding sites. The K_d values are K_{d1} = 1.5 μM and K_{d2} = 50 μM. The curve drawn is the sum of the two sets of binding sites.

decrease in pH (Table I). Titration studies of PGA were performed using many of the buffers chosen for the pH studies, and the data were fit to determine the values for K_3 and the enhancement of the ternary enolase–Mn–PGA complex (ϵ_t). The K_3 values increased by a factor of 2–4 over the pH range studied, and the values for ϵ_t did not change regardless of the buffer used.

Most of the studies showed little substantial difference in kinetic parameters or in binding parameters with various buffers. One exception is the difference in ϵ_b observed with DMG. This buffer gave ϵ_b values significantly lower than those observed with other buffers (12.4). The other anomalous behavior was observed at pH 5.8 with PIPES. At this pH, the V_m measured by variation in Mn^{2+} or PGA concentration is 40% or 30%, respectively, of that measured from the same experiments with three other buffers at the same pH. This relatively low value for V_m was observed even if the buffer mixture was adjusted to a final concentration of 50 mM and the ratio of cacodylate to PIPES was 1:2, 1:1, or 2:1. Under analogous conditions in the presence of PGA, Mn^{2+} binding studies showed only a single Mn^{2+} binding site per subunit with a K_d of 1.5 μM. All of the other buffers at the same pH value or PIPES at pH 5.6 and 6.2 showed two Mn^{2+} sites per subunit. Both the kinetic and the binding studies were repeated at least three times with the same results. The energy of activation of the enolase-catalyzed reaction was measured at pH 5.8 in cacodylate and in PIPES buffers. The assays were both performed with 0.1 mM $MnCl_2$, and V_m was obtained by extrapolation to infinite PGA concentrations. These values for V_m were measured at six temperatures from 8 to 38 °C, and the Arrhenius plots for each experiment gave linear data (results not shown). Activation energies of 17.4 ± 0.2 and 17.6 ± 0.3 kcal/mol were calculated for the reactions in PIPES and cacodylate, respectively. To further understand this behavior, the primary kinetic isotope effect was measured both in PIPES and in cacodylate. From the data, a primary isotope effect of 1.95 is measured with cacodylate and 1.60 with PIPES. Analogous experiments were performed with 1 mM $MgCl_2$ (saturating) at the same pH and buffers. An isotope effect of 2.0 was determined with cacodylate and an effect of 2.1 was determined with PIPES. In each set of experiments, there was no effect of deuterium substitution at C-2 on the K_m. The isotope effects in cacodylate or in PIPES buffers were not significantly different.

Discussion

The kinetic responses of yeast enolase were measured as a function of pH using Mn^{2+} as the activating cation. Although Mn^{2+} stimulates activity at about 40% the rate of Mg^{2+}, it has a lower activator constant (K_a). Since Mn^{2+} is paramagnetic, its physical properties are such that it serves as a good probe to study cation interactions with proteins and ligand interactions with the protein–Mn^{2+} complex (Nowak, 1981; Mildvan & Gupta, 1977). Experiments were designed to compare the activation of enolase by Mn^{2+} and the binding of Mn^{2+} to enolase and to clarify the roles of the cations at the various sites on the enzyme. Recent reviews have outlined the physical and catalytic properties of enolase (Brewer, 1981) and have led to hypotheses for roles for the cations at the two sites in the protein (Brewer, 1985). Studies have shown that binding of the cations Mg^{2+} or Mn^{2+} or other activating cations to the enzyme induces a conformational change in the protein. This cation site has been labeled the "conformational" site (Brewer, 1981, and references therein). The Mn^{2+} that binds at this site has been shown to have two rapidly exchangeable water molecules (Nowak et al., 1973; Lee & Nowak, 1992), is at the catalytic site, and interacts with the substrate via the hydroxyl group of the C-3 hydroxymethyl group of PGA (Nowak et al., 1973). This cation plays a direct role in the catalytic process. Binding studies of Mg^{2+} concomitant with kinetic studies have been used to suggest that enolase is not catalytically active until the second cation site is occupied (Faller et al., 1977). Thus, the second site has been termed "catalytic". Since the location of the second cation site relative to the first site has not been determined and its role in catalytic activity has not yet been clarified, the nomenclature for the cation sites used in this and subsequent publications (Lee & Nowak, 1992) will be descriptive and based on relative binding affinities. Thus, the sites are termed I, II, and III in the order of the relative binding affinities for Mn^{2+}.

Some initial kinetic and binding studies were performed with commercially purchased enolase. When these data (not shown) were compared to the results obtained with enolase

purified in our laboratory, some discrepancies were evident. It was much more difficult to obtain cation-free enolase from the commercial source, K_m values were higher, and K_i values did not agree. The values for binding constants were more erratic; K_d values were sometimes higher, and stoichiometric binding was not always obtained. Thus, all results reported in this paper were obtained with our preparations of yeast enolase.

Steady-state kinetics of the activation of enolase by a variety of cations, including Mn^{2+}, show that at higher pH values inhibition is observed at higher cation concentrations. When parallel Mn^{2+} binding studies are performed, three cation sites per monomer are measured in the presence of PGA. The value for K_i for Mn^{2+} is of the same order of magnitude as K_{d3}. When conditions are such that Mn^{2+} inhibition is minimized, at pH < 6.8, the third of the three cation sites disappears. This is the first direct experimental evidence of a specific site on the enzyme that is the inhibitory site. This site has been postulated to exist from many reported observations of cation inhibition (Elliot & Brewer, 1980). This site appears to be titrated at a pH value of about 6.5–7.0 and may be located either at a histidine-containing site, at the phosphoryl group of the substrate at the catalytic site, or at both. There is reasonable evidence that one or two arginine residues are at the phosphate site (Elliot & Brewer, 1978; Borders et al., 1978; B. H. Lee and T. Nowak, unpublished observations). The location of the "critical" histidine residue (Elliot & Brewer, 1979) has not been determined. Recent X-ray results of the enolase–substrate complex indicate that His373 is close to the catalytic site (Lebioda & Stec, 1991).

The binding of Mn^{2+} to sites I and II of enolase in the presence of PGA shows little change over the pH range from 7.5 to 5.15. This suggests that the ligands that bind the cations are not affected over this pH range and indicates that neither histidines, lysines, nor cysteine tholates are at the binding sites. The most probable ligands are carboxylates from aspartate and/or glutamate and/or backbone carbonyl groups. The suggestion that there are all oxygen-containing ligands to the metals is consistent with X-ray results that indicate Asp246, Glu295, and Asp320 as ligands at site I (Lebioda & Stec, 1989; Lebioda et al., 1991), with EPR spectroscopic data with Cu^{2+} (Dickenson et al., 1980), or with ^{113}Cd NMR (Spencer et al., 1985).

There are some buffer effects on the kinetic and physical properties of enolase. There appear to be differences in some of the kinetic properties, primarily K_a and K_i values for cation activation, with different buffers and at different pH values. The only buffer that alters the environment at the bound Mn^{2+}, as reflected in ϵ_b, is DMG. This buffer may perhaps form a weak but significant ternary enolase–Mn–buffer complex, thus altering the enhancement. This is surprising since the buffers were, in part, chosen because they are very weak ligands for Mn^{2+}. Prior measurement indicated that these buffers have K_d values with free Mn^{2+} of 0.5 M or greater. Nonetheless, DMG does not affect the V_m or V/K for enolase. Tris and imidazole buffers have been reported to alter the inhibition of enolase by Zn^{2+} (Elliot & Brewer, 1980). The most striking buffer effect, however, is with PIPES at pH 5.8. This effect is not observed with PIPES at any other pH value or with any other buffer, including HEPES, which is structurally related but is a monosulfonic acid buffer. Under experimental conditions, the results indicate that with PIPES, pH 5.8, only a single cation site (site I) exists. Under these conditions, enolase still has catalytic activity but only 30–40% that of the enzyme when both sites I and II are occupied (with other buffers). PIPES appears to have the same inhibitory function at pH 5.8 when used as a mixture with cacodylate. These results imply that the site II cation may not be mandatory for catalytic activity but may serve only in a stimulatory role, perhaps by alteration of the conformation at the catalytic site. These results are consistent with mixed metal activation studies (Lee & Nowak, 1992) and cation labeling studies[2] that suggest that only the site I cation modulates k_{cat} and site II cation has no chemical activity, respectively. Although a decrease in k_{cat} is measured when site II does not exist, this appears not to be due to a significant change in the rate-determining step in this reaction. There is no change in the activation energy of the reaction or any significant change in the primary isotope effect. The measured isotope effect is consistent with earlier reports (Dinovo & Boyer, 1971; Shen & Westhead, 1973) that indicate that the rate-determining step in this reaction at pH <8.5 is the removal of the C-2 proton to generate the anion intermediate. If any change in the rate of this step has been made, it is only about 20%. The nature of this buffer effect is not clear, although we have consistently verified this unusual behavior. These results suggest a specific protein–buffer interaction. Caution should be taken in overinterpretation of this observation.

The binding of PGA to the enolase–Mn^{2+} complex is also not significantly affected by pH over the range measured, although the K_m value increases as the pH decreases. The binding data indicate that amino acid residues important for PGA binding do not have a pK_a in this pH range. Two pK_a values for PGA are 3.5 and 7.0 (Wold & Ballou, 1957). The pH effects indicate that enolase does not discriminate between the phospho monoanion and phospho dianion forms of the substrate in binding. The decrease in k_{cat} with pH, however, suggests that the amino acid(s) that is (are) important in catalysis is (are) affected by pH over this range. The lack of correlation of K_m and K_3 for PGA demonstrates that the kinetic constant is certainly not a thermodynamic constant with enolase. The same correlations can be drawn with Mn^{2+} as the activator. The K_a for Mn^{2+}, based on calculations for Mn^{2+}_{free}, does not simply correspond to either K_{d1} or K_{d2}. In most cases, the value for K_a falls midway between the values for the dissociation constants for sites I and II. This suggests that the activator constant is not a thermodynamic constant but a kinetic constant and it could not be used to unequivocally assign a catalytic role for either of the two cations. The large discrepancy in K_a and K_d for Mn^{2+} at pH 5.8 with PIPES confirms this conclusion.

In summary, these results demonstrate that there are three cation sites in yeast enolase in the presence of PGA. Site I is at the catalytic site and is the critical cation site responsible for catalytic activity. Site II may be an activator site, but it apparently is not critical for activity. Both sites I and II contain carboxyl and/or carbonyl groups as the cation-binding ligands. Site III is the inhibitory cation site and can be eliminated at lower pH values. This site may be at the phosphate of the bound substrate. The binding of PGA to enolase–Mn^{2+} is nearly pH independent. The kinetic constants are not thermodynamic constants with Mn^{2+} as the activator. The unusual decrease of catalytic activity observed with PIPES buffer only at pH 5.8 indicates that buffer effects, normally assumed not to be appreciable, should be addressed in the study of kinetic and thermodynamic effects of enzymes.

Registry No. PGA, 3443-57-0; PIPES, 5625-37-6; Mn, 7439-96-5; enolase, 9014-08-8.

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

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