Role of the Divalent Metal Cation in the Pyruvate Oxidase Reaction*

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Purified pyruvate oxidase requires a divalent metal cation for enzymatic activity. The function of the divalent metal cation was studied for unactivated, dodecyl sulfate-activated, and phosphatidylglycerol-activated oxidase. Assays performed in the presence of Mg"+, Ca"+, Zn"+, Mn"+, Bax", Ni"+, Co"+, Cu"+, and Cr"+ in each of four different buffers, phosphate, 1,4-piperazinediethanesulfonic acid, imidazole, and citrate, indicate that any of these metal cations will fulfill the pyruvate oxidase requirement. Extensive steady state kinetics data were obtained with both Mg"+ and Mn"+. All the data are consistent with the proposition that the only role of the metal is to bind to the cofactor thiamin pyrophosphate (TPP) and that it is the Me"+-TPP complex which is the true cofactor. Values of the Mg"+ and Mn"+ dissociation constants with TPP were determined by EPR spectroscopy and these data were used to calculate the Michaelis constant for the Me"+-TPP complexes. The results show that the Michaelis constants for the metal"+-TPP complexes are independent of the pyruvate oxidase. All samples contained 100 mM MgCl₂.

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Pyruvate oxidase is a peripheral membrane enzyme from Escherichia coli (1). It catalyzes the oxidative decarboxylation of pyruvate to form acetate and CO₂ (2). The purified enzyme, a tetramer, contains 4 molecules of noncovalently bound FAD and has an absolute requirement for added thiamin pyrophosphate and divalent metal cations (3). The purified enzyme also has a rather low turnover number, which can be stimulated 20-50-fold in the presence of appropriate amphi-

that there are basically 3 kinetic forms of the oxidase: unactivated, monomeric amphiphile-activated, and aggregated amphiphile-activated. The two activated forms of the enzyme are very similar; the only difference is that enzyme activated by aggregated amphiphiles displays cooperative kinetics with respect to the TPP cofactor, while enzyme activated by monomer amphiphiles does not (6).

In this paper, attention is drawn to the role of the divalent metal cation in the pyruvate oxidase reaction. The requirement for a divalent metal cation was recognized early in the purification of the enzyme (1). Historically, Mg"+ has been the metal cation of choice in TPP-catalyzed reactions. All previous work with pyruvate oxidase has been conducted in the presence of a saturating concentration of Mg"+ (10 mM). Like many TPP-requiring enzymes (9-11), however, the oxidase will permit other divalent metal cations to substitute for Mg"+. As part of the goal of determining the mechanism of amphiphsle activation of pyruvate oxidase, the function of the divalent metal cation was studied for all three kinetic forms of the enzyme using steady state kinetics, fluorescence, and EPR spectroscopy.

EXPERIMENTAL PROCEDURES

Materials—All metal cations investigated were obtained as their chloride salts. Gadolinium" and europium" were purchased from Apache Chemicals, Inc. Terbium" was purchased from ICN Pharmaceuticals, Inc. All other metal cations were reagent grade. Magnesium" was obtained from Mallinckrodt Chemical Works. Nickel", cobalt", and manganese" were obtained from J. T. Baker Chemical Co. Copper" and zinc" were obtained from Fisher, Barium" and chromium" were obtained from Allied Chemical Co. Finally, calcium" was obtained from Matheson, Coleman, and Bell.

Thiamin pyrophosphate, sodium pyruvate, Pipes, and phosphatidylglycerol were purchased from Sigma. Sodium dodecyl sulfate was obtained from Eastman. Sodium ferricyanide was obtained from K & K Laboratories, Inc. All other chemicals were reagent grade.

Pyruvate oxidase was purified according to previously published procedures (12). Enzyme assays were conducted as previously described (6).

EPR Experiments—The EPR experiments were conducted at room temperature on a Varian E-9 EPR spectrometer. The EPR spectrum of 1 mM MnCl₂ was recorded in 1) distilled water; 2) 0.05 M Pipes buffer, pH 6.0; 3) Pipes buffer plus 0.5, 1.0, 2.0, and 4.0 mM TPP; 4) Pipes buffer plus 4 mM TPP and 5, 10, 20, 40, 80, 160, and 200 mM MgCl₂; 5) Pipes buffer plus 25, 50, 100, 150, and 200 mM pyruvate; and 6) Pipes buffer plus 10 mM pyruvate and 25, 50, and 100 mM MgCl₂.

EPR spectroscopy was also used to examine the binding of Mn"+ to pyruvate oxidase. All samples contained 100 pm MnCl₂ and 0.1 M Pipes buffer, pH 5.7. Spectra were recorded and compared for samples containing 1) no additions, 2) 78 PM pyruvate oxidase subunits, 3) 100 PM TPP, 4) 100 PM TPP plus 78 PM pyruvate oxidase subunits. All spectra were recorded at 9.5 GHz at room temperature, 25°C.

All fluorescence measurements were made with a Perkin-Elmer MFP-44A fluorimeter equipped with a circulating constant temperature bath. The temperature of the sample is measured using a YSI

*The abbreviations used are: TPP, thiamin pyrophosphate; Pipes, 1,4-piperazinediethanesulfonic acid.

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model 42SC telethermometer. To avoid large inner filter corrections due to TPP absorption, all fluorescence titrations were done with excitation at 300 nm. Fluorescence intensity was followed at 333 nm using an excitation band pass of 6 nm and an emission band pass of 10 nm. The Raman scattering intensity was negligible.

At the end of a titration, the fluorescence intensity values were corrected for small volume changes (always less than 5%) and for TPP inner filter effects. Since thiamin does not bind to pyruvate oxidase as judged from steady state kinetics, a thiamin quenching curve can be used as an empirical inner filter correction for the TPP titration data.

RESULTS

Survey of Metal Cations—Eight divalent cations and one trivalent cation were surveyed for their ability to fulfill the metal cation requirement shown by the oxidase. The results are shown in Table I. In the appropriate buffer, all of the metals could be observed to support pyruvate oxidase activity. In many cases, attempts to reach saturating concentrations of the metal cation led to precipitation and loss of activity. In those cases where the concentration of the metal cation could be increased to apparent saturation without precipitation occurring, the maximum velocity of the oxidase showed no apparent fluctuation. Thus, neither the metal ion nor the buffer appeared to influence the maximum velocity of the enzyme.

| Metal cation | Apparent Michaelis constanta (M) | Apparent Michaelis constantb (M) | Apparent Michaelis constantc (M) |
|-------------|---------------------------------|---------------------------------|---------------------------------|
| Mg2+        | 0.05 mM | 0.05 mM | 0.05 mM | 0.05 mM |
| Ca2+        | 10 mM   | 22 mM   | 12.5 mM | 12.5 mM |
| Zn2+        | +       | +       | +       | +       |
| Mn2+        | +       | +       | +       | +       |
| Ba2+        | 33 mM   | -       | -       | -       |
| N2+         | +       | +       | +       | +       |
| Co2+        | 100 µM  | +       | +       | +       |
| Cu2+        | 150 µM  | -       | -       | -       |
| Cr3+        | 85 µM   | -       | -       | -       |

— designates that no pyruvate oxidase activity was observed. + designates that pyruvate oxidase activity was present, but precipitation prevented full activity from being seen.

Repeated efforts to support pyruvate oxidase activity with the trivalent Tb3+, Eu3+, or Gd3+ ions were unsuccessful. All three ions at very low concentrations (1 µM or less) caused precipitation of the enzyme.

Kinetic Studies with Mg2+—Initial velocity experiments were conducted on all three kinetic forms of the enzyme with Mg2+ in 0.1 M phosphate buffer. Lineweaver-Burk plots of velocity1 versus TPP−1 at fixed levels of Mg2+ and the alternate plots of velocity1 versus (Mg2+)−1 at fixed levels of TPP were presented for unactivated, dodecyl sulfate-activated, and phosphatidylglycerol-activated pyruvate oxidase in Figs. 1, 2, and 3, respectively. The concentrations of each component were chosen such that the same data points could be used for each pair of plots. In all cases, the plots of velocity1 versus the varied substrate−1 at different levels of the changing fixed substrate yielded a family of lines that intersected at a common point on the velocity−1 axis. Secondary plots of the slope versus the changing fixed substrate−1 were linear and passed through the origin.

Similar experiments with Mg2+ and pyruvate or ferricyanide yielded the data summarized in Table II. All these plots gave families of lines that intersected at a common point to the left of the velocity−1 axis. Secondary plots in all cases were linear and did not pass through the origin (data not shown).

Kinetic Studies with Mn2+—Initial velocity experiments similar to those just described were performed with Mn2+ in 0.05 M Pipes buffer. Lineweaver-Burk plots of velocity1 versus TPP−1 at fixed levels of Mn2+ and the alternate plots of velocity1 versus (Mn2+)−1 at fixed levels of TPP were constructed for unactivated, dodecyl sulfate-activated, and phosphatidylglycerol-activated pyruvate oxidase (data not shown). The concentrations of each component were again chosen such that the same data points could be used for each pair of plots. As was the case when Mg2+ was the cation, all plots of velocity1 versus the varied substrate−1 at different levels of the changing fixed substrate yielded a family of lines that intersected at a common point on the velocity−1 axis. Secondary plots of the slope versus the changing fixed substrate−1 were linear and passed through the origin.

Similar experiments with Mn2+ and ferricyanide yielded the data also summarized in Table II. All these plots gave families of lines that intersected at a common point to the left of the
FIG. 2. Initial velocity kinetic experiments on dodecyl sulfate-activated pyruvate oxidase with TPP and Mg++. Enzyme assays were conducted as described under “Experimental Procedures.” The enzyme activity units are defined as micromoles of pyruvate decarboxylated/min. A is a double reciprocal plot with TPP as the varied substrate and Mg++ as the changing fixed substrate. The Mg++ concentrations were 5 mM (•), 3 mM (○), 2 mM (□), 1 mM (■), and 0.6 mM (▲). B is a double reciprocal plot with Mg++ as the varied substrate and TPP as the changing fixed substrate. The TPP concentrations were 10 µM (●), 8 µM (○), 6 µM (△), 4 µM (□), 2 µM (▲), and 1 µM (■). The concentrations of pyruvate and ferricyanide were 200 mM and 10 mM, respectively, in both panels. The inset in each panel shows the dependence of the slope upon the reciprocal of the concentration of the changing fixed substrate.

FIG. 3. Initial velocity kinetic experiments on phosphatidylglycerol-activated pyruvate oxidase with TPP and Mg++. Enzyme assays were conducted as described under “Experimental Procedures.” The enzyme activity units are defined as micromoles of pyruvate decarboxylated/min. A is a double reciprocal plot with TPP as the varied substrate and Mg++ as the changing fixed substrate. The Mg++ concentrations were 2.0 mM (●), 1.6 mM (○), 1.0 mM (□), 0.75 mM (△), and 0.5 mM (▲). B is a double reciprocal plot with Mg++ as the varied substrate and TPP as the changing fixed substrate. The TPP concentrations were 10 µM (●), 8 µM (○), 6 µM (△), 4 µM (□), 2 µM (▲), and 1 µM (■). The concentrations of pyruvate and ferricyanide were 200 mM and 10 mM, respectively, in both panels. The inset in each panel shows the dependence of the slope upon the reciprocal of the concentration of the changing fixed substrate. In the presence of phosphatidylglycerol, the binding of TPP to the oxidase is cooperative. The data are plotted as a function of TPP−1 to take into account this cooperativity.

Table II
Lineweaver-Burk patterns obtained with Mg++ and Mn++

| Varied substrate | Changing fixed substrate | Pattern observed for unactivated pyruvate oxidase | Pattern observed for dodecyl sulfate-activated pyruvate oxidase | Pattern observed for phosphatidylglycerol-activated pyruvate oxidase |
|------------------|-------------------------|---------------------------------------------|-----------------------------------------------|--------------------------------------------------|
| Mg++             | Pyruvate                | 1NT                                        | 1NT                                            | 1NT                                             |
| Pyruvate         | Mg++                   | 1NT                                        | 1NT                                            | 1NT                                             |
| Mg++             | Ferricyanide            | 1NT                                        | 1NT                                            | 1NT                                             |
| Ferricyanide     | Mg++                   | 1NT                                        | 1NT                                            | 1NT                                             |
| Mn++             | Ferricyanide            | 1NT                                        | 1NT                                            | 1NT                                             |
| Ferricyanide     | Mn++                   | 1NT                                        | 1NT                                            | 1NT                                             |

*1NT designates a family of lines that intersect at a common point to the left of the velocity−1 axis.

Similar experiments conducted with Mn++ and pyruvate resulted in data quite different from that obtained in the presence of Mg++. Lineweaver-Burk plots of velocity−1 versus pyruvate−1 at fixed levels of Mn++ and the alternate plots of velocity−1 versus Mn++−1 at fixed levels of pyruvate were constructed for unactivated (Fig. 4), dodecyl sulfate-activated, and phosphatidylglycerol-activated pyruvate oxidase. For all three kinetic forms of the oxidase, the plots of velocity−1 versus pyruvate−1 at fixed levels of Mn++ were nonlinear and concave up. Thus, at low levels of Mn++, pyruvate was apparently a very strong substrate inhibitor. The plots of velocity−1 versus Mn++−1 at fixed levels of pyruvate yielded families of lines that were linear, but that did not pass through a common velocity−1 axis. Secondary plots in all cases were linear and did not pass through the origin (data not shown).
Mg$_2^+$-TPP binding, and a dissociation constant of 5 mM was used to monitor the intensity of the Mn$^{2+}$ EPR spectra was used to monitor the dissociation constant obtained is 0.49 PM for the Mn$^{2+}$-TPP complex. Both the dissociation constant and Michaelis constant for TPP were determined in the presence of 50 mM Mn$^{2+}$ in the form of a double reciprocal plot (Fig. 4). The data are plotted during the fluorescence quenching of the tryptophan emission which accompanies cofactor binding (14). The data are plotted in the form of a double reciprocal plot (Fig. 5A). The dissociation constant obtained is 0.49 PM for the Mn$^{2+}$-TPP complex to pyruvate oxidase. The concentrations of TPP and ferricyanide were 10 mM and 50 mM, respectively. The inset in B shows the dependence of the slope (○) and intercept (∆) upon pyruvate $^{-1}$.

**EPR and Fluorescence Experiments**—In order to facilitate the interpretation of the steady state kinetic experiments, various metal-ligand dissociation constants were determined by EPR spectroscopy. The intensity of the Mn$^{2+}$ EPR spectra was utilized as a measure of the uncomplexed Mn$^{2+}$. Thus, by adding increasing concentrations of TPP to an aqueous solution of Mn$^{2+}$ in 0.05 M Pipes buffer, pH 6.0, and monitoring the decrease in the intensity of the Mn$^{2+}$ EPR spectra, a dissociation constant of 0.5 mM was determined for the Mn$^{2+}$-TPP complex. This compares favorably with the value of 0.2 mM in the literature for the dissociation constant of Mn$^{2+}$-TPP in 0.1 M Tris buffer, pH 7.2 (13). Control experiments in distilled water showed no discernible interaction between Mn$^{2+}$ and Pipes at the concentrations used in these experiments (see “Experimental Procedures”). A solution containing 10% free Mn$^{2+}$ and 90% Mn$^{2+}$-TPP complex was then titrated with increasing concentrations of Mg$^{2+}$. The resulting increase in the intensity of the Mn$^{2+}$ EPR spectra was used to monitor Mg$^{2+}$-TPP binding, and a dissociation constant of 5 mM was determined for the Mg$^{2+}$-TPP complex.

The strong binding between Mn$^{2+}$ and TPP makes it possible to do both kinetic and binding experiments under conditions where virtually all the TPP exists as a metal-TPP complex. Both the dissociation constant and Michaelis constant for TPP were determined in the presence of 50 mM Mn$^{2+}$ (Fig. 5). A Michaelis constant for the Mn$^{2+}$-TPP complex determined in this way is 0.88 μM. The dissociation constant of Mn$^{2+}$-TPP with pyruvate oxidase was determined by measuring the fluorescence quenching of the tryptophan emission which accompanies cofactor binding (14). The data are plotted in the form of a double reciprocal plot (Fig. 5B). The dissociation constant obtained is 0.49 PM for the Mn$^{2+}$-TPP complex to pyruvate oxidase. The close agreement between the dissociation constant and the Michaelis constant for TPP is consistent with earlier studies performed in the presence of Mg$^{2+}$ (14).

Fluorescence quenching of pyruvate oxidase was also used to determine the stoichiometry of Mn$^{2+}$ binding to the enzyme.
should bind to the enzyme in the form of a Mn$^{2+}$-TPP complex. Fig. 6 demonstrates a linear quenching curve up to a point where 0.8 Mn$^{2+}$ has been added per pyruvate oxidase subunit. Further additions of MnCl$_2$ have no effect on the fluorescence emission from pyruvate oxidase. These results strongly indicate the existence of one metal binding site per pyruvate oxidase subunit. Previously reported results have indicated one TPP binding site per pyruvate oxidase subunit (14).

It has already been demonstrated that TPP does not bind to the enzyme in the absence of a divalent cation (14). EPR experiments clearly demonstrate that Mn$^{2+}$ will not bind to pyruvate oxidase in the absence of the cofactor TPP. All experiments were performed in the presence of 0.1 M Pipes buffer, pH 5.7. The results (Table III) indicate that pyruvate oxidase has a substantial effect on the signal intensity from Mn$^{2+}$ only in the presence of TPP. Little if any interaction between the metal and the enzyme is observed in the absence of the cofactor.

When the survey experiments were being conducted, it was observed that the colored cations (Ni$^{2+}$, Co$^{2+}$, Cu$^{2+}$, and Cr$^{3+}$) all exhibited subtle changes in their visible spectra when mixed with pyruvate. This observation indicated the possibility of an interaction between the metal cations and pyruvate.

EPR experiments identical to those described above were performed with pyruvate instead of TPP, and indeed, at high concentrations of pyruvate, a Mn$^{2+}$-pyruvate complex was detected. Titration with various concentrations of pyruvate yielded a dissociation constant of 71 mM for the Mn$^{2+}$-pyruvate complex. Addition of Mg$^{2+}$ up to 100 mM did not displace Mn$^{2+}$ from the Mn$^{2+}$-pyruvate complex. Therefore, under the conditions of the steady state assay, it can be assumed that no complex between Mg$^{2+}$ and pyruvate exists. The data derived from EPR are compared to the steady state Michaelis constants in Table IV.

**DISCUSSION**

Pyruvate oxidase displays a lack of specificity for the metal cation required for enzymatic activity. The group of metal cations that will replace Mg$^{2+}$ in the assay differ sufficiently in their properties to suggest that perhaps the metal cation is not directly involved in the catalytic process. Its function, then, could perhaps be that of binding to the TPP cofactor. The resulting metal-cofactor complex would then be the actual cofactor. This rather passive role for the metal cation is quite common in enzymatic reactions in which nucleotides or triphosphates participate. Alternatively, the metal cation could bind directly to the enzyme and promote subsequent TPP binding or act as an essential activator of the enzyme.

The experiments presented in this paper were performed to distinguish between these alternatives. All the data are consistent with the proposition that the true cofactor is a metal-TPP complex and indicate no other role for the metal. The steady state kinetics data are described using the equations derived under "Appendix," based on the model of the metal-TPP complex serving as the cofactor. The family of curves (e.g. Figs. 1–3) are consistent with Equations 4 and 8 (see "Appendix"). Various other schemes (Segal (15) lists seven such schemes) all lead to initial velocity equations that are more complex than Equation 4 and do not fit the data.

The only complication arises when steady state kinetics are performed with variable concentrations of Mn$^{2+}$ and pyruvate. At low Mn$^{2+}$ concentrations, pyruvate acts as a substrate inhibitor (Fig. 4) and effectively removes free Mn$^{2+}$ from the solution. EPR demonstrates that, in fact, Mn$^{2+}$ can form a complex with pyruvate with a dissociation constant of 71 mM. The apparent inhibitory effect of pyruvate can thus be explained and the kinetics data are consistent with Equation 8 (see "Appendix"). Equation 8 predicts the observed nonlinear behavior for the plots of velocity$^{-1}$ versus pyruvate$^{-1}$ at changing fixed levels of Mn$^{2+}$. Furthermore, Equation 8 predicts

![Graph](image)

**TABLE III**

| Sample                     | Relative signal intensity |
|----------------------------|---------------------------|
| 100 $\mu$M Mn$^{2+}$       | 1.00                      |
| 100 $\mu$M Mn$^{2+}$ + 78 $\mu$M pyruvate oxidase | 0.94                      |
| 100 $\mu$M Mn$^{2+}$ + 100 $\mu$M TPP          | 0.85                      |
| 100 $\mu$M Mn$^{2+}$ + 100 $\mu$M TPP + 78 $\mu$M pyruvate oxidase | 0.41                      |

**TABLE IV**

| Kinetic constant                  | EPR experiment | Fluorescence experiment | Steady state kinetics |
|-----------------------------------|----------------|-------------------------|-----------------------|
| Dissociation constant Mn$^{2+}$ and pyruvate | 71 mM         | 64 mM                   | 68 mM                 |
| Dissociation constant Mn$^{2+}$ and TPP       | 0.5 mM        | 75 mM                   |                       |
| Dissociation constant Mg$^{2+}$ and TPP      | 3 mM          | 83 $\mu$M               |                       |
| Dissociation constant Mn$^{2+}$-TPP and pyruvate oxidase | 0.49 $\mu$M | 88 $\mu$M               |                       |
| Michaelis constant Mg$^{2+}$-TPP            | 0.61 $\mu$M   | 1.0 $\mu$M              |                       |
| Michaelis constant Mn$^{2+}$-TPP            | (1.04 $\mu$M)$^a$ | 0.61 $\mu$M            |                       |

$^a$ Obtained with a saturating value of TPP by varying the concentration of Mn$^{2+}$.

$^b$ Obtained by analysis using Equation 4 under Appendix. Details are given in the text.
that plots of velocity ^ -1 versus manganese ^ -1 at changing fixed levels of pyruvate will consist of a family of straight lines that do not have a common interception point. Equation 8 also predicts that secondary plots of the slope versus pyruvate ^ -1 will be nonlinear and that secondary plots of the intercept versus pyruvate ^ -1 will be linear. This is in fact what is observed and, thus, the proposed scheme explains the unusual nature of the data.

The K, (Mn^2+-pyruvate dissociation constant) values calculated from unactivated, dodecyl sulfate-activated, and phosphatidylglycerol-activated pyruvate oxidase are listed in Table IV, along with the value obtained from the EPR titration. These calculations are based on Equation 8 (see "Appendix"). The close agreement in these values indicates that the proposed explanation for the Mn^2+ versus pyruvate kinetics is probably valid. The fact that no Mg^2+-pyruvate complex could be detected by EPR spectroscopy is consistent with the kinetic behavior observed between Mg^2+ and pyruvate.

The EPR experiments also provided values for the dissociation constants of Mg^2+-TPP and Mn^2+-TPP. Consideration of these values and the various concentrations of Mg^2+, Mn^2+, and TPP used in the steady state experiments indicates that the concentration of the metal-TPP complex is usually well below that of the free metal or free TPP concentration.

The values for the metal-TPP dissociation constants also provide the information necessary to calculate the actual Michaelis constants for the metal-TPP complexes. It is evident from Equation 4 (see "Appendix") that the slopes of the secondary plots in Figs. 1-3 are (K/KM+-TPP)/Vmax. Since Vmax is known from the primary plot and the K, values are known from the EPR experiments, KM+-TPP can be calculated. The results of these calculations are also shown in Table IV. These calculations are based on data collected using Pipes buffer, since phosphate buffer can bind Mg^2+.

Table IV that the Michaelis constants for the metal-TPP ccmplexes. It is evident constant, V,,;,, is known from the primary plot and the K, values are available from the data.

Relevant Steady State Kinetics Equations

The observed kinetic behavior is easily explained by the proposal that only the Mg^2+-TPP complex binds to the enzyme and functions as a cofactor. The initial velocity, v, can be expressed simply as:

\[ v = \frac{V_{\text{max}}[\text{Mg}^{2+}\text{TPP}]}{K_{M_{\text{Mg}^{2+}}\text{TPP}} + [\text{Mg}^{2+}\text{TPP}]} \]  

where Vmax is the maximum velocity, [Mg^{2+}.TPP] is the concentration of metal-TPP complex, KM_{Mg^{2+}TPP} is the apparent Michaelis constant for the metal-TPP complex, and the concentrations of pyruvate and ferricyanide are assumed to be saturating. The equilibrium between Me^{2+} and TPP is represented as:

\[ K' = \frac{[\text{Me}^{2+}][\text{TPP}]}{[\text{Mg}^{2+}\text{TPP}]} \]  

where [Me^{2+}] and [TPP] represent the concentrations of free metal cation and free TPP, respectively. Substitution of Equation 2 into Equation 1 yields an expression relating the initial velocity to the concentration of free Me^{2+} and TPP.

\[ v = \frac{V_{\text{max}}[\text{Me}^{2+}][\text{TPP}]}{K'(K_{M_{\text{Me}^{2+}}\text{TPP}} + [\text{Me}^{2+}] + [\text{TPP}] + [\text{Me}^{2+}][\text{TPP}])} \]  

or

\[ \frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{K'K_{M_{\text{Me}^{2+}}\text{TPP}}}{V_{\text{max}}[\text{Me}^{2+}] + [\text{TPP}]} \]  

Equation 4 is consistent with the data obtained in Figs. 1-3. Some caution must be observed in adopting this equation, however, since the concentrations of free Me^{2+} or TPP may not necessarily be approximated by the total concentrations of Me^{2+} or TPP. If this situation prevails, then Equation 4 will become more complex but will remain symmetrical with respect to the total concentrations of Me^{2+} and TPP.

EPR evidence indicates that Mn^{2+} can form a complex with pyruvate. Assuming 1) that pyruvate and Mn^{2+}-TPP combine with the oxidase in a random fashion and 2) that a Mn^{2+}-pyruvate complex that forms does not bind to the enzyme. The initial velocity is given by:

\[ \frac{V_{\text{max}}}{v} = \frac{[\text{Mn}^{2+}\text{TPP}][\text{pyruvate}]}{K_{M_{\text{Mn}^{2+}}\text{TPP}}[\text{pyruvate}] + [\text{Mn}^{2+}\text{TPP}] + [\text{pyruvate}][\text{Mn}^{2+}\text{TPP}]} \]  

where [P] and K, are the free pyruvate concentration and dissociation constant with the enzyme. This equation assumes rapid equilibrium of both pyruvate and the metal-TPP complex, but the final form of the equation is not dependent on this assumption. Define the dissociation constant, K, as:

\[ K_{o} = \frac{[\text{Mn}^{2+}][\text{pyruvate}]}{[\text{Mn}^{2+}\text{TPP}]} \]  

If the amount of pyruvate bound to metal is negligible:

\[ [\text{Me}^{2+}] = \frac{[\text{Mn}^{2+}]}{1 + [\text{pyruvate}]} \]  

where the total metal ion concentration is [Me^{2+}]_{tot}. Substituting equations 2 and 7 into 5 yields the following after rearranging:

\[ \frac{V_{\text{max}}}{v} = \frac{K_{o}K_{o}K_{\text{Mn}^{2+}\text{TPP}}(1 + [\text{pyruvate}])}{[\text{pyruvate}][\text{Mn}^{2+}\text{TPP}]} \]  

Equation 8 reduces to Equation 4 at saturating pyruvate ([P] \gg K,) and if pyruvate does not bind to the metal (K_{o} \gg [P]). All the steady state data with both Mg^{2+} and Mn^{2+} are consistent with Equation 8. The value of K, can be calculated from the kinetic data. Plotting velocity ^ -1 versus [Mg^{2+}]_{tot} yields a curve whose slope is given by:
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Taking the first derivative with respect to \([P]\) yields:

\[
\frac{d \text{slope}}{d[P]} = \frac{K_o K_{M_o^2} \cdot \text{TPP}}{V_{\text{max}} \cdot \text{TPP}} \left[ 1 + \frac{K_o}{[P]} \right] \left[ 1 + \frac{P}{K_o} \right]
\]

Setting this equal to zero, and solving for \(K_o\):

\[
K_o = \left( \frac{[P]_{\text{em}}}{K_p} \right)
\]

The value of \(K_o\) is obtained from the intercept secondary plot, and, thus, the value of \(K_o\) can be obtained if the value of \([P]\) is known where the change in slope of the slope secondary plot equals zero, i.e. at the minimum of the concave-up secondary plot (Fig. 4). Values obtained using this calculation are included in Table IV.

REFERENCES

1. Hager, L. F. (1957) J. Biol. Chem. 229, 251–263
2. Hager, L. P. (1957) J. Am. Chem. Soc. 79, 5575–5576
3. Williams, P. R., and Hager, L. P. (1966) Arch. Biochem. Biophys. 116, 168–175
4. Cunningham, C. C., and Hager, L. P. (1971) J. Biol. Chem. 246, 1575–1582
5. Cunningham, C. C., and Hager, L. P. (1971) J. Biol. Chem. 246, 1583–1589
6. Blake, R. II, Hager, L. P., and Gennis, R. B. (1978) J. Biol. Chem. 253, 1963–1971
7. Schrock, H. L., and Gennis, R. B. (1980) Biochim. Biophys. Acta 614, 225–229
8. Schrock, H. L., and Gennis, R. B. (1980) Biochim. Biophys. Acta 615, 10–18
9. Singer, T. F., and Pensky, J. (1952) J. Biol. Chem. 196, 375–388
10. Green, D. E., Herbert, D., and Subrahmanyan, V. (1941) J. Biol. Chem. 138, 327–339
11. Kochetov, G. A., Filippov, P. P., and Usmanov, R. A. (1969) Biochim. Biophys. Acta 434, 810
12. O’Brien, T. A., Schrock, H. L., Russell, P., Blake, R. II, and Gennis, R. B. (1976) Biochim. Biophys. Acta 452, 13–39
13. Grande, H. J., Houghton, R. L., and Veeger, C. (1973) Eur. J. Biochem. 37, 563–569
14. O’Brien, T. A., Blake, R. II, and Gennis, R. B. (1978) Biochemistry 16, 3105–3109
15. Segel, D. H. (1975) Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems, pp. 242–271, John Wiley and Sons, New York
16. Smith, R. M., and Alberty, R. A. (1956) J. Am. Chem. Soc. 78, 2376–2380