Magnesium Ion Requirements for Yeast Enolase Activity†

Larry D. Faller,* Bahige M. Baroudy, Alan M. Johnson, and Ralph X. Ewall

ABSTRACT: It has generally been concluded that two divalent cations are required for enolase activity, even though the enzyme is a homodimer that specifically binds four metal ions in the presence of substrate. This paper reports a reinvestigation of the stoichiometry of enolase activation. Specific ion electrode measurements of Mg^{2+} binding in the presence and absence of substrate are compared with stopped-flow measurements of the velocity of 2-phosphoglycerate dehydration. It is concluded that the enzyme is inactive when only two metal-binding sites are filled and that four sites must be populated with Mg^{2+} for full activity. An ordered binding mechanism is proposed that quantitatively predicts the activation of enolase by the four Mg^{2+} ions from their measured dissociation constants and the Michaelis constant for the dehydration reaction. To explain the loss of enzymatic activity at still higher metal concentrations, the binding of additional, inhibitory Mg^{2+} ions is postulated.

Enolase (2-phospho-D-glycerate hydro-lyase, EC 4.2.1.11) catalyzes the dehydration of PGA[1] to PEP in glycolysis. The yeast enzyme is a dimer (Brewer and Weber, 1968) composed of apparently identical subunits (Brewer et al., 1970). Two Mg^{2+} ions bind tightly to the apoenzyme (Hanlon and Westhead, 1969a) and are required for substrate binding (Faller and Johnson, 1974a). Two additional Mg^{2+} ions bind loosely to the metalloenzyme–substrate complex (Hanlon and Westhead, 1969a), but it has been concluded repeatedly that they are kinetically irrelevant.

The evidence that only the two tight metal-binding sites are kinetically relevant comes from quantitative comparisons of equilibrium binding data with kinetic measurements of metal activation. The most careful comparison was made by Hanlon and Westhead (1969a,b). They measured the binding of radioactive Mg^{2+} and Mn^{2+} ions to enolase by equilibrium dialysis, both in the presence and in the absence of substrate. Kinetic measurements under experimentally comparable conditions gave monophasic activation curves for both metals. Hanlon and Westhead interpreted the quantitative similarity between the dissociation constant for the second equivalent of each metal ion bound (Mg^{2+}, K_2 = 170 μM; Mn^{2+}, K_2 = 4.2 μM) and the single kinetic activation constant for that metal (Mg^{2+}, K_A = 450 μM; Mn^{2+}, K_A = 6.3 μM) to mean that population of both metal-binding sites one and two is necessary and sufficient for catalytic activity. With the exception of an abstract claiming biphasic activation (Hanlon and Feins, 1969), all subsequent studies of metal binding to yeast enolase and/or activation of the enzyme (Brewer, 1971; Nowak et al., 1973; Brewer, 1974a) have reached the same conclusion. Two metal ions, putatively one on each of the identical subunits, are required to activate the dimer. The stoichiometry of metal ion activation is the basis for including enolase in tables of enzymes exhibiting half-of-the-sites reactivity (Matthews and Bernhard, 1973).

This model was disturbing, not simply because it ignores metals three and four, but because two separate laboratories have reported that under some conditions enolase monomers are fully active (Keresztes-Nagy and Orman, 1971; Holleman, 1973). Closer examination of the evidence that the second metal ion bound "turns enolase on" raised further doubts. First, Hanlon and Westhead compared the metal binding and kinetic properties of enolase in 0.5 M KCl. Difference spectra (L. D. Faller, to be published) indicate that apoenolase is half-dissociated at the enzyme and chloride ion concentrations they used, so the metal dissociation constants Hanlon and Westhead reported are complex quantities that reflect both metal binding and subunit association. They were compared to metal activation constants that were not affected by the monomer–dimer equilibrium, because only the holodimer is catalytically competent in high concentrations of halide ions (O'Flaherty, 1965; Brewer and Weber, 1968; Gawronski and Westhead, 1969). Second, Hanlon and Westhead assumed the molecular weight of yeast enolase is 67 000. The correct value has since been shown to be 88 000 (Mann et al., 1970). Wold (1971) has recalculated the Mg^{2+} dissociation constants from Hanlon and Westhead's binding data using the higher value of the molecular weight. When the values he obtained are compared to the Mg^{2+} activation constant reported by Hanlon and Westhead (450 μM), it more nearly equals the dissociation constant from site three (320 μM) than it does the dissociation constant from site two (40 μM).

Preliminary evidence that enolase is inactive when only sites one and two are filled with Mg^{2+} ions has been presented (Faller et al., 1974). This paper documents evidence that sites three and four must both be populated for full activity.[2]

Experimental Section

Materials. Enolase was purified from baker's yeast and made metal-free as previously described (Faller and Johnson, 1974a). An optical factor of 0.89 cm^{-1} mg^{-1} mL (Warburg and Christian, 1941) and a molecular weight of 88 000 were used to convert absorbance readings at 280 nm to the concentration of dimeric enzyme. The specific activity at 240 nm and 30 °C (Westhead, 1966) of the enzyme used in the experiments reported was 300 ± 30 units. Endogenous metal was monitored with a Perkin-Elmer Model 303 atomic absorption

† From the Department of Biochemistry, George Washington University School of Medicine, and the National Science Foundation, Washington, D.C. 20550. *Received September 28, 1976; revised manuscript received May 24, 1977.* Supported by National Science Foundation Grant No. GB-36704.

[1] Abbreviations used are: PGA, D-2-phosphoglycerate; PEP, 2-phosphoenolpyruvate; Tris, tris(hydroxymethyl)aminomethane; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

[2] This work has been communicated in preliminary form (Faller, 1976).

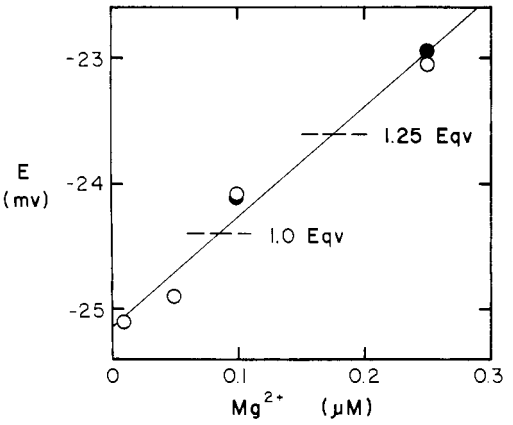

FIGURE 1: Electrode potentials (E) measured in standard Mg^{2+} solutions for two (○, ●) overlapping calibration curves. The best straight line through the points was determined by the method of least squares. The dashed horizontal lines are the potentials measured in apoenolase solutions to which 1.0 and 1.25 equiv of $Mg(C_2H_3O_2)_2$ had been added. Both the standard and the unknown solutions were buffered by K^+-Pipes at pH 7.5, 25 °C, and μ = 0.05 M.

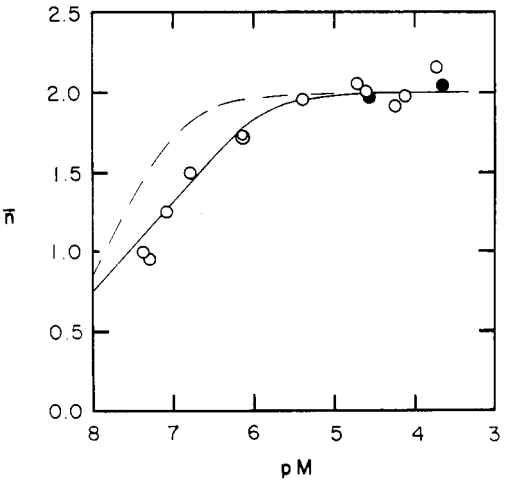

FIGURE 2: Average number of Mg^{2+} ions bound ($\bar{n}$) to enolase (0.1 mM) as a function of free Mg^{2+} concentration. Measurements were made in K^+-Pipes (○) and Tris-acetate buffers (●) at pH 7.5, 25 °C, and μ = 0.05 M. The dashed line was calculated for binding to two identical sites ($K_1 = K_2 = 0.017\ \mu M$) and the solid line for binding to the weaker of two sites ($K_1 < K_2 = 0.2\ \mu M$).

spectrophotometer. The instrument was calibrated with commercially available primary metal standards.

Tris and Pipes buffers were used, because neither binds Mg^{2+} ions significantly (Good et al., 1966). All measurements were made at 0.05 M ionic strength, pH 7.5, and 25 °C. Both the apo- and the holoenzyme have been shown by sedimentation equilibrium experiments to be dimeric under these conditions (L. D. Faller, to be published). PGA and PEP were obtained as the trisodium salt hexahydrates and magnesium acetate as the tetrahydrate. The conductivity of house distilled water was reduced to less than 1 $\mu\Omega^{-1}$ by passage through an ion exchange unit.

Binding Measurements. The concentration of free Mg^{2+} ions was measured directly with an Orion divalent cation electrode. An Orion single junction Ag|AgCl reference electrode was used with a Beckman Research Model pH meter. Measurements down to 0.01 μM free Mg^{2+} ion were possible in the K^+ salt of Pipes. The electrode was recalibrated by measuring several standard solutions on either side of the anticipated free metal ion concentration before each measurement on an enzyme solution. Above 0.1 mM the measured potential was proportional to the logarithm of the divalent cation concentration with an average slope of 20 mV per decade. Below 0.1 mM the electrode response is proportional to the concentration of Mg^{2+} ions over a sufficiently narrow concentration range. Figure 1 shows the calibration curve and two measurements of Mg^{2+} binding to apoenolase in the concentration range in which dissociation from site two is occurring.

Kinetic Measurements. Kinetic measurements were made by following the appearance of PEP with a Beckman Acta CV spectrophotometer, or with a prototype Beckman stopped-flow instrument. To increase the light intensity of the latter in the 220–240-nm wavelength region, the Littrow double prism monochromator and deuterium source were replaced by a Bausch and Lomb high intensity grating monochromator and a 75-W xenon arc lamp powered by a PEK low ripple supply.

Results

Mg^{2+} Binding. Apoenolase was titrated with $Mg(C_2H_3O_2)_2$. No free metal was detected when 0.75 or fewer equiv of metal, both added plus endogenous, were present. Assuming 0.01 μM is the lowest concentration of free Mg^{2+} that can be measured (Figure 1) gives an upper estimate of 0.003 μM for the dissociation constant from the tighter of two binding sites, or 0.017 μM from two identical sites. The results of adding 1 or more equiv of metal are shown in Figure 2. The filled circles demonstrate that $\bar{n}$ is independent of buffer. Either underestimation of the endogenous metal in apoenzyme samples, or contamination from divalent metal ions adsorbed to the glassware and in the reagents that were used, would imply tighter binding. The reciprocal slope of the calibration curve in Figure 1 is 0.11 μM/mV, so that a millivolt uncertainty in the measurement of the specific ion electrode potential represents a 50% uncertainty in the value of K_2.

In the presence of substrate it was necessary to add 1.5 equiv of $Mg(C_2H_3O_2)_2$ to obtain a measurable concentration of free Mg^{2+} ions. The measured free Mg^{2+} concentration (0.062 μM) equals the apparent value of K_2 in the presence of substrate estimated by assuming that only Mg^{2+} binding to the enzyme need be considered and that site one was filled.

The results of adding 2 or more equiv of $Mg(C_2H_3O_2)_2$ in the presence of substrate are shown in Figure 3. Calculation of $\bar{n}$ is complicated by the fact that the equilibrium constant for the dehydration reaction is near unity, and the binding of Mg^{2+} ions to PGA and PEP cannot be ignored. Expressing the conservation equations for substrate and enzyme in terms of the equilibrium constant between substrate and product,

$$K_{sp} = \frac{[P]}{[S]} = 4.55 \tag{1}$$

the substrate and product constants,

$$K_s = \frac{[E][S]}{[ES]} = 21.2\ \mu M \tag{2}$$

$$K_p = \frac{[E][P]}{[EP]} = 127\ \mu M \tag{3}$$

and the dissociation constants for Mg^{2+} from substrate and product corrected to pH 7.5

$$K_{sm}' = K_{sm}\left(1 + \frac{[H^+]}{K_a^s}\right) = 4.69\ mM \tag{4}$$

$$K_{pm}' = K_{pm}\left(1 + \frac{[H^+]}{K_a^p}\right) = 5.95\ mM \tag{5}$$

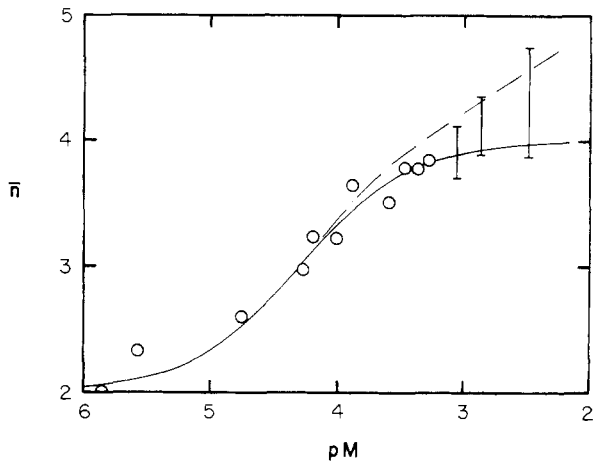

FIGURE 3: Average number of Mg^{2+} ions bound ($\bar{n}$) to enolase (0.1 mM) in the presence of substrate (1.2 mM) as a function of free Mg^{2+} concentration. Measurements were made in K^+-Pipes buffer at pH 7.5, 25 °C, and μ = 0.05 M. $\bar{n}$ was calculated as described in the text. The bars indicate the uncertainty in its value at high metal concentrations. The solid line was drawn for independent binding of two additional Mg^{2+} ions to enolase in the presence of substrate ($K_3' = K_4' = 50\ \mu M$). The dashed line assumes a fifth binding site ($K_5' = 2$ mM).

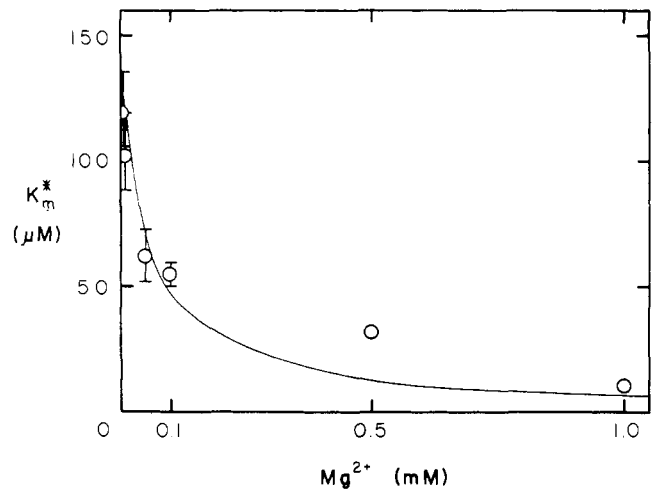

FIGURE 4: Variation of the apparent Michaelis constant (K_m*) for the dehydration of PGA with Mg^{2+} concentration. Measurements were made in Tris buffer at pH 7.5, 25 °C, and μ = 0.05 M; 5 μM ≤ $[PGA]_0$ ≤ 1 mM; 16 ≤ $[E]_0$ ≤ 56 nM. The bars represent the standard deviations. The solid line was calculated using eq 11, as explained in the text.

gives a quadratic equation in the free substrate concentration:

$$\langle K_{sm}'K_{pm}'[K_p + K_{sp}(K_s + K_p) + K_sK_{sp}^2] + \{K_pK_{pm}' + K_{sp}(K_pK_{sm}' + K_sK_{pm}') + K_sK_{sm}'K_{sp}^2\}[M]\rangle[S]^2 + \{K_sK_pK_{sm}'K_{pm}'(1 + K_{sp}) + K_{sm}'K_{pm}'(K_p + K_sK_{sp})([E]_0 - [S]_0) + K_sK_p(K_{pm}' + K_{sm}'K_{sp})[M]\}[S] - K_sK_pK_{sm}'K_{pm}'[S]_0 = 0 \quad (6)$$

Solving for [S] permits the average number of Mg^{2+} ions bound to the enzyme to be calculated using an expression derived from the conservation equation for metal (eq 7).

$$\bar{n} = \frac{[M]_0 - [M]\left\{1 + \left(\frac{1}{K_{sm}'} + \frac{K_{sp}}{K_{pm}'}\right)[S]\right\}}{[E]_0} \quad (7)$$

Equation 6 contains seven constants that must be determined independently. The values of five of them (K_{sp}, K_{sm}, K_{pm}, K_a^s, and K_a^p) were available in a paper by Wold and Ballou (1957a). Slight differences between the conditions used to measure them and those employed in this study were shown experimentally to be immaterial. The two remaining constants (K_s and K_p) can be calculated from the Michaelis and velocity constants for the dehydration and hydration reactions as described by Hanlon and Westhead (1969b). Significantly different experimental conditions were used to obtain the published values, so the steady-state parameters for the enolase reaction had to be reevaluated.

Steady-State Kinetic Parameters. The apparent Michaelis constants (K_m*) and maximum velocity were obtained from double reciprocal plots of the measured initial velocities by the method of least squares. The dependence of K_m* for the dehydration reaction on Mg^{2+} concentration is shown in Figure 4. The ordinate intercept gives the true Michaelis constant (140 μM), as explained in the Discussion section. The maximum velocities gave a dehydration velocity constant for the active enzyme that is independent of the Mg^{2+} ion concentration and equal to 280 s^{-1}.

The velocity of the hydration reaction is more difficult to measure, since only 18% of the PEP present initially is converted to PGA. However, by working at 220 nm and using longer path length cells enough measurements were made to

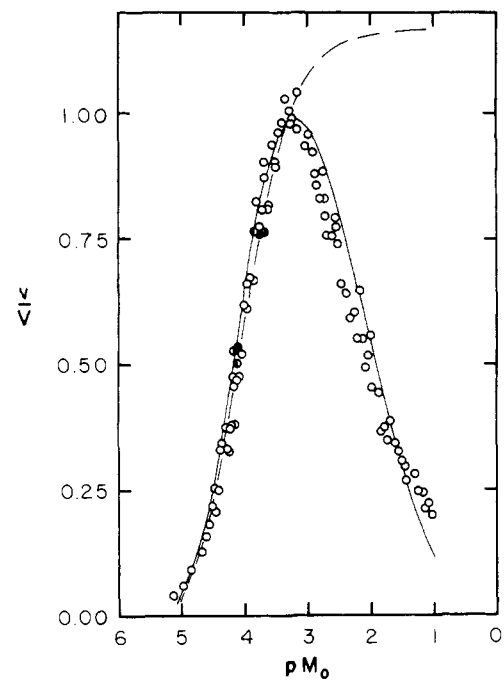

FIGURE 5: Relative initial velocity (v/V) as a function of total Mg^{2+} concentration. Measurements were made at pH 7.5, 25 °C, and μ = 0.05 M; $[PGA]_0$ = 0.2 mM and $[E]_0$ = 3.5 μM: (○) substrate mixed with enzyme and Mg^{2+} in Tris; (●) substrate mixed with enzyme and Mg^{2+} in Pipes; (◐) enzyme mixed with substrate and Mg^{2+} in Tris; (◑) Mg^{2+} mixed with enzyme and substrate in Tris. The dashed line was calculated using eq 8-11 and the solid line using eq 12, as explained in the text.

show that the hydration reaction parallels the dehydration reaction. The apparent Michaelis constant increases with decreasing Mg^{2+} concentration, extrapolating to 150 μM. The hydration velocity constant is 50 s^{-1}.

Activation of Enolase by Mg^{2+} Ions. The effect of Mg^{2+} ion concentration on the velocity at which enolase catalyzes the dehydration of PGA is shown in Figure 5. Stopped-flow measurements were made to permit work at a sufficiently high enzyme concentration so that any contamination by divalent metal ions during the course of an experiment could be detected, while still being assured of measuring initial steady-

state velocities (less than 3% of the reaction). The measured change in absorbance was corrected for the effect of Mg^{2+} ions on the absorptivity of PEP at 240 nm (Wold and Ballou, 1957a). The measured velocity was shown to be independent of the order in which the reagents were mixed.

Discussion

The advantage of studying metal binding in the absence of substrate is that the interpretation of the measurements is unambiguous. The average number of Mg^{2+} ions bound to enolase is given directly by the quantities measured in the specific ion electrode experiments, and the only independently determined number needed to calculate the dissociation constants is the enzyme molecular weight. Three conclusions can be drawn from the titration curve shown in Figure 2. First, two Mg^{2+} ions bind. Second, they bind nonequivalently, the second less tightly than the first. Third, the dissociation constant for Mg^{2+} from the weaker site (K_2) is 0.2 μM. These conclusions are consistent with those drawn previously from measurements of the heat evolved (Faller and Johnson, 1974a) and the number of protons released (Faller and Johnson, 1974b) when Mg^{2+} ions bind to enolase. The question of whether more than two Mg^{2+} ions can bind in the absence of substrate is mechanistically important. The maximum free Mg^{2+} concentration measured in this study was 0.22 mM, but the complexometric titration was extended to 0.81 mM and the thermal titration to 1.6 mM total Mg^{2+} concentration. No indication that more than two Mg^{2+} ions bind was observed in any of the studies, so if additional Mg^{2+} ions do bind in the absence of substrate, their dissociation constants must exceed 5 mM. The dashed line in Figure 2 is the titration curve that would be expected if the two Mg^{2+} binding sites were equivalent. More compelling evidence that the sites are not equivalent is the difference in the enthalpies of binding to them. The values reported by Faller and Johnson (1974a) for binding to site one (−8.8 kcal/mol) and to site two (−2.6 kcal/mol) in Tris buffer have been confirmed by Brewer (1974b). Even the larger of the two dissociation constants was too small to actually measure in either of the two previous studies. K_2 values of 0.1 and 1 μM, respectively, were used to fit the thermal and the complexometric titration curves. It is apparent from Figures 1 and 2 that K_2 can be measured potentiometrically. Its value (0.2 μM) is more than three orders of magnitude smaller than that reported by Hanlon and Westhead (1969a) (470 μM). Tighter binding of divalent cations is qualitatively what would be expected at the lower ionic strength used in this study (0.05 M compared with 0.5 M).

The disadvantage of measuring Mg^{2+} binding in the absence of substrate is that it can only tentatively be concluded from the more than 2 pM unit difference between the midpoints of the titration curve for site two (Figure 2) and the activation curve (Figure 5) that enolase is inactive when just two Mg^{2+} ions are bound, because bound substrate might increase the apparent dissociation constants from sites one and two. Experimentally, the second Mg^{2+} ion binds tighter in the presence of substrate, so that it cannot possibly "turn enolase on". One and a half equivalents of $Mg(C_2H_3O_2)_2$ was required to obtain a measurable concentration of free Mg^{2+} in the presence of substrate, compared with only 1 equiv in its absence, and complexation with substrate could account for at most 0.4% of this difference. Hanlon and Westhead (1969a) also concluded that binding to site two is tighter in the presence of substrate.

In order to decide how many additional Mg^{2+} ions are required to activate enolase, it is necessary to know the number of additional Mg^{2+} ions that bind in the presence of substrate and their dissociation constants. The problem is to sort out how Mg^{2+} partitions among enzyme, substrate, and product. The solution described in the Results section does not assume, as Hanlon and Westhead (1969a) did, that substrate is present in large excess. Four conclusions can be drawn from the resulting titration curve (Figure 3). First, at least two additional Mg^{2+} ions bind in the presence of substrate. Second, they bind equivalently. Third, their dissociation constants are 50 μM. Fourth, weak binding of additional Mg^{2+} ions is not precluded. Hanlon and Westhead (1969a) concluded that just two additional Mg^{2+} ions bind and that they bind equivalently, but much weaker ($K_3 = K_4 = 1.28$ mM) at high ionic strength, as would be expected. The reason weak binding of more than four Mg^{2+} ions cannot be excluded is that an uncertainty of only 2.5% in the highest measured value of the free Mg^{2+} ion concentration translates into calculated $\bar{n}$ values ranging from 3.9 to 4.8. The dashed line in Figure 3 shows that a fifth Mg^{2+} binding site with a dissociation constant of 2 mM is compatible with the experimental data.

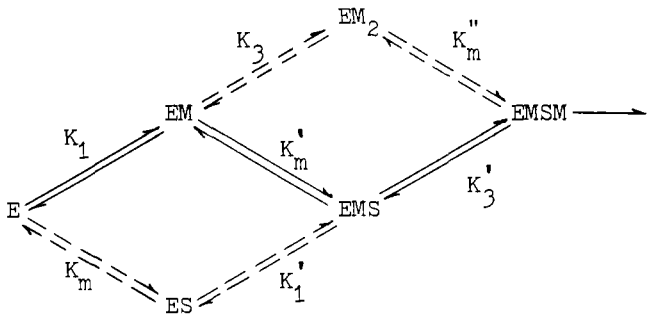

FIGURE 6: Generalized scheme for the binding of two metal ions and a substrate molecule to an enzyme. The solid arrows indicate the proposed ordered pathway for formation of an active quaternary complex of Mg^{2+} and PGA with one protomer of enolase.

Comparison of Figures 3 and 5 suggests that metals three and four activate enolase and that both are required for full activity. This hypothesis was tested more rigorously by deriving the expected rate equation and showing that the observed activation curve can be fitted with the dissociation constants measured in the binding experiments. A generalized scheme for the binding of two metal ions and one substrate molecule to one of the protomers is shown in Figure 6. Assuming that the subunits act independently, the relative velocity is given by the expression:

$$\frac{v}{V} = \frac{1}{2}\left[\frac{[M]}{([M] + K_1^*)} + \frac{[M]}{([M] + K_2^*)}\right] \times \frac{[M]}{([M] + K_3')} \frac{[S]}{([S] + K_m^*)} \quad (8)$$

There is abundant evidence that Mg^{2+} ions one and two bind nonequivalently (Hanlon and Westhead, 1969a; Faller and Johnson, 1974a,b; Brewer, 1974b), but no compelling reason for attributing the nonequivalence to an anticooperative interaction between the protomers in preference to alternative explanations, such as the formation of an asymmetric dimer. Both the spectrophotometric (Spring and Wold, 1971) and thermal (Faller and Johnson, 1974a) titrations of enolase with competitive inhibitors indicate that there are two equivalent substrate binding sites. Equivalent binding of Mg^{2+} ions to sites three and four adequately explains the variation of $\bar{n}$ with Mg^{2+} concentration shown in Figure 3.

The free substrate concentration is approximately given by eq 9:

$$[S] = [S]_0\left[1 - \frac{[M]}{([M] + K_{sm}')}\right] \quad (9)$$

in which substrate complexed to the enzyme has been neglected, because substrate was present in excess (more than 50-fold), and the metal-substrate complex does not bind to the enzyme (Wold and Ballou, 1957b; Hanlon and Westhead, 1969b). The percentage error in [S] that results from this approximation becomes substantial at high metal concentrations, but nowhere is the error introduced into the predicted relative velocity more than 1.7%. The expressions for the apparent dissociation constants of Mg^{2+} from site one and from site two, which is analogous, and for the apparent Michaelis constant are:

$$K_1^* = \frac{K_1}{\left(1 + \frac{[M]}{K_3}\right)}\frac{\left(1 + \frac{[S]}{K_m}\right)}{\left(1 + \frac{[S]}{K_m^*}\right)} = \frac{K_1}{\left(1 + \frac{[S]}{K_m^*}\right)} \quad (10)$$

and

$$K_m^* = K_m'\frac{\left(1 + \frac{[M]}{K_3}\right)}{\left(1 + \frac{[M]}{K_3'}\right)} = \frac{K_m'}{\left(1 + \frac{[M]}{K_3'}\right)} \quad (11)$$

They simplify as shown if binding is ordered, that is, if the pathways denoted by dashed arrows in Figure 6 are insignificant. The preponderance of evidence indicates that binding is ordered. Both spectrophotometric titrations with chromophoric substrate analogues (Spring and Wold, 1971) and thermal titration with a competitive inhibitor (Faller and Johnson, 1974a) have shown that metal is required for substrate binding. The thermal titrations also demonstrated that it is the Mg^{2+} ions at sites one and two that are necessary. This conclusion is supported by nuclear magnetic resonance (NMR) measurements of the effect of bound Mn^{2+} on the relaxation rates of solvent protons (Cohn and Leigh, 1962; Cohn, 1963) and of magnetic nuclei in a bound PEP analogue (Nowak et al., 1973), because fewer than 2 equiv of metal was present in those experiments. Evidence that Mg^{2+} ions three and four bind weakly, if at all, in the absence of substrate has already been discussed.

The principal evidence against an ordered binding mechanism is the independence of the Michaelis constant from metal concentration and of the metal activation constant from substrate concentration reported by Hanlon and Westhead (1969b). Both of the dependencies predicted by an ordered binding scheme have since been reported for activation of yeast enolase by Mn^{2+} (Nowak et al., 1973). The Michaelis constant for the rabbit muscle enzyme has been reported to depend on Mg^{2+} concentration (Wang and Himoe, 1974). The variation of the apparent Michaelis constant with Mg^{2+} concentration found in the present study is shown in Figure 4. The solid line was calculated using eq 11 with $K_3' = 50\ \mu M$ and $K_m' = 140\ \mu M$. In contrast to mechanisms in which activating metal binds first and then substrate (Dixon and Webb, 1958), in the proposed scheme the true Michaelis constant (K_m') is found by extrapolating to zero metal concentration (eq 11). The tighter binding of Mg^{2+} two in the presence of substrate that was observed is qualitatively predicted by the analogue of eq 10 for K_2^*. Quantitatively eq 10 predicts ten times tighter binding compared with the three times tighter binding that was estimated in this study and reported by Hanlon and Westhead (1969a).

According to eq 8 the relative velocity should equal the probability that both metal-binding sites and the substrate site on each protomer are simultaneously filled. The dashed line in Figure 5 was calculated using eq 8–11, the measured metal dissociation ($K_1 < K_2 < 0.2\ \mu M$, $K_3' = 50\ \mu M$) and Michaelis ($K_m' = 140\ \mu M$) constants, and the literature value of K_{sm}' (4.7 mM). Although the mechanism in Figure 6 adequately accounts for the activation of enolase by Mg^{2+} ions, it does not predict the observed loss of activity above 500 μM Mg^{2+}. If an additional Mg^{2+} binding site on each protomer is assumed that "turns it off", eq 8 becomes:

$$\frac{v}{V} = \frac{1}{2}[Y_1(1 - Y_1) + Y_2(1 - Y_{11})]Y_3Y_s \quad (12)$$

in which the shorthand notation $Y_i = [L]/([L] + K_i)$ has been used to denote the fraction of site i binding ligand L that is populated. The solid line in Figure 5 was calculated for $K_1 = 2$ mM and $K_{11} = 20$ mM. It has already been explained that the measurements of Mg^{2+} binding in the presence of substrate reported in this study neither provide convincing evidence that more than four Mg^{2+} ions bind to enolase, nor preclude weak binding of additional Mg^{2+} ions. A weak binding site (dissociation constant 500 μM) has been observed by fluorometric titration and postulated to be inhibitory (Brewer, 1971). An inhibitory site with a dissociation constant of 2 mM has been inferred from kinetic measurements of the inactivation of rabbit muscle enolase by high concentrations of Mg^{2+} (Wang and Himoe, 1974). It is necessary to postulate at least two additional inhibitory sites to explain the gradual loss of activity at high Mg^{2+} concentrations shown in Figure 5.

An alternative explanation of Mg^{2+} inactivation suggested by Hanlon and Westhead (1969b) is formation of the inactive Mg^{2+}-PGA complex (Wold and Ballou, 1957b), since the concentration of Mg^{2+} at which the velocity is halved (8.7 mM in Figure 5) is comparable to K_{sm}' (4.7 mM). This cannot be the explanation of inactivation by high concentrations of Mn^{2+} (Hanlon and Westhead, 1969b) or Zn^{2+}. The relative velocity drops to half for both metals at a concentration of roughly 75 μM (L. D. Faller, to be published), compared with PGA dissociation constants of 816 μM for Mn^{2+} and 400 μM for Zn^{2+} (Wold and Ballou, 1957a). It can be categorically ruled out as the explanation of Mg^{2+} inhibition by the present study, because the apparent Michaelis constant at high substrate concentration is more than an order of magnitude smaller at $\mu = 0.05$ M (10 μM) than the value reported by Hanlon and Westhead for $\mu = 0.5$ M (200 μM). As a consequence of this tighter binding the substrate sites remain saturated, and the predicted velocity continues to increase (dashed line in Figure 5), even though the substrate concentration is reduced 20-fold at the highest Mg^{2+} concentration studied. Tighter binding would be expected at lower ionic strength, because the substrate is negatively charged.

In contrast, the dehydration velocity constant is not affected by ionic strength. Correction of the value found in this study at 25 °C in 0.05 M ionic strength buffer (280 s^{-1}) to 30 °C using the published activation enthalpy of 14.27 kcal/mol (Malmström, 1961) gives 416 s^{-1}, the same value reported by Hanlon and Westhead (1969b) for the higher temperature and $\mu = 0.5$ M. The velocity constant would not be expected to depend on ionic strength, unless the rate-determining step involved charge separation.

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