activity, and that the more weakly bound metal ions are, to a greater or lesser extent, inhibitory in action.

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Kinetic Studies on the Activation of Yeast Enolase by Divalent Cations*

David P. Hanlon and E. W. Westhead

ABSTRACT: Activation constants for Mg^{2+} and Mn^{2+} have been determined for yeast enolase at several concentrations of 2-phosphoglyceric acid in the forward reaction and phosphoenolpyruvic acid in the reverse direction. Michaelis constants for both substrates have been determined at several concentrations of the activating cations. The activation constants are shown to be independent of substrate concentration and the Michaelis constants are independent of metal ion concentration. It is concluded that activating ions and substrates bind to the critical sites independently, ruling out the possibility that the metal functions as a bridge to bind substrate and enzyme. The kinetically determined activation constants were found to be nearly the same as the binding constants for the two cations at the weak sites, determined by equilibrium dialysis. Inhibition by excess Mn^{2+} is shown to be dependent upon the concentration of substrate. The inhibition constant is similar to the binding constant for the binding of a third Mn^{2+} in the presence of substrate.

In the preceding paper (Hanlon and Westhead, 1969) we have established that the interaction of yeast enolase (phosphoenolpyruvate hydratase EC 4.2.1.11) with metal ion activators involves a number of different metal ion acceptor sites. Since the presence of divalent metal cations is an absolute requirement for enolase activity, it is obvious that one or more of these metal ion binding sites must be filled to effect catalysis.

In this paper we report enzyme kinetic studies of both the forward and reverse reaction and correlate these findings with information obtained from our investigation of the equilibrium system.

Experimental Section

Materials

Buffer, 2-D-(+)-PGA,[1] and the enzyme preparation used in these studies have been described in the preceding paper

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[1] Abbreviations used are: PGA, phosphoglyceric acid; PEP, phosphoenolpyruvate; K_A, kinetically determined activation constant for metal ion; K_m, Michaelis constant for substrate; K_S, dissociation constant for enzyme–substrate complex; K_A and K_S are constants for the dissociation of the binary enzyme–metal and enzyme–substrate com-

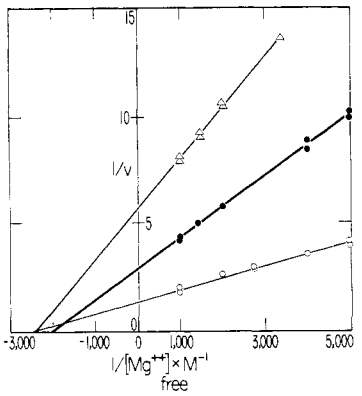

FIGURE 1: The activation of yeast enolase by Mg^{2+} at three different concentrations of 2-PGA: (○—○) 1.2×10^{-3} M, (●—●) 2.0×10^{-4} M, and (△—△) 1.0×10^{-4} M. Line slopes were obtained from a weighted least-squares treatment of the data as described in Results. Conditions were as described in the Experimental Section.

(Hanlon and Westhead, 1969). PEP was obtained from Calbiochem Corp. as the tricyclohexylammonium salt (Grade A). Stock solutions of 2-PGA and PEP of known concentrations were prepared using a spectrophotometric assay (Westhead, 1966). Metal salts were Baker Analyzed Reagent grade which were obtained in the form of their most stable hydrate.

Methods

Kinetic measurements were made by following the increase or decrease in absorbance in the ultraviolet region due to the formation or loss of PEP. Initial velocities were measured during the first 5% of the total reaction. Most measurements were made at 230 mμ, but for velocity studies performed with low concentrations of 2-PGA, 220 mμ was used. In several of the experiments where PEP served as the substrate it was necessary to use 5-cm path-length cells as well as 220 mμ in order to obtain measurable initial rates because the reaction is at equilibrium when only 20% of the initial amount of PEP is converted into 2-PGA. All data were gathered at 30°, using a Zeiss PMQ II spectrophotometer with a thermostatted cell holder, and a Photovolt log–linear recorder.

Stock enzyme solutions at 2–5 mg/ml were diluted to 0.1–0.3 mg/ml with assay buffer before use. To initiate the reaction, microliter amounts of the diluted enzyme solution were added to the otherwise completed assay mixture and the solution was agitated for a few seconds. A check for residual activity due to contamination by traces of metal ion was made by omitting metal ion from the assay. Only stock enzyme solutions giving a velocity of not more than 0.005 A/min per 10 μl in 1 ml of assay solution lacking metal ion were used.

Results

Mg^{2+} and Mn^{2+} Activation at Various Substrate Concentrations. K_A values were obtained for Mg^{2+} and Mn^{2+} in the presence of several concentrations of 2-PGA. K_A's for Mg^{2+} were also determined in the presence of two concentrations of PEP. The velocity data for the Mg^{2+}-activated system, plotted in double-reciprocal fashion, are shown in Figures 1 and 2. Corrections have been made for the interactions of 2-PGA with Mn^{2+} and Mg^{2+} (using the dissociation constants of Wold and Ballou, 1957). No correction has been made for Tris–metal ion interactions which are negligible in the cases of Mg^{2+} and Mn^{2+} under our conditions (Hanlon *et al.*, 1966). A least-squares fit of the velocity data, weighted according to the method of Wilkinson (1961), was used to calculate all kinetic constants reported in this paper. The results of these experiments are summarized in Table I.

Measurements of the Effect of the Concentration of Mg^{2+} and Mn^{2+} on K_m for 2-PGA and PEP. Michaelis constants for both substrates were measured at several concentrations of the metal ion activators. Velocity data for 2-PGA and PEP in the Mg^{2+} system are shown in Figures 3 and 4 in the form of Lineweaver–Burk plots. These data and data from studies where Mn^{2+} served as activator are listed in Table II.

TABLE I: Metal Ion Activation Constants for Mg^{2+} and Mn^{2+}.

Substrate	Metal Ion Activator	[Substrate] (M)	K_A (M)
2-PGA	Mg^{2+}	1.00×10^{-4}	4.30×10^{-4}
		2.00×10^{-4}	5.18×10^{-4}
		1.20×10^{-3}	4.20×10^{-4}
	Mn^{2+}	1.00×10^{-4}	6.80×10^{-6}
		1.20×10^{-3}	5.80×10^{-6}
2-PEP	Mg^{2+}	1.57×10^{-4}	5.38×10^{-4}
		8.80×10^{-4}	5.48×10^{-4}
	Mn^{2+}	7.50×10^{-5}	4.50×10^{-6}
		1.50×10^{-4}	5.20×10^{-6}
		3.64×10^{-4}	4.80×10^{-6}
		1.11×10^{-3}	4.30×10^{-6}

TABLE II: Michaelis Constants for 2-PGA and 2-PEP in the Mg^{2+}- and Mn^{2+}-Activated Systems of Yeast Enolase.

Substrate	Metal Ion Activator	$[M^{2+}]$ (M)	K_m (M)
2-PGA	Mg^{2+}	1.00×10^{-4}	1.60×10^{-4}
		2.00×10^{-4}	1.85×10^{-4}
		5.00×10^{-4}	1.72×10^{-4}
		1.00×10^{-3}	1.85×10^{-4}
	Mn^{2+}	1.00×10^{-5}	1.50×10^{-4}
2-PEP	Mg^{2+}	5.00×10^{-4}	2.00×10^{-4}
		2.00×10^{-3}	2.20×10^{-4}
	Mn^{2+}		1.24×10^{-4} [a]

[a] This K_m value was obtained from a double-reciprocal plot of V_{max} for the Mn^{2+} system at four different concentrations of 2-PEP using data in Table I.

plexes; K_A' and K_S' are constants for the dissociation of metal ion and substrate, respectively, from the ternary enzyme–metal–substrate complex.

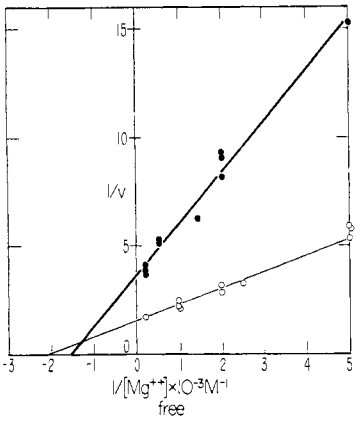

FIGURE 2: The activation of yeast enolase by Mg^{2+} at two different concentrations of 2-PEP: (○—○) 8.80×10^{-4} M and (●—●) 1.57×10^{-4} M. Line slopes were obtained from a weighted least-squares treatment of the data as described in Results. Conditions were as described in the Experimental Section.

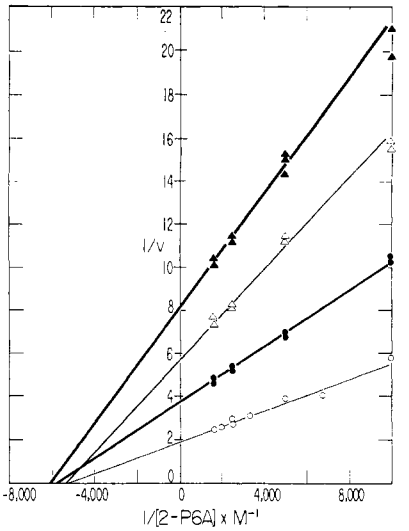

FIGURE 3: A double-reciprocal plot of the effect of 2-PGA on the velocity of the yeast enolase catalyzed reaction measured at four different concentrations of Mg^{2+}: (○—○) 1.00×10^{-3} M, (●—●) 5.00×10^{-4} M, (△—△) 2.00×10^{-4} M, and (▲—▲) 1.00×10^{-4} M. Line slopes were obtained from a weighted least-squares treatment of the data as described in Results. Conditions were as described in the Experimental Section.

Inhibition of Enolase Activity by High Substrate and Mn^{2+} Concentrations. A decrease in velocity is observed at a concentration of 10^{-5} M Mn^{2+} (total) when the concentration of 2-PGA (total) is greater than about 6×10^{-4} M. We have made calculations of the free concentration of Mn^{2+} at various concentrations of 2-PGA using the Mn^{2+}–2-PGA interaction constants obtained by Wold and Ballou (1957). Data presented in Table III indicate excellent agreement between theoretical velocity values and observed velocity values making the assumption that the inhibition results *only* from the loss of free Mn^{2+}.

Inhibition is also observed when, at any lower concentration of 2-PGA, the concentration of free Mn^{2+} is much above 10^{-5} M. Pertinent data appear in Figure 5.

When the concentration of free Mn^{2+} is increased beyond 10^{-5} M, one notes a decrease in velocity at both high ($6 \times K_m$) and at low ($K_m/2$) concentrations of 2-PGA (Figure 5). In neither case can the observed velocity result entirely from a loss of free substrate as consideration of the concentrations involved will show. To estimate K_i values, a calculated hyperbolic saturation plot was constructed from data at low concentrations of Mn^{2+}. K_i was taken as that concentration of free Mn^{2+} which yielded an experimental velocity equal to one-half the calculated value (see Figure 5). At a total 2-PGA concentration of 10^{-3} M, $K_i = 4.2 \times 10^{-5}$ M. At 10^{-4} M total 2-PGA, $K_i = 10 \times 10^{-5}$ M. Where necessary, appropriate corrections have been made for velocity decreases due to decreases in the concentration of free 2-PGA.

Discussion

In discussing the data of this paper, we will refer to the cation binding data of the previous paper (Hanlon and Westhead, 1969). The data of the present paper were obtained at 30° and those of the previous paper were obtained at 22°. The data are comparable because K_m(PGA) and $K_A(Mg^{2+})$ at 20° are identical with the 30° values (D. P. Hanlon and E. W. Westhead, unpublished data) within experimental error. Also, a few binding measurements for Mn^{2+} at 30° gave results identical with the more extensive data at 22°.

The several reactions for the activation of enzymes by metal ions can be listed as follows.

$$E + M \rightleftarrows EM + S \rightleftarrows EMS \quad (1)$$

$$E + S \rightleftarrows ES + M \rightleftarrows EMS \quad (2)$$

$$M + S \rightleftarrows MS + E \rightleftarrows EMS \quad (3)$$

$$E + M + S \rightleftarrows EMS \quad (4)$$

Reaction 1 involves the obligatory formation of an enzyme–metal complex in order for substrate to bind. Reaction 2 requires that substrate and enzyme form a complex in order for metal ion to bind. In reaction 3, the enzyme interacts

TABLE III: The Effect of High Concentration of 2-PGA on the Velocity of the Mn^{2+}-Activated Yeast Enolase System.[a]

$[2\text{-PGA}]_{total}$ × 10^4 M	$[2\text{-PGA}]_{free}$ × 10^4 M	$[Mn^{2+}]_{free}$ × 10^6 M	Velocity Obsd	Velocity Theor
6.00	5.96	5.78	0.210[b]	
12.0	11.9	4.06	0.195	0.178
24.0	23.9	2.54	0.150	0.156
60.0	59.9	1.20	0.095	0.089

[a] Theoretical velocities were calculated assuming $K_A = 6.0 \times 10^{-6}$ M and $K_m = 1.50 \times 10^{-4}$ M. The total concentration of Mn^{2+} was set at 10^{-5} M. [b] A V_{max} value for the system when saturated with 2-PGA and Mn^{2+} was calculated from this velocity. $V_{max} = 0.52$. Theoretical velocities were obtained from this V_{max} value.

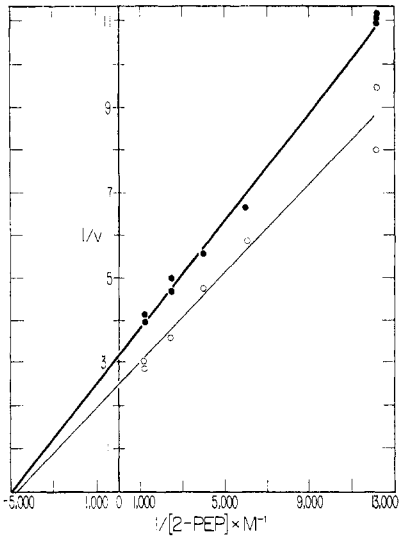

FIGURE 4: A double-reciprocal plot of the effect of 2-PEP on the velocity of the yeast enolase catalyzed reaction measured at two different concentrations of Mg^{2+}: (O—O) 2.00×10^{-3} M and (●—●) 5.00×10^{-4} M. Line slopes were obtained from a weighted least-squares treatment of the data as described under Results. Conditions were as described in the Experimental Section.

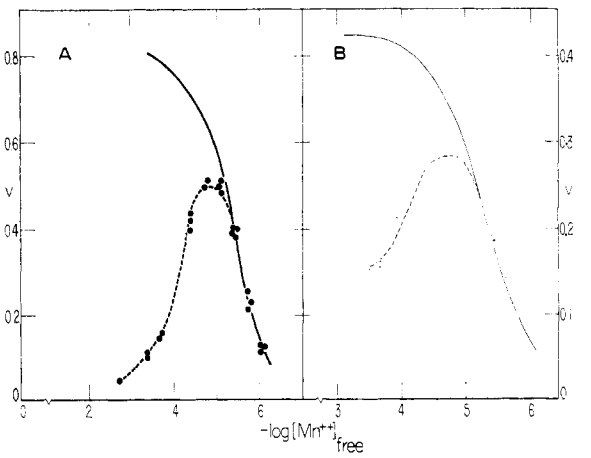

FIGURE 5: The activation of yeast enolase by Mn^{2+}, showing the inhibition of activity at higher concentrations of Mn^{2+}. Plot A represents data obtained in the presence of 1.2×10^{-3} M 2-PGA (total) and plot B shows data obtained using 1.0×10^{-4} M 2-PGA (total). The solid lines are hyperbolic curves constructed from points in the activating region of Mn^{2+} concentration and illustrate the theoretical activation curve for Mn^{2+} at each concentration of 2-PGA. The dashed lines are theoretical curves for inhibition by Mn^{2+} with a $K_i = 4.5 \times 10^{-5}$ M in plot A and $K_i = 10 \times 10^{-5}$ M in plot B.

with a preformed metal ion–substrate complex. In reaction 4, metal ion and substrate can interact with enzyme independently of the other component. We will examine each of these possible schemes in light of our kinetic data to rule out unlikely modes of metal ion activation of yeast enolase.

Reactions 1 and 2 represent an ordered addition wherein either the ES or EM complex must form first, to allow binding of the second ligand. In either case formation of the EMS complex will be enhanced by the presence of the second adduct. Thus K_A for metal ion activator and K_M for substrate will be a function of the concentration of substrate and metal ion, respectively.

Dixon and Webb (1964) give the following velocity expression covering all degrees of interdependency of substrate and metal ion activator, where E and EM have different affinities for substrate.

$$v = V_{max}/[1 + K_S/S][1 + (K_A/M)(1 + S/K_S)/(1 + S/K_S')] \quad (1)$$

where K_S and K_S' are substrate dissociation constants for ES and EMS complexes, respectively.

Reactions 1 and 2 obviously have a symmetrical relationship to each other and it is only necessary to look at the kinetic expressions for one case to see what will happen in the other. Using Dixon and Webb's example which corresponds to reaction 1 above, we find that the apparent dissociation constants are related to ligand concentrations as follows

$$K_A(app) = K_A/(1 + S/K_S') \quad (2)$$

$$K_S(app) = K_S'(1 + K_A/A) \quad (3)$$

Two features stand out: as substrate gets much larger than K_S', the apparent dissociation constant for metal ion decreases without limit, becoming inversely proportional to the substrate concentration; the apparent dissociation constant for substrate is relatively constant only when the concentration of metal ion is well above its dissociation constant—as the activator ion concentration is reduced below this level the apparent K_s is increased indefinitely.

Data in Tables I and II, derived from Figures 1 and 2, show the independence of K_A for Mg^{2+} from the concentration of 2-PGA at 2-PGA concentrations up to ten times K_S, and show the independence of K_M from Mg^{2+} concentrations down to $K_A/4$.

Since K_A and K_S are not interdependent in the fashion described by eq 2 and 3, we can rule out reaction 1 and, by analogy, reaction 2. It is important to note that although the above equations were derived using the Michaelis–Menten equilibrium conditions, exactly similar equations are obtained with steady-state kinetics. In the reverse reaction, K_A for Mg^{2+} and K_S for PEP are likewise independent of ligand concentrations (Tables I and II).

In reaction 3 the true substrate is a metal ion–substrate complex. Implicit in this argument is a requirement that neither metal ion nor substrate can interact with the enzyme in the absence of the other. Dixon and Webb (1964) show that this type of mechanism requires the apparent K_M for substrate at a fixed metal ion concentration to be identical with the apparent K_A for the metal ion activator at the same fixed total concentration of substrate. Again, independence of K_M from the concentration of metal ion and independence of K_A from the total concentration of 2-PGA rule out this possibility.

By elimination we are left with reaction 4 and will show that kinetic data agree best with this general mechanism for yeast enolase. Reaction 4 is analogous to the case of two-substrate–enzyme systems in which each substrate interacts with the enzyme in an independent, nonordered, fashion. The observed velocities should be in accord with the following velocity expression taken from Dixon and Webb (1964),

TABLE IV: Rate Constants and Substrate Dissociation Constants in the Mg^{2+}- and Mn^{2+}-Activated Yeast Enolase Systems.[a]

Metal Ion Activator	k_1 M^{-1} sec^{-1}	k_{-1} sec^{-1}	k_3 sec^{-1}	k_{-3} M^{-1} sec^{-1}	$K_{S(2\text{-PGA})}$ (M)	$K_{S(2\text{-PEP})}$ (M)
Mg^{2+}	2.4×10^6	70	420	2.7×10^6	2.9×10^{-5}	1.5×10^{-4}
Mn^{2+}	2.1×10^6	27	280	2.5×10^6	1.3×10^{-5}	1.1×10^{-4}

[a] Constants relate to the model presented in the text. K_S is the dissociation constant for substrate and enzyme.

which includes constants for metal ion–enzyme and substrate–enzyme interactions

$$v = \frac{V_{max}}{(1 + K_m/S)(1 + K_A/(M^{2+}))} \quad (4)$$

Double-reciprocal plots of velocity *vs.* substrate or velocity *vs.* metal ion will be linear whether the system is one in which the enzyme–substrate complexes are in equilibrium or one in which steady-state kinetics prevail, *provided* the interaction of metal ion with both free enzyme and the ES complex occurs *via* a rapid adjustment step (Frieden, 1964). Since K_A values for metal ion activators are independent of substrate concentration and K_A values for a given activator are unchanged by substituting PEP for 2-PGA, a rapid metal ion interaction with E and ES is indicated. Although every metal ion activator of yeast enolase shows some inhibition at high concentrations, the effect is least for Mg^{2+}-activated systems (Westhead and Malmström, 1957) and these can be used most fairly to test for linearity in the double-reciprocal plots. Figures 1–4 indicate that linearity does hold for the enolase system. One can then distinguish between a rapid equilibrium system and a steady-state system in which only the metal ions are in true equilibrium with their complexes utilizing data in Table IV. If we describe the reversible reaction catalyzed by enolase by the model

$$EM + PGA \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EM(PGA) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EM(PEP) \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EM + PEP$$

and assume that k_2 and k_{-2} are much larger than k_3 and k_{-1}, respectively, we can determine the nature of the Michaelis constants. The catalytic rate constants, k_3 and k_{-1}, are derived directly from V_{max} values obtained by extrapolating observed V_{max} values in the forward and reverse directions to conditions of infinite substrate and metal ion concentration. With the assumptions above, the Michaelis constants for the forward and reverse reactions become, respectively, $K_m(PGA) = (k_{-1} + k_3)/k_1$ and $K_m(PEP) = (k_{-1} + k_3)/k_{-3}$. The data in Table IV show that k_3 is four to six times as large as k_{-1} for the two metal–enzyme complexes so steady-state kinetics appear to prevail in the conversion of 2-PGA into PEP. Conversely, K_m for the reverse reaction approaches the value of K_S, *i.e.*, k_3/k_{-3}.

The rate constants for magnesium and manganese dissociation from the enzyme occur too rapidly to be measured by dilution in the assay system, unlike the finding of Ray and Roscelli (1966) for phosphoglucomutase. Under our assay conditions the rate constant for the dissociation of both Mg^{2+} and Mn^{2+} from enolase must be greater than 1 sec^{-1}.

Inhibition by Substrate and Metal Ions. Substrate inhibition is explained quantitatively by the complexing of activating cations with consequent lowering of their concentration (Table III).

Mn^{2+} inhibition, in contrast, must result from direct interaction with the protein. K_i for Mn^{2+} inhibition is in excellent agreement with the dissociation constants for the third and fourth Mn^{2+} ions bound in the presence of substrate as measured by equilibrium dialysis (Hanlon and Westhead, 1969; Table I).

The fact that inhibition, seen in the case of increasing Mn^{2+} concentration, is not also observed in the studies where substrate is increased can readily be explained. Substrate inhibition studies were made using a total concentration of Mn^{2+} which never exceeded 10^{-5} M. The addition of 2-PGA to the assay decreases the concentration of free Mn^{2+}. Thus, even though substrate enhances the binding of Mn^{2+} at inhibitory sites, the free Mn^{2+} has fallen from 10^{-5} to 4.0×10^{-6} M at a 2-PGA concentration of 10^{-3} M. At this point the concentration of free Mn^{2+} is less than $0.1 \times K_i$ and no inhibition would be observed.

Inhibition by excess Mg^{2+} can be obtained, but, calculation of a reasonable K_i value is complicated by the high concentration of Mg^{2+} and 2-PGA required plus the uncertainty in the value of the equilibrium constant for the association of 2-PGA and Mg^{2+}. Mn^{2+} appears to be more suitable for kinetically probing inhibition by activating metal ions.

The Role of the Divalent Cations. We have shown in the preceding paper that two metal ions are bound in the absence of substrate. Dissociation constants for the tightest bound Mg^{2+} and Mn^{2+} are unchanged in the presence of substrate, but $K_{d(2)}$ appears to decrease threefold for Mg^{2+} and fivefold for Mn^{2+} in the presence of substrate. This apparent dependence upon substrate concentration may result from the difficulty of obtaining an accurate $K_{d(2)}$ when substrate induces the binding of two additional divalent cations. Comparison of K_d values for Mg^{2+} and Mn^{2+} with K_A values show that the kinetic constants approximate the $K_{d(2)}$ values in each system, but are somewhat larger. (The enzyme dissociation phenomena discussed in the following paper (Gawronski and Westhead, 1969) suggest the possibility that the kinetic and equilibrium constants differ because of the vastly different enzyme concentrations used in the two techniques, but both equilibrium constants and kinetic constants have been obtained over manyfold enzyme concentrations with no observable variation in the constants.) The similarity between K_A and the second dissociation constant for both metals implies that the weaker

bound metal ion is essential for activity. In support of this point is the fact that K_A for both metal ion activators is the same in the forward and reverse reaction.

Of the first two Mn^{2+} ions bound, the weaker bound interacts at the same site as the stronger bound Mg^{2+} and probably *vice versa* (Hanlon and Westhead, 1969; Figure 5). In combination with the fact that K_A values for Mg^{2+} and Mn^{2+} are close to $K_{d(2)}$ values in each system, this indicates that both sites must be filled for catalytic activity. Independence of K_A and K_m from the concentration of the second component rules out a bridge mechanism at the kinetically important site as well as a mechanism wherein the true substrate is a metal–substrate complex. The binding of additional metal ions in the presence of substrate shown in the preceding paper (Hanlon and Westhead, 1969) implies the binding of substrate to the enzyme in a complex influenced by the metal ion binding, very possibly a "bridge" complex. This complex could certainly be the one shown to exist by Cohn (1963) with electron spin resonance and proton magnetic resonance techniques. Data presented here show that for the Mg^{2+}-activated enzyme, this site must be kinetically irrelevant, whereas for the Mn^{2+}-activated enzyme it appears to be inhibitory.

Summary

Yeast enolase appears to require two ions of a divalent cation per molecule of enzyme (67,000 molecular weight). This enzyme is dimeric but the two sites have distinctly different affinities for the divalent cation.

The activation constants determined kinetically are independent of substrate concentration and K_m values for PGA and PEP are independent of metal ion concentration; therefore the activating cations and substrate must bind independently at the kinetically relevant sites and a "bridge" role for the cation is ruled out.

The higher affinity site for Mg^{2+} appears to be the lower affinity site for Mn^{2+}. Since activation constants in both systems approximate the weaker site dissociation constants, cations are apparently required at both sites for catalytic activity.

The substrate-dependent binding of more than two atoms of Mg^{2+} or Mn^{2+} seen by direct binding measurements seems to have no effect in the Mg^{2+} system and to lead to inhibition in the Mn^{2+} system.

In both the forward and reverse directions, equilibration with activating metal ion is rapid; in the forward reaction, the K_m for PGA is a steady-state constant; the K_m for PEP approximates the dissociation constant for the PEP–enzyme complex.

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