Yeast Inorganic Pyrophosphatase

III. KINETICS OF Ca\(^{2+}\) INHIBITION*

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SUMMARY

The magnesium ion-dependent hydrolysis of pyrophosphate catalyzed by yeast inorganic pyrophosphatase is markedly inhibited by calcium ion. The kinetics of inhibition have been measured and analyzed in terms of a model involving strong interactions between CaPPi and two metal-complexed forms of the enzyme, EMg and ECa. Inhibition constants for these interactions have been estimated by nonlinear regression and are compared with those obtained by equilibrium measurements. These results are discussed in terms of several theories for specificity in metal ion activation of catalysis.

Inorganic pyrophosphatase (pyrophosphate phosphohydrolase (EC 3.6.1.1)) from yeast provides several advantages for a study of divalent metal ion specificity (1-3) in hydrolytic and phosphoryl transfer reactions involving high energy phosphates. The reaction catalyzed by this enzyme involves a structurally simple substrate, pyrophosphate (PPi), for which stability constants for many metal complexes have been well defined (4). Moreover, the enzyme has been purified (5-7) in amounts sufficient for metal and ligand binding studies. Finally, the yeast enzyme exhibits a rather broad metal ion specificity (6) and hence lends itself to a comprehensive study of metal ion effects.

This communication presents a study of the kinetics of Ca\(^{2+}\) inhibition of the Mg\(^{2+}\)-activated reaction. Ca\(^{2+}\) was chosen because it is so effective an inhibitor that significant inhibition can be obtained at concentrations low enough that the state of the Mg\(^{2+}\)-PPi equilibrium system is not appreciably disturbed, considerably reducing the complexity of the kinetic analysis. Moreover, both Mg\(^{2+}\) and Ca\(^{2+}\) belong to the alkaline earth group (Group IIa) and thus form complexes predominantly through electrostatic interactions (1). These metals are relatively uncomplicated by the covalent bonding and crystal field effects which play an important role in the chemistry of the transition metals (8). In addition, it is probable that Mg\(^{2+}\) and Ca\(^{2+}\) both form complexes of the same octahedral geometry (9). Thus inhibition due to gross differences in the chemistry of the two ions can be assigned a minimal importance.

The preceding paper (10) presented the results of a detailed kinetic study of the Mg\(^{2+}\) activation of the yeast pyrophosphatase reaction. A kinetic mechanism was proposed in which Mg\(^{2+}\) is required to bind to the enzyme before pyrophosphate ligands can bind. According to this mechanism, inhibition by other metal ions could be due to either competition with free Mg\(^{2+}\) for the enzyme, or to formation of inhibitory complexes with PPi. Equilibrium measurements described in the first paper of this series (11) presented evidence for a very strong ternary complex between CaPPi and a Ca\(^{2+}\)-enzyme (ECa) binary complex. This finding suggests another possible mode of inhibition and has been considered in the analysis of the inhibition kinetics.

METHODS

With certain specific exceptions described below, materials and assay method were as described in the previous paper (10). The conditions for the rate measurements were 30°, 0.45 M Tris-HCl, pH 7.40 and ionic strength, 1.0 (KCl). EGTA\(^{1}\) was omitted from the assay solutions because of its high affinity for calcium ion (4).

CALCULATIONS

Equations relating the added concentrations of Ca\(^{2+}\), Mg\(^{2+}\), K\(^{+}\), and PPi to the equilibrium concentrations of the various ligand, metal, and metal-ligand species are extremely complex but the complexity of the system is greatly reduced if the equations are derived under the assumption that [MgPPi] \(\gg [Mg_2PPi]\).

Under this assumption, the following scheme can depict the important equilibria in the pH range of 6 to 9 for this system.

\[
\begin{align*}
K_{dx} & \xrightarrow{K_{xa}} \text{PPi}^- \\
K_{ha} & \xrightarrow{K_{hb}} \text{PPi}^- \\
K_{k} & \xrightarrow{K_{k}} \text{PPi}^- \\
K_{dx} & \xrightarrow{K_{xa}} \text{PPi}^- \\
K_{ha} & \xrightarrow{K_{hb}} \text{PPi}^- \\
K_{k} & \xrightarrow{K_{k}} \text{PPi}^- \\
\end{align*}
\]

\[\text{SCHEME 1}\]

\(^{1}\) The abbreviation used is: EGTA, ethylene glycol bis(\(\beta\)-aminoethyl ether)-N, N\(^{-}\)-tetraacetic acid.

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For the above scheme, $K$ represents $K^+$, $X$ can be either $Ca^{2+}$ or $Mg^{2+}$ and $K_{app}$ is defined as:

$$k_{app} = \frac{[X][PPi]}{[PPi]^t} = \frac{[X][PPi]^t - [PPi]^t}{[PPi]^t - [PPi]^t}$$

Under experimental conditions, $[K^+]$, $[PPi]$, and $[K^+]$ is therefore closely approximated by $[K^+]$. When this is the case,

$$k_{app} = \frac{[K^+]_t[PPi] + [K^+]_t[PPi]^t + [K^+]_t[PPi]^t}{[PPi]^t}$$

Literature values for the acid dissociation and $Mg^{2+}$ complex stability constants were shown in Fig. 1 of the previous paper (10); the corresponding values for the $Ca^{2+}$ complex stability constants are $K_{df}, 1.12 \times 10^{-6} M$ and $K_{df}, 5.02 \times 10^{-7} M$ (12). These values were used to calculate the pH-dependent (apparent) metal ion-PPi stability constants. At pH 7.40, and $0.60 M KCl$, $K_{app}^{Ca^{2+}}$, $1.37 \times 10^{-4} M$ and $K_{app}^{Mg^{2+}}$, $4.21 \times 10^{-4} M$.

When $Ca^{2+}$, $Mg^{2+}$, and $PPi$ are all present in the system, the following equations hold.

$$[PPi]^t = [PPi]_t + [MgPPi]_t + [CaPPi]_t$$

$$[Mg^{2+}]_t = [Mg^{2+}]_t + [MgPPi]_t$$

$$[Ca^{2+}]_t = [Ca^{2+}]_t + [CaPPi]_t$$

Hence,

$$[Mg^{2+}]_t = [Mg^{2+}]_t + [PPi]_t$$

$$[Ca^{2+}]_t = [Ca^{2+}]_t + [PPi]_t$$

Incorporating these expressions into the equation for $[PPi]_t$ yields the following equation:

$$[PPi]_t = [PPi]_t \left[ \frac{[Mg^{2+}]_t}{[PPi]_t} + \frac{[Ca^{2+}]_t}{[PPi]_t} \right]$$

This equation can be arranged into one of the form:

$$aY^2 + bY + cY + d = 0$$

$$Y = [PPi]_t$$

$$a = 1$$

$$b = \frac{[Mg^{2+}]_t + [Ca^{2+}]_t}{[PPi]_t} - [PPi]_t$$

$$c = \frac{[Mg^{2+}]_t}{[PPi]_t} + \frac{[Ca^{2+}]_t}{[PPi]_t}$$

$$d = -[PPi]_t \frac{[Mg^{2+}]_t + [Ca^{2+}]_t}{[PPi]_t}$$

This equation can be solved by the Newton-Raphson method (13) and the value for $[PPi]_t$ can be used to calculate the concentrations of the other equilibrium species using the equations for $[Mg^{2+}]_t$ and $[Ca^{2+}]_t$. A sample calculation for one experiment is given in Table I.

### RESULTS

Since all calculations were made under the assumption that $[MgPPi]_t \gg [CaPPi]_t$, care was taken to ensure that this condition would exist experimentally. Five combinations of $[MgCl_2]$ and $[PPi]_t$, were selected such that $MgPPi$ comprised only a small percentage of the total metal ion-PPi species (i.e. always less than 8% at zero $[CaCl_2]$). At each of these five combinations of $[MgCl_2]$ and $[PPi]_t$, the rate of enzyme-catalyzed hydrolysis of substrate was measured as a function of $[CaCl_2]$. In no case did $[CaCl_2]$ exceed $[MgCl_2]$; in most cases the highest $[CaCl_2]$ was approximately one-half that of $[MgCl_2]$.

If $[CaCl_2]$ is varied under conditions where $[CaPPi]_t$ is only a small fraction of $[PPi]_t$, then $[MgPPi]_t$, $[PPi]_t$, and $[Mg^{2+}]_t$ remain essentially constant. In addition, $[Ca^{2+}]_t$ and $[CaPPi]_t$ remain in constant ratio (let $Z = [CaPPi]_t/[CaPPi]_t$) as $[CaCl_2]$ is varied. However, as $[CaCl_2]$ begins to comprise a larger fraction of $[PPi]_t$, the approximations listed above no longer hold. An example of this can be seen in the calculations for a typical experiment, listed in Table I. At $[CaPPi]_t$ from 0 to 54 $\mu M$, $[MgPPi]_t$, $[PPi]_t$, and $[Mg^{2+}]_t$ as well as the value for $Z$ vary less than 4%. As $[CaPPi]_t$ approaches 54 $\mu M$, the variation is 20 to 30%.

The kinetic data for the experiment listed in Table I is displayed in Fig. 1 in the form of a l/v versus $[CaPPi]_t$ plot. Note that there are two distinct regions in this graph. By expanding the graph it can be seen that at relatively low $[CaPPi]_t$, l/v is a linear function of $[CaPPi]_t$. At higher $[CaPPi]_t$, the data deviate appreciably from linearity.

Qualitatively similar behavior was observed in the experiments run at other combinations of $[MgCl_2]$ and $[PPi]_t$. Representative results under two other sets of experimental conditions are shown in Fig. 2. Significant deviation from linearity usually did not occur until approximately 50 to 60% inhibition had been reached. These data are analyzed in terms of a model for calcium inhibition in the next section.

### Analysis of Data

The results obtained in the previously described equilibrium binding (11) and steady state kinetic (10) studies suggest Scheme 2 as a possible explanation for the kinetic behavior of $Ca^{2+}$ inhibition.
Fig. 1. Inhibition of PP₁ hydrolysis as a function of [CaPP₁]. Experimental conditions were described under “Methods,” with 1.0 × 10⁻⁵ M MgCl₂ and 2.0 × 10⁻⁵ M Na₄P₂O₇. ——, reciprocal velocity predicted from the kinetic analysis of the data (see text for discussion).

Fig. 2. Inhibition of PP₁ hydrolysis as a function of [CaPP₁]. Conditions: 5.0 × 10⁻⁴ M MgCl₂, 1.0 × 10⁻⁴ M Na₄P₂O₇ (▲) and 5.0 × 10⁻⁴ M MgCl₂, 5.3 × 10⁻⁵ M Na₄P₂O₇ (●). The lines have the same significance as in Fig. 1.

This model postulates that PP₁ ligands bind only to metal ion-complexed enzyme and that only the EMg(MgPP₁) complex is catalytically active. The rate law for this model, derived under a steady state assumption, is:

\[
\frac{1}{V} = \frac{1}{V_{\text{mpp}}} + \frac{[\text{CaPP₁}]K_{\text{mpp}}}{V_{\text{mpp}}[\text{MgPP₁}]} + \frac{1}{k_4} + \frac{K_m}{k_{[Mg^2+]}} + \frac{[\text{CaPP₁}]K_{\text{mpp}}}{V_{\text{mpp}}[\text{MgPP₁}]} + \frac{1}{k_4} + \frac{K_m}{k_{[Mg^2+]}}
\]

\[
\text{Equation 1}
\]

where \( V_{\text{mpp}} = k_1E_0 \), \( K_{\text{mpp}} = (k_{-4} + k_4)/k_5 \), \( K_{\text{mpp}} = k_{-4}/k_5 \), \( K_{\text{mpp}} = k_{-5}/k_6 \), \( K_{\text{mpp}} = k_{-5}/k_6 \), \( K_{\text{mpp}} = k_{-5}/k_6 \), \( K_{\text{mpp}} = k_{-5}/k_6 \), \( K_{\text{mpp}} = k_{-5}/k_6 \), and \( Z = [\text{CaPP₁}] / [\text{MgPP₁}] \).

For each set of experimental conditions (see Table II for the five different sets of [MgCl₂]₁ and [PP₁]₁), the slope of the initial linear region in each 1/v versus [CaPP₁] plot can be estimated and analyzed in terms of Equation 1. In this linear region of each graph, the calculated concentrations of Mg²⁺, MgPP₁, and PP₁ as well as the calculated value of Z, are essentially constant. Equation 1, then, predicts a linear relationship between 1/v and [CaPP₁] at concentrations of the latter which are low enough to make the term \( K_m[\text{CaPP₁}] / K_{\text{mpp}}[\text{MgPP₁}] \) negligible in comparison to \( (1/K_{\text{mpp}} + K_mZ/K_{\text{mpp}}) \). In this case, the slope of the linear region in each plot (designated slope₁, or the slope from the primary plots) is given by:

\[
\text{slopel} = \frac{1}{k_4} + \frac{K_m}{k_{[Mg^2+]}} + \frac{[\text{CaPP₁}]K_{\text{mpp}}}{V_{\text{mpp}}[\text{MgPP₁}]} + \frac{1}{k_4} + \frac{K_m}{k_{[Mg^2+]}}
\]

\[
\text{Equation 2}
\]

A secondary plot demonstrating the dependence of slope₁ (as estimated for each experiment) on the reciprocal of the constant [MgPP₁] in each experiment is shown in Fig. 3. From Equation 2, this observed linear relationship would be possible only if \( K_mZ/K_{\text{mpp}}[\text{MgPP₁}] \ll 1/K_{\text{mpp}} \), or, if the numerical value of the former term were fortuitously identical for each experiment. As can be seen in Table II, the ratio \( Z/[\text{MgPP₁}] \), and therefore the term \( K_mZ/K_{\text{mpp}}[\text{MgPP₁}] \) varies 20-fold under the five different sets of experimental conditions. Thus, in subsequent analysis, this term can be considered to be negligibly small in comparison to \( 1/K_{\text{mpp}} \).

Therefore, the slope of the secondary plot (slope₂) can be expressed as:

\[
\text{slopel} = \frac{k_{\text{mpp}}}{V_{\text{mpp}}} \left( \frac{1}{k_{\text{mpp}}} + \frac{K_m}{k_{[Mg^2+]}} \right)
\]

\[
\text{Equation 3}
\]

Since \( K_{\text{mpp}} \) and \( V_{\text{mpp}} \) have been estimated under these conditions (10), a value for \( K_{\text{mpp}} \), the dissociation constant for the interaction of CaPP₁ with EMg, can be calculated to be \( 1.2 \times 10^{-7} \) M.

The deviation from linearity observed in these experiments (see Figs. 1 and 2) can be predicted by Equation 1 when, due to
FIG. 3. Dependence of the slopes of the linear regions of the 1/v versus [CaPP_i] plots for five different combinations of [MgCl_2] and [PP_i] on the reciprocal of the MgPP_i concentration at which they were determined.

an increase in the CaPP_i concentration, the term $K_m$ (CaPP_i)-Z/$K_eK'_cpp$ ($Mg^{2+}$) becomes equal and greater in magnitude than 1/$K_{cpp}$. Since $V_{mapp}$, $K_{mapp}$, $K_m$, and $K_p$ were estimated earlier (10), it is possible to fit Equation 1 (excluding the term $K_eZ/K_e(Mg^{2+})$ as previously discussed) to the entire set of inhibition data (40 points) using nonlinear regression (14). This allows estimation of the product $K_eK'_cpp$ and refinement of the value for $K_{cpp}$. The numerical values for these parameters, as well as estimates of their standard deviation (14), are:

$$K_{cpp} = 1.0 \pm 0.2 \times 10^{-7} M$$

$$K_eK'_cpp = 1.4 \pm 0.5 \times 10^{-18} M^2$$

The multiple correlation statistic, $R^2$ (15), for the data fitting by nonlinear regression was 0.90, indicating reasonable fit. The major difficulty in fitting the data to Equation 1 occurred in the two experiments at lowest MgCl_2 concentration where a small, constant concentration of contaminating metal ion lowered the predicted rate. This experiment difficulty was encountered because EGTA could not be added to the assay mixtures to remove the traces of highly inhibitory metal ion (see the previous paper in this series (10) for discussion of this problem). The relationship between 1/v and (CaPP_i) as predicted by the nonlinear regression is shown by the lines in Figs. 1 and 2.

Several other simpler models were also tested as possible explanations of the inhibition kinetics. Models involving CaPP_i or Ca^{2+}, either alone or together, as inhibitors competitive with MgPP_i or Mg^{2+} gave a poor fit of the data; inclusion of CaPP_i binding by ECa was necessary to provide a good fit.

DISCUSSION

The equilibrium studies described in the first paper of this series (11) demonstrated a very tight, nonreactive complex between CaPP_i and the ECa binary complex. A model postulating the existence of such an inhibitory complex has been shown to provide a reasonably good fit to the inhibition data. Comparison of the kinetic value for the product $K_eK'_cpp$ with an upper limit of this same product derived from static measurements is given in Table III. These values agree easily within one order of magnitude which must be considered good for the product of two separate constants, each having been measured by different techniques in the equilibrium studies (11). This qualitative and quantitative agreement between the kinetic and static studies provides good evidence for the model outlined here.

Although this study compares the relative effects of only two metal ions, several important points can be made concerning the effect of the metal ion in this reaction. First, the metal ion chelated with the PP_i anion has a very large and critical effect on the rate of the hydrolytic reaction and a smaller but measurable effect on the binding of the chelate to the enzymes. Thus, while both CaPP_i and MgPP_i interact with EMg, the former binds much more strongly (compare $K_{mapp}$ and $K_{cpp}$ in Table III) but the latter is at least 10^5 times more reactive.

Second, in this particular system, the interaction of free metal ion with the enzyme is necessary for the binding of PP_i ligands. In the case of the binding of CaPP_i, the metal ion bound to the enzyme appears to make little difference (compare $K_{mapp}$ and $K_{cpp}$ in Table III).

The question concerning the astonishingly large differences in reactivity between the different metal ion-pyrophosphate chelates is a critical one in understanding the mechanisms of reactions of this type. This question has not been resolved; several possible chemical roles for the metal ion in the catalysis of polyphosphate hydrolysis have been postulated.

Hammes and Levison (16) have suggested that since the actual role for the metal ion may be involved in bond breaking, its efficiency might then be related to its ability to dissociate a proton from its innermost hydration shell. In this case, the metal ion would be acting as a general acid catalyst. They noted that since the reverse protolytic reaction is diffusion

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Kinetic determination</th>
<th>Static determination</th>
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</thead>
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<tr>
<td>$V_{mapp}$</td>
<td>1170 units/mg</td>
<td>200 units/mg</td>
</tr>
<tr>
<td>$V_{mcpp}$</td>
<td>6.3 x 10^-4 M</td>
<td>6.3 x 10^-4 M</td>
</tr>
<tr>
<td>$K_{cpp}$</td>
<td>7.9 x 10^-4 M</td>
<td>7.9 x 10^-4 M</td>
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<td>$K_{mapp}$</td>
<td>1.3 x 10^-3 M</td>
<td>1.3 x 10^-3 M</td>
</tr>
<tr>
<td>$K_{mcpp}$</td>
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<td>0.8 x 10^-4 M</td>
</tr>
<tr>
<td>$K'_cpp$</td>
<td>9.0 x 10^-4 M</td>
<td>9.0 x 10^-4 M</td>
</tr>
<tr>
<td>$K'_cpp$</td>
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<td>0.8 x 10^-8 M</td>
</tr>
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<td>7 x 10^-8 M</td>
</tr>
<tr>
<td>$K_{cpp}$</td>
<td>0.6 x 10^-10 M^2</td>
<td>0.6 x 10^-10 M^2</td>
</tr>
</tbody>
</table>
controlled and therefore roughly the same for all divalent metal ions, the catalytic efficiency would parallel the acid ionization constant. If this were the case, Mg$^{2+}$ would be expected to be a somewhat better catalyst than Ca$^{2+}$ since it is the more acidic (17).

In this last respect the smaller size of Mg$^{2+}$ than Ca$^{2+}$ is important. Mutual charge repulsion between incoming ligand groups plays an important role in the chemistry of Mg$^{2+}$ complexes, especially when these complexes involve binding of multidentate or multiple ligands (1). Because of this mutual charge repulsion, it is sometimes energetically more favorable for Mg$^{2+}$ to bind fewer than the total possible ligand groups and remain partially hydrated. Williams (1) suggested that in hydrolytic reactions such additional water molecules could be the source of specificity of Mg$^{2+}$ action as compared with Ca$^{2+}$.

Another possibility is metal ion assistance of the nucleophilic attack of water or some enzyme nucleophile on the phosphorous atom. This type of metal activation could be achieved in several ways: by shielding the negative charges on the pyrophosphate, thus allowing the nucleophile to approach more readily; by increasing the reactivity of the phosphorous atom itself by withdrawing electrons; and by making the leaving group a better leaving group (18, 19).

Because Mg$^{2+}$ has a higher charge density (i.e. a greater charge to radius ratio) (20) than Ca$^{2+}$, it is able to exert a relatively larger electrostatic effect on the pyrophosphate oxygens. This should enable it to more strongly activate the phosphorous atom toward nucleophilic attack. This difference in electron withdrawing capability was invoked to explain Mg$^{2+}$ activation and Ca$^{2+}$ inhibition of β-methyl aspartase (21).

In addition to these possible rationalizations for the differing abilities of Mg$^{2+}$ and Ca$^{2+}$ to activate the yeast pyrophosphatase, the geometry of the metal-PP complexes both in solution and on the enzyme surface may well play an important role in binding and catalysis (22).

It is of interest to note that Cohn (23) found that the exchange between $[^{18}O]$orthophosphate and water catalyzed by this same yeast pyrophosphatase is dependent on Mg$^{2+}$ for activity and inhibited by Ca$^{2+}$. Moreover, the results suggested that only MgPP, is able to undergo exchange. This is further evidence that a metal ion is required for activation of the phosphate group toward water substitution, but that a strict specificity for metal ion exists.

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