Yeast Inorganic Pyrophosphatase

II. KINETICS OF Mg2+ ACTIVATION*

Owen A. Moe and Larry G. Butler†

From the Department of Biochemistry, Purdue University, Lafayette, Indiana 47907

SUMMARY

The kinetics of the Mg2+ activation of crystalline yeast inorganic pyrophosphatase have been thoroughly investigated from pH 7.40 to pH 9.05 using a sensitive isotope assay in order to determine the role of the divalent metal ion activator in the reaction. A computer program has been devised for calculating the concentration of each of the various components of the complex equilibrium involving Mg2+ and inorganic pyrophosphate (PPi). The reaction rate was measured over a wide range of Mg2+ and PPi concentrations, and the concentration dependence of the measured rate was kinetically analyzed by a computerized algorithm for nonlinear regression. The computer analysis included the testing of several plausible kinetic models for goodness of fit to the data, and determination of best values of kinetic parameters for the various models.

The simplest kinetic model which provides a good fit to all of the data involves binding of free Mg2+ by the enzyme followed by binding of PPi ligands. Both MgPPi and Mg2PPi are substrates; at pH 7.40 the latter is hydrolyzed 22 % as rapidly as the former. Free PPi is bound but is not hydrolyzed at a significant rate.

On the basis of these kinetic studies we propose two roles for the metal ion in this reaction: activation of the enzyme to a form which binds substrate, and formation of substrate by complexing with PPi. These roles are entirely consistent with our previously reported equilibrium binding studies of this enzyme.

Inorganic pyrophosphatase (pyrophosphate phosphohydrolase (EC 3.6.1.1)) from bakers' yeast exhibits a strict requirement for added divalent metal ion. The relative catalytic efficiency of different metals that were tested is: Mg2+ > Zn2+ > Co2+ > Mn2+ (1, 2). Several other divalent metal ions (Cd2+, Ca2+, and Ni2+) that do not activate the enzyme (i.e. yield hydrolysis rates less than 0.01 % of that measured in the presence of Mg2+) can, nonetheless, act as inhibitors of the magnesium-dependent activity.

Early kinetic evidence with the yeast enzyme was qualitatively suggestive of a catalytic role for a magnesium-pprophosphate complex (3). An attempt was made to study the dependence of the reaction rate on [PPi] (1), but the data obtained did not follow normal Michaelis-Menten kinetics (i.e. 1/v versus 1/[PPi]). Plots were markedly nonlinear. Studies of the effect of pH on the enzyme activity showed that pH optimum was a function of the Mg2+ and PPi concentrations (1).

A rigorous kinetic analysis of the reaction catalyzed by this enzyme requires knowledge of the equilibrium concentrations of the different types of pyrophosphate, metal ion, and metal-pyrophosphate species as a function of total metal ion and pyrophosphate concentrations as well as pH. This report describes a thorough kinetic study of the concentration dependence of the magnesium-activated reaction, which was conducted in an attempt to deduce the nature and catalytic importance of possible enzyme-ligand complexes.

MATERIALS AND METHODS

Imidazole, crystalline and designated "Grade 1," and EGTA2 were obtained from Sigma Chemical Company. The source of all other materials including 32PPi and crystalline enzyme was described in the preceding publication (4). For the kinetic assays, the enzyme crystals were dissolved in appropriate buffer, followed by extensive dialysis versus several changes of buffer.

Conditions and Methods of Rate Measurement—All measurements of the rate of PPi hydrolysis were carried out at an ionic strength of unity and at 30°. Although the temperature at which the metal ion-PPi stability constants were measured was 25°, there is good evidence that there is a negligible temperature dependence of the constants from 25-30° (5, 6).

The progress of the hydrolytic reaction was followed by measuring the production of [32P]orthophosphate ([32P]) from [32P]-pyrophosphate ([32P]PPi). [32P]Pi was separated from [32P]PPi by selective extraction of a [32P]molybdate complex into an organic solvent using a modification (7) of the method of Martin and Doty (8). The assay solutions for a typical experiment consisted of buffer (Tris-HCl), KCl (used to adjust ionic strength to 1.0), EGTA (see below), MgCl2, and Na2HPO4 at appropriate concentrations. [32P]Pi was added to give from 0.5 to 2.5 million cpm per ml of assay solution.

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‡ J. Sperom and L. G. Butler, unpublished observations.
§ The abbreviation used is: EGTA, ethylene glycol bis(γ-aminoethyl ether) N,N'- tetraacetic acid.
Plots of $P_i$ formed versus time were strictly linear for initial velocity measurements (less than 10% hydrolysis). The arithmetic mean of the values and the average absolute deviation of these values from the mean were calculated. The latter parameter is displayed as error bars in the graphs in order to give some indication of the amount of variance in the measured values (usually 8 to 16 measurements per point). The average deviation from the mean usually ranged between 3 and 5% of the mean value.

During initial experiments with this system it was found that inhibition occurred at low $\text{MgCl}_2$ concentrations, presumably caused by contaminating metal ions in the assay solution, unless special care was taken to remove these contaminants. For this reason, the Tris buffer and $\text{KCl}$ stock solutions were extracted with 0.005% diphenylthiocarbazone in $\text{CCl}_4$ in order to reduce metal ion contamination (9, 10).

EGTA was also added to the assay solutions because its unique complexation properties allow it to reduce interference by extraneous metal ions. This chelator binds $\text{Mg}^{2+}$ very weakly ($K_{\text{diss}} = 7.8 \times 10^{-4}$ M at pH 7.40) while forming complexes with other metal ions approximately as strongly as does EDTA (11). At the concentration at which EGTA was present it exerted a negligible effect on the concentration of free $\text{Mg}^{2+}$.

The individual rate measurements were normalized to the following defined set of standard conditions: pH, 7.40; ionic strength, 1.0; temperature, 30$^\circ$C; and $\text{MgCl}_2$ and $P_P$ present at concentrations of $2.0 \times 10^{-3}$ M and $3.0 \times 10^{-4}$ M, respectively. The absolute rate measured under the standard conditions is 650 pmoles of $P_P$ hydrolyzed per min per mg of protein for the purest enzyme preparations. The rate of standard conditions was given a relative value of unity. This normalization procedure proved to be the simplest and most straightforward approach since many different enzyme preparations of slightly varying specific activity (580 to 650) were used.

Calculations—A schematic representation of the important equilibria involved when $\text{Mg}^{2+}$, $K^+$, and $P_P$ are present in solution in the pH range of 6 to 9 is shown in Fig. 1. Values for the constants governing the equilibria shown in this scheme were selected from the literature (11) and are listed in Table 1, for convenience in the graphing and analysis which follow, these concentration terms are expressed as the sums of

\[ \left[ P_P \right] = \left[ P_P^{-1} \right] + \left[ P_P^{-2} \right] + \left[ P_P^{-3} \right] + \left[ P_P^{-4} \right] 
\]

The conservation of mass equations for this system are:

\[ \left[ P_P \right] = \left[ P_P^{-1} \right] + \left[ P_P^{-2} \right] + \left[ P_P^{-3} \right] + \left[ P_P^{-4} \right] 
\]

The cubic equations can be solved for $\left[ P_P^{-4} \right]$ by the Newton-Raphson method (13) and the value obtained can be used to calculate the equilibrium concentrations of the other species. A computer program designed to carry out all necessary computations was written in Fortran IV and implemented on the Control Data Corporation 6500 Computer. At a given pH, the ratios of the different ionic forms of $\text{Mg}PP_i$ and $P_P$ are constant. Therefore, for convenience in the graphing and analysis which follow, these concentration terms are expressed as the sums of

\[ \left[ P_P^{-4} \right] = \left[ P_P^{-1} \right] + \left[ P_P^{-2} \right] + \left[ P_P^{-3} \right] + \left[ P_P^{-4} \right] 
\]

For all experimental conditions, $[K^+]_t \gg [P_P]_t$ and therefore, $[K^+]_t$ is a good approximation of $[K^+]_{\text{free}}$. Using this relationship, the equation for $[P_P]_t$ can be written as follows:

\[ [P_P]_t = [P_P^{-4}] \left( 1 + \frac{[H^+]_t}{K_{\text{ha}}} + \frac{[H^+]^2_t}{K_{\text{ha}^2}} \right) 
\]

\[ [K^+]_t = [K^+]_t 
\]

Combining these two equations yields a third equation of the form:

\[ aX^3 + bX^2 + cX + d = 0, \]

where

\[ a = \frac{1}{K_{\text{ma}}K_{\text{mc}}} \]
\[ b = \frac{2[P_P]_t - [Mg^{2+}]_t}{K_{\text{ma}}K_{\text{mc}}} \]
\[ c = \frac{[H^+]_t}{K_{\text{ma}}} + \frac{[H^+]^2_t}{K_{\text{ma}^2}} + \frac{[K^+]_t}{K_{\text{ka}}} + \frac{[H^+]_t[P_P]_t - [Mg^{2+}]_t}{K_{\text{ma}K_{\text{ma}^2}}} \]
\[ d = -[Mg^{2+}]_t \left( 1 + \frac{[H^+]_t}{K_{\text{ha}}} + \frac{[H^+]^2_t}{K_{\text{ha}^2}} + \frac{[K^+]_t}{K_{\text{ka}}} \right) \]

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their respective ionic forms:

\[ [\text{MgPPi}] = [\text{MgHPPi}^-] + [\text{MgPPi}^2] \]

\[ [\text{PPi}] = [\text{PPi}^-] + [\text{HPPi}^3] + [\text{H}_2\text{PPi}^4] + [\text{KPPi}^5]. \]

**RESULTS**

A comparison of the measured rate of PP\(_i\) hydrolysis with the calculated concentrations of the different equilibrium species at pH 7.40 is shown in Fig. 2 as a function of [Mg\(^{2+}\)] at constant [PP\(_i\)]. The rate profile is similar in appearance to the curve depicting [MgPP\(_i\)] variation except for the behavior at highest and lowest [MgCl\(_2\)]. As [MgCl\(_2\)] approaches its highest values, the reaction rate appears to level off, approaching a constant value as does [MgPP\(_i\)] rather than approaching zero, as does [MgPP\(_i\)]. As the [MgCl\(_2\)] drops from \(1 \times 10^{-3}\) M to \(1 \times 10^{-4}\) M, the rate of enzymatic hydrolysis shows a considerably sharper decrease in value than does [MgPP\(_i\)].

Fig. 3 presents the catalytic rate as function of [MgCl\(_2\)] at four different PP\(_i\) concentrations at pH 7.40. In each case it was found that the observed decrease in reaction rate at lower [MgCl\(_2\)] was greater than the corresponding decrease in [MgPP\(_i\)]. In one experiment (Fig. 3A, [PP\(_i\)] \(_t\) = \(2.6 \times 10^{-5}\) M) the rate decreased approximately 60% in a range of [MgCl\(_2\)] variation where [MgPP\(_i\)] showed no significant decrease. The shapes of such rate profiles at pH 8.10 and 9.05 (not shown) were found to be qualitatively very similar to pH 7.40.

Representative results of a different type of experiment are shown in Fig. 4. In these experiments, [PP\(_i\)]\(_t\) was varied at a constant [MgCl\(_2\)], with [Mg\(^{2+}\)] always much less than [MgCl\(_2\)]. In this situation, [Mg\(^{2+}\)] and the ratios of [MgPP\(_i\):[MgPP\(_i\)] and [PP\(_i\):[MgPP\(_i\)] are essentially constants. In each case linear \(1/v\) versus \(1/\text{[MgPP\(_i\)]}\) plots were observed, and \(V_{\text{app}}^\text{max}\) and \(K_{\text{app}}^\text{max}\) values were estimated for each graph. \(K_{\text{app}}^\text{app}\) was defined as slope/(ordinate intercept) and \(V_{\text{app}}^\text{app}\) as 1/(ordinate intercept). A summary of values from pH 7.40, 8.10, and 9.05 is given in Table I. As can be seen, both \(V_{\text{app}}^\text{max}\) and \(K_{\text{app}}^\text{max}\) vary greatly with [MgCl\(_2\)]. If MgPP\(_i\) were the only species affecting the...
rate of reaction, the \( V_{\text{app}} \) and \( K_{\text{app}} \) values at each pH should have been independent of [MgCl\(_2\)].

**Analysis of pH 7.40 Data**—It is apparent from a qualitative examination of the kinetic data presented above that several or all of the different types of equilibrium species (Mg\(^{2+}\), MgPP\(_i\), MgPP\(_i\), and PP\(_i\)) may affect the rate of the catalyzed reaction. Several models can be suggested as possible qualitative explanations for the observed kinetic behavior. The approach taken toward interpretation of this system has consisted of an analysis of the concentration dependence of the measured reaction rate in terms of the rate law derived for each model.

For systems where interdependent equilibria determine the concentrations of the species which affect the reaction rate, the concentration dependence of the rate can be analyzed readily by using data fitting procedures. An algorithm for nonlinear regression (14) written in the form of a computer program in Fortran IV (15) was chosen for data analysis.

The criteria used to test goodness of fit for any given model is \( R^2 \), the multiple correlation statistic (15), which measures the proportion of the total measured velocity about its mean which can be accounted for by the predicted velocity, for that particular model. As \( R^2 \) approaches 1.0, the observed and predicted velocity values become identical. More detailed discussions of the use of this algorithm in nonlinear parameter estimation (16, 17) and its application to enzyme kinetics (5, 7, 18) are available.

Several models which included, in varying combinations, roles for all of the different types of equilibrium species were tested for the ability of their respective rate laws to predict the observed kinetic behavior. The simplest model which was found to give a good fit to the experimental data (\( R^2 = 0.98 \)) is presented in Table II. In this model, the interaction of free Mg\(^{2+}\) with the enzyme is required for the binding of the pyrophosphate ligands. Both MgPP\(_i\) and MgPP\(_i\) are substrates and PP\(_i\) is an inhibitor. The estimated values for the kinetic parameters at pH 7.40 are given in Table III. The solid lines in Figs. 3 and 4 represent velocity values predicted by this analysis. No simpler models were able to provide a good fit to the data (i.e. the \( R^2 \) values were \( \leq 0.91 \)). Especially poor were those models which postulated only one reactive species (\( R^2 = 0.45 \) to 0.58).

Results of the previously described equilibrium studies (4) indicated that free Mg\(^{2+}\) is necessary for the binding of PP\(_i\) to the yeast enzyme. The kinetic model presented here provides support for this contention. Requirement of free Mg\(^{2+}\) for the binding of pyrophosphate ligands (consider MgPP\(_i\) for example) is reflected in the expression for the experimentally measured parameter (see Table I), \( K_{\text{pp}}^{\text{app}} \).

\[
K_{\text{pp}}^{\text{app}} = \frac{1 + [\text{Mg}^{2+}] \cdot [\text{Mg}^{2+}]}{1/K_{\text{pp}} + x/K_{\text{pp}} + y/K_{\text{pp}}}
\]

\[
x = \frac{[\text{MgPP}_i]}{[\text{MgPP}_i]} \quad \text{and} \quad y = \frac{[\text{PP}_i]}{[\text{PP}_i]}.
\]

\( x \) and \( y \) are constant for any given [MgCl\(_2\)], since [MgCl\(_2\)] \( \gg \) [PP\(_i\)].
TABLE II

\[
\begin{align*}
\text{Mg} + E & \xrightarrow[k_1]{k_{-1}} \text{MgE} \\
\text{MgE} + \text{PPi} & \xrightarrow[k_2]{k_{-2}} \text{MgE(PPi)} \\
\text{MgE} + \text{MgPPi} & \xrightarrow[k_3]{k_{-3}} \text{MgE(MgPPi)} \\
\text{MgE} + \text{MgPi} & \xrightarrow[k_4]{k_{-4}} \text{MgE(MgPi)} \\
\text{MgE(MgPPi)} & \xrightarrow[k_5]{k_{-5}} \text{MgE + products} \\
\text{MgE(MgPi)} & \xrightarrow[k_6]{k_{-6}} \text{MgE + products}
\end{align*}
\]

Rate Law

\[
v = \frac{V_{\text{mpp}}[\text{MgPPi}]/K_{\text{mpp}} + V_{\text{mapp}}[\text{MgPPi}]/K_{\text{mapp}}}{1 + [\text{MgPPi}]/K_{\text{mpp}} + [\text{MgPPi}]/K_{\text{mapp}} + [\text{PPi}]/K_{\text{pp}} + [\text{MgPi}]/[\text{Mg}^{2+}]}
\]

\[
V_{\text{mpp}} = k_3E_f \\
V_{\text{mapp}} = k_4E_f \\
K_{\text{mpp}} = (k_{-5} + k_3)/k_3 \\
K_{\text{mapp}} = (k_{-4} + k_4)/k_4 \\
K_{\text{pp}} = k_{-2}/k_2 \\
K_m = k_{-1}/k_1
\]

TABLE III

Computed values of kinetic parameters

| pH   | Data points | \( R^2 \) | \( V_{\text{mpp}} \) (relative) | \( V_{\text{mapp}} \) (relative) | \( K_{\text{mpp}} \) | \( K_{\text{mapp}} \) | \( K_{\text{pp}} \) | \( K_m \) | \( V_{\text{mapp}}/V_{\text{mpp}} \) |
|------|-------------|----------|-------------------------------|-------------------------------|-----------------|-----------------|-----------------|----------|----------------|
| 7.40 | 69          | 0.981    | 1.81                          | 0.406                         | 16.6            | 6.20            | 7.88            | 1.28     | 0.221           |
| 8.10 | 31          | 0.986    | 1.03                          | 0.272                         | 9.70            | 5.08            | 4.86            | 1.23     | 0.264           |
| 9.05 | 27          | 0.972    | 0.480                         | 0.207                         | 3.30            | 2.14            | 2.88            | 2.10     | 0.430           |

Models not requiring this role for free \( \text{Mg}^{2+} \) do not include the \( K_m/(\text{Mg}^{2+}) \) term in the numerator of the above expression and therefore cannot predict the dramatic increase in \( K_{\text{mpp}} \) that is experimentally observed at low concentration of free \( \text{Mg}^{2+} \) (see Fig. 5). The solid line in Fig. 5 represents the behavior predicted by the model postulated here. The dashed line was predicted by a model (\( R^2 = 0.91 \)) similar to the one in Table II except that the binding of the different \( \text{PPi} \) ligands did not require previous interaction of free metal ion with the enzyme. This observation clearly demonstrates the kinetic necessity for this role for free \( \text{Mg}^{2+} \).

Analysis of pH Dependence of Kinetic Parameters—The \( R^2 \) and kinetic parameter values which were obtained for the proposed model at the three pH values are given in Table III. The model provides an excellent fit to the data at all three pH values although definite differences are observed in the numerical values for the kinetic parameters. As the pH is raised, several trends become apparent. \( V_{\text{mpp}} \) and \( V_{\text{mapp}} \) both decrease but not to the same extent, as can be seen in the increasing \( V_{\text{mapp}}/V_{\text{mpp}} \) ratio. The constants describing the interaction of the pyrophosphate species with the enzyme (\( K_{\text{pp}} \), \( K_{\text{mapp}} \), and \( K_{\text{mapp}} \)) all decrease. \( K_m \) increases only slightly as the pH is raised.

Trial and error variation of the estimated parameter values revealed that the fit to the data was somewhat insensitive to the precise values of several parameters. Thus, 2- to 3-fold variation in some parameter values could occur without substantially affecting the fit to the data. Because of this problem, exact analysis of the effect of pH on the enzyme, or, estimation of the relative catalytic importance of the different ionic forms of \( \text{MgPPi} \) and \( \text{PPi} \) was not feasible. However, the large changes in the parameter values at pH 9.05 suggest significant differences in the enzyme structure from that at the lower pH.

DISCUSSION

Two limitations of this type of study should be noted. First, the model which has been selected was the simplest one to provide a good fit of the kinetic data. More complicated models cannot be excluded, however, on the basis of the observed data. Secondly, the equations derived for the various models were of the same form whether obtained under a steady state or rapid equilib-
solid and are true equilibrium constants or not, and the pathways to the concentration
What the kinetic study does provide is a quantitative descrip-
tion of the different stoichiometric enzyme-ligand complexes
EMg(PPi) for example, could conceivably be formed by either
random or ordered addition of hIg2f and PPi to the enzyme or,
alternatively, by direct addition of 1IgPPi to the enzyme.

Qualitative agreement of the kinetic results with the equilib-
rium assumption. Thus, it is not known if the kinetic constants
are true equilibrium constants or not, and the pathways to the
different enzyme complexes are not defined. The complex
EMg(PPi) for example, could conceivably be formed by either
random or ordered addition of Mg2+ and PPi to the enzyme or,
alternatively, by direct addition of MgPPi to the enzyme.

Thus, it is possible for the proposed kinetic model to provide a
good fit of the data using $K_m$ values which are reasonably close to
those determined by static methods.

The apparent reactivity of MgPPi warrants special considera-
tion. It could be conceptualized most simply as enzyme-cata-
yzed hydrolysis of this species at a rate approximately 4- to 5-
fold slower than that of the MgPPi species. Complexes of quite
different geometry proposed for these two species could be in-
voked to account for the rate difference (5). Alternatively,
EMg(MgPPi) could rearrange, either rapidly or in a partially
rate determining step, to form an EMg$_2$(Mg$_3$PPi) complex
which could react in a manner similar to EMg(MgPPi) except for some
alteration in catalytic efficiency. It is also possible that an
EMg$_3$(Mg$_4$PPi) complex could be formed through either ordered
or random addition of Mg2+ and MgPPi to EMg and not directly
involve the binding of the MgPPi species. The available data
do not distinguish between these possibilities.

Most of the features of the model proposed here for yeast
pyrophosphatase have previously been separately suggested for
pyrophosphatases from other sources: MgPPi as alternative
substrate (19); inhibition by free PPi (21); and activation by free
Mg2+ (20, 21).

During final preparation of this manuscript two reports on
the kinetics of yeast inorganic pyrophosphatase have appeared
(22, 23). One of these (23) differs on several major points from
what is reported here. Specifically, a model is proposed which
involves direct interaction of pyrophosphate species with the
free enzyme (i.e. prior activation by free Mg2+ is not required)
and it is suggested that free Mg2+ does not interact with the
enzyme (i.e. $K_m = \infty$). In contrast, the report of Rapoport
et al. (23) is in close qualitative and quantitative agreement with
our findings at lower Mg2+ concentrations. These authors pro-
pose as kinetically important the same enzyme-ligand complexes
as do we in this paper with the exception that MgPPi plays no
role in their model. As the relatively low MgCl2 concentrations
employed in their experiments the MgPPi species is unimportant.

The observation of a dual role for divalent metal ion in this rea-
tion raises the question of the nature of catalytic differences
between the metal ions bound only to the enzyme and those
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metal ion specificity for these two roles. A study of the inhibi-
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