ACTIVATION OF YEAST INORGANIC PYROPHOSPHATASE BY MAGNESIUM

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SUMMARY - An analysis of the kinetic mechanism of pyrophosphate hydrolysis by inorganic pyrophosphatase has been carried out with the highly purified enzyme from yeast. The initial velocity, as a function of free pyrophosphate and Mg^{2+} ion concentration over a wide reactant concentration range at pH 6.5 and 7.2 has been investigated. The involvement of three metal atoms in the formation of the active complex has been supported. The enzyme requires $MgPP_i$ as a substrate and free Mg^{2+} ion as an essential activator. Kinetic schemes for the enzymatic reaction at pH 6.5 and 7.2 are presented; the dissociation constants of intermediate complexes are given.

Yeast inorganic pyrophosphatase (E.C.3.6.1.1) requires a bivalent metal ion for its activity (1). The authors have previously reported (2,3) on the kinetics of pyrophosphate hydrolysis at pH 7.2 at a relatively high concentration of magnesium ions ($\geqslant 10^{-4}$M). It has been shown that in these conditions the enzyme interacts with one substrate molecule and one metal atom per active site. The present study of this reaction at pH 6.5 and 7.2 over a wide range of magnesium concentrations shows the role of the metal ions in the enzymic reaction to be much more complex and points to the metal ion interaction with the free enzyme.

MATERIALS AND METHODS - The inorganic pyrophosphatase was isolated from baker's yeast according to Kunitz (4) and purified by chromatography on DEAE Sephadex A-50. Its specific activity was estimated to be 1500 I.U./mg. The homogeneity

of the preparation was checked by electrophoresis, as described by Davis (5). The enzyme concentration was determined by absorbance at 280 nm; according to (1,6) the molar extinction was assumed to be 8.7×10^4 M^{-1} cm^{-1}. In order to obtain necessary concentrations of free forms of pyrophosphate and magnesium, their total concentrations were calculated using the values for $K_o = [Mg^{2+}][PP_i] / [MgPP_i]$ and $K'_o = [Mg^{2+}][MgPP_i] / [Mg_2PP_i]$ equal to 2.56×10^{-4}M and 1×10^{-2}M at pH 6.5 and to 1.07×10^{-4}M and 0.33×10^{-2}M at pH 7.2, respectively, according to (3). The enzymic reaction was carried out at 25° in 0.05 M imidazole-HCl buffer at pH 7.2 and 6.5. The ionic strength (μ=0.1) was maintained by adding NaCl. The enzyme concentration was varied in the 0.15×10^{-10} to 0.6×10^{-10}M range at pH 7.2 and in the 0.25×10^{-10} to 2×10^{-10} M range at pH 6.5.A linear dependence of the reaction rate on enzyme concentration was thereby found to exist in these conditions.

Kinetic measurements were made by following the liberation of P_i due to the hydrolysis of PP_i during the first 5-10% of the total reaction (3). The standard error of the calculated reciprocal velocity was only 2-3% of the average of the observed reciprocal velocity.

RESULTS AND DISCUSSION - Fig. 1 and 2 show the initial reaction rate as a function of free magnesium and pyrophosphate concentrations. It is seen in the figures that the reciprocal rate is linearly dependent on the reciprocal PP_i concentration, whereas the dependence of v^{-1} on $[Mg^{2+}]^{-1}$ is nonlinear and can be expressed by a polynomial. This means that the rate equation is, in the most general case, as follows:

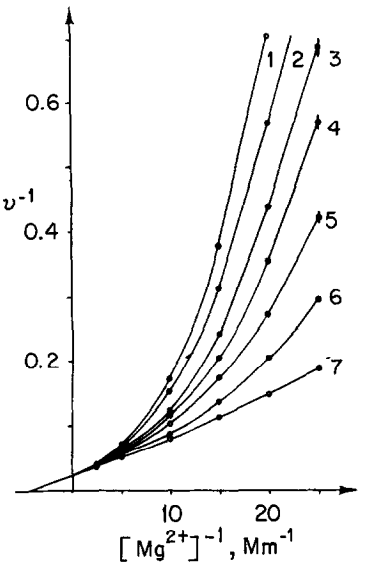


Fig.1. The initial velocity of pyrophosphate hydrolysis by yeast inorganic pyrophosphatase as a function of the free Mg^{2+} concentration at various fixed concentrations of pyrophosphate at pH 6.5. Free PP_i concentrations were: (1), 0.025 mM; (2), 0.033 mM; (3), 0.05 mM; (4), 0.067 mM; (5), 0.1 mM; (6), 0.2 mM; (7), 1.0 mM. Velocity is given in μmoles PP_i per min per nmole enzyme.

$$v^{-1} = \sum_{i=0}^{n} \frac{A_i}{[M]^i} + \frac{I}{[S]} \sum_{i=0}^{k} \frac{A'_i}{[M]^i} \qquad (I)$$

where S and M refer to pyrophosphate and magnesium, respectively, A_i and A'_i are constants.

The v^{-1} on $[Mg^{2+}]^{-1}$ dependence curves intercept at one point on the ordinate. Hence, $A'_0=0$. The values of other coefficients of Equation (I) were determined from the slopes and intercepts in Fig.1A and B. The dependence of intercepts and slopes on free Mg^{2+} concentration (Fig.2 C,D) were expressed by a polynomial of two and three terms, whose coefficients were also parameters of Equation (I) at n=2, k=3:

$$\text{Intercept} = \sum_{i=0}^{2} \frac{A_i}{[M]^i}, \quad \text{Slope} = \sum_{i=0}^{3} \frac{A'_i}{[M]^i}$$

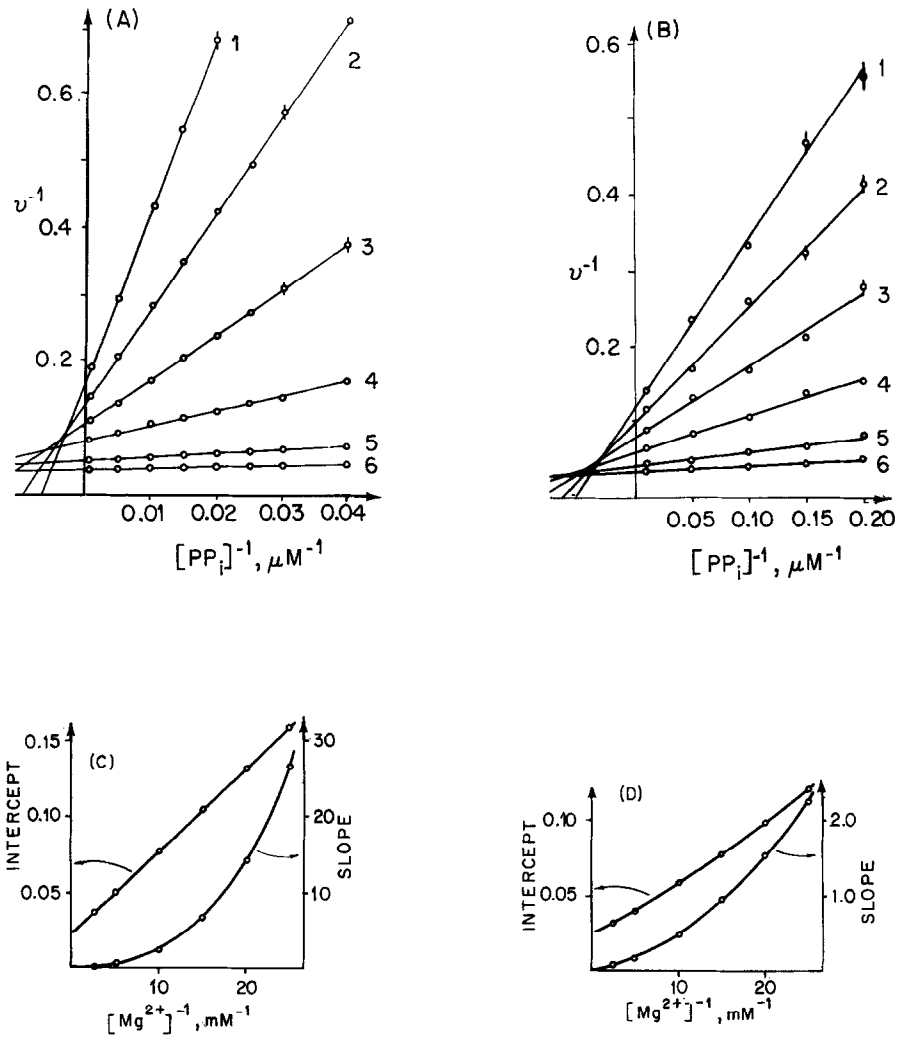
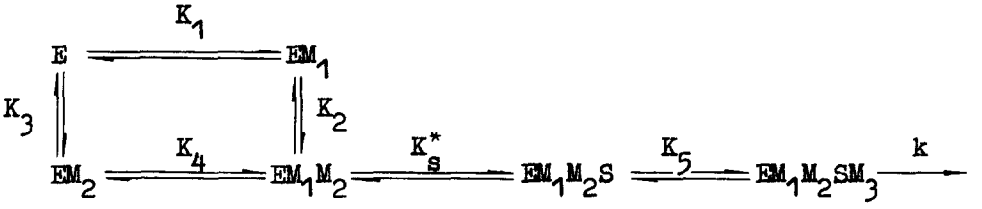


Fig.2. The effect of free $[Mg^{2+}]$ on the initial velocity pattern at varying free concentrations of pyrophosphate. Free Mg^{2+} concentrations were: (1), 0.04 mM; (2), 0.05 mM; (3), 0.067 mM; (4), 0.1 mM; (5), 0.2 mM; (6), 0.4 mM. A, a double reciprocal plot of the initial velocity data at pH 6.5; B- the same at pH 7.2; C and D, replots of intercepts and slopes of Fig.2A and B, respectively, versus reciprocal free Mg^{2+} concentration, Velocity is given in μmoles PP_i per min per nmole enzyme.

The values of A_i, A_i', which were determined from least squares fitting of the data to Equation (I) are given in Table I.

The analysis of the results reveals the active complex to contain at least three magnesium atoms and a pyrophosphate molecule ($EM_1M_2SM_3$). This is true if one assumes that the equilibrium mechanism of the active complex formation is a rapid one.

The comparative evaluation of the coefficients in Equation (I) points to the kinetic scheme of the reaction. The A_0' coefficient being equal to zero, serves as evidence that no intermediate complex $EM_1M_2M_3$ is formed, that is one of magnesium atoms is added only after the pyrophosphate molecule has been bound. It might be suggested that this magnesium atom promotes the formation of a pyrophosphate complex capable of undergoing enzymic hydrolysis. The zero values of coefficients A_2 and A_3 in Equation (1) for the reaction rate dependence at pH 6.5, indicate the absence of the intermediate complexes ESM and ES, or show that the reaction proceeds through complex EM_1M_2, according to Scheme I:

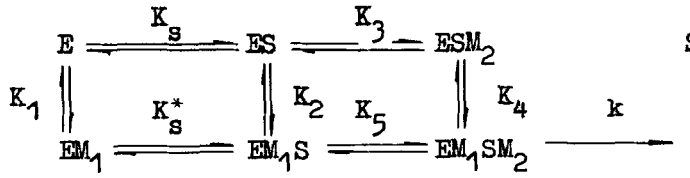


Scheme 1

Hence, the equation for the reaction rate at pH 6.5 can be written as:

$$\frac{I}{v} = \frac{I}{V}\left\{1 + \frac{K_5}{[M]}\right\} + \frac{I}{V[S]}\left\{\frac{K_s^* K_5}{[M]} + \frac{K_s^* K_5 (K_2+K_4)}{[M]^2} + \frac{K_s^* K_5 K_1 K_2}{[M]^3}\right\} \quad (2)$$

A comparison of A_i, A_i' coefficients in the rate equation at pH 7.2 leads to Scheme 2 and the corresponding expression for the reaction rate (3).



Scheme 2

$$\frac{I}{v} = \frac{I}{V}\left\{I + \frac{K_4+K_5}{[M]} + \frac{K_3K_4}{[M]^2}\right\} + \frac{I}{V[S]}\left\{\frac{K^*_sK_5}{[M]} + \frac{K^*_sK_5K_1}{[M]^2}\right\} \quad (3)$$

The appearance of the enzymic interaction with two magnesium atoms at pH 7.2 seems to be due to one of the enzymatic complexes being more stable at this pH. Thus, even at low concentrations of Mg^{2+} the enzyme is completely coordinated at one of the binding sites. It should be noted that the kinetic study of this reaction at pH 5.6 substantiates the participation of three metal atoms in the formation of the active complex (7).

The parameters of Equations (2) and (3), obtained from the numerical values of A_i and A'_i (Table I) are given in Table II. When calculating the dissociation constants of enzyme-magnesium complexes at pH 6.5, two extreme cases for the K_2 and K_4 ratio were considered, namely: 1) $K_2 \gg K_4$ and 2) $K_2 = K_4$. Some reaction parameters were calculated at pH 7.2 by assuming $K_3 = K_5$. Indeed, if the last metal ion being added (see Scheme 2) is bonded only to pyrophosphate in the active complex, it can be logically deduced that the values of K_3 and K_5 equal K_o.

The results obtained reveal the following regularity. The magnesium ion interaction with free enzyme enables or considerably enhances pyrophosphate addition. On the other hand, the PP_i bonding makes the coordination of the enzyme with Mg^{2+} much stronger. These effects can be due either to additional contacts in the enzyme-pyrophosphate-metal ions

Table I - Values of Parameters of Equation (I) at n=2, k=3 (V and [S] are expressed as shown in Fig.2 and [M] is given in 0.1mM)

pH	A_o	A_1	A_2	A'_o	A'_1	A'_2	A'_3
6.5	0.024	0.054	0.000	0.00	0.40	0.60	1.40
7.2	0.024	0.032	0.002	0.00	0.24	0.264	0.00

Table II - Values of Kinetic Parameters at pH 6.5 and 7.2 (0.05 M imidazole - HCl, NaCl, μ = 0.1, 25°)

pH 6.5, Scheme 1, Eq. (3)		pH 7.2, Scheme 2, Eq. (4)	
Constant	Value	Constant	Value
V	42 $\frac{\mu\text{mole } PP_i}{\text{nmole E min}}$	V	42 $\frac{\mu\text{mole } PP_i}{\text{nmole E min}}$
K_1	(2.3-4.7) x 10^{-4}M	K_1	1.1 x 10^{-4}M
K_2	(0.75-1.5)x 10^{-4}M	K_S	1.1 x 10^{-4}M
K_3	$\geqslant$ 4.7 x 10^{-4} M	K_2	0.8 x 10^{-5} M
K_4	$\leqslant$ 0.75 x 10^{-4}M	K_3	1.2 x 10^{-4} M
K_S^*	0.74 x 10^{-5} M	K_4	0.8 x 10^{-5} M
K_5	2.2 x 10^{-4} M	K_S^*	0.8 x 10^{-5} M
		K_5	1.2 x 10^{-4} M

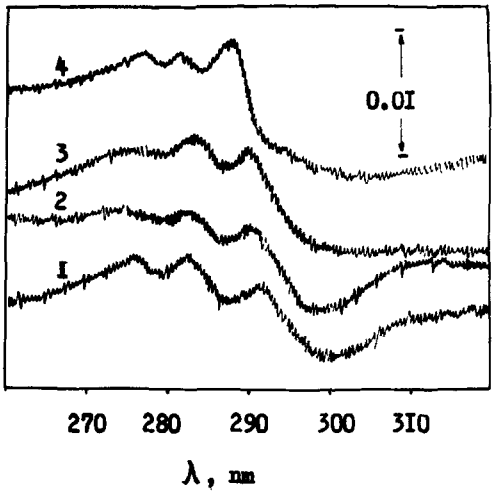


Fig.3. The ultraviolet difference spectra of inorganic pyrophosphatase with bivalent metal ions. Both the sample and reference cell (20°) contained 0.07 M cholamine chloride-HCl (pH 6.5) and 0.5 to 0.9 x 10^{-5}M enzyme. The total concentrations of cations in the sample were: (1), 3 mM Mg^{2+}; (2), 0.44 mM Mn^{2+}; (3), 1 mM Ca^{2+}; (4), 0.05 mM Zn^{2+}. The difference spectra were obtained on a Cary 15 spectrophotometer using an 0,1 absorbance scale.

system, or to conformational changes in the protein molecule on enzyme interaction with the metal or the substrate.

The spectrophotometric study also substantiates the binding of metal ions to the enzyme in the absence of the substrate (see Fig.3) and points to a great increase in the PP_i association constant when the enzyme is saturated with cations. The data on stoichiometry and binding constants will be published elsewhere.

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