THE INTERACTION OF YEAST INORGANIC PYROPHOSPHATASE WITH BIVALENT CATIONS AND PYROPHOSPHATE

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1. Introduction

Inorganic pyrophosphatase from yeast (EC 3.6.1.1) requires a bivalent metal ion for its activity [1]. A kinetic study of the enzymic hydrolysis of inorganic pyrophosphate (PP_i) in the presence of magnesium has shown that one molecule of PP_i and three atoms of magnesium are involved in the reaction. The metal ions activate both the substrate and the enzyme [2–6]. In the present paper difference spectroscopy and equilibrium dialysis were used to measure the metal and substrate binding properties. The enzyme has been demonstrated to interact with metal in the absence of the substrate and to increase the affinity for the substrate when it is saturated with cations.

2. Materials and methods

Inorganic pyrophosphatase was isolated from yeast and assayed as described in [2, 5, 6]. The enzyme with a specific activity of 1200 I.U./mg was used and corrections for a decrease in the concentration of the active enzyme were made. Imidazole (Reanal), Pipes (Piperazine-*NN'*-bis-2-ethane-sulphonic acid, BDH) and cholamine chloride, obtained as described in [7] were used to prepare buffer solutions. Stock solutions of $CaCl_2$, $MgSO_4$, $MnCl_2$ and $ZnCl_2$ (reagent grade or analytical grade) were standardized by EDTA titration [8].

The UV difference spectra were determined at 20° on a Cary Model 15 spectrophotometer with a 0 to 0.1 absorbance scale and cells of 1 cm light path. The spectra did not change during 30 min of incubation at 20°. The experiments with Mg^{2+}, Ca^{2+} and PP_i were carried out in 50 mM imidazole-HCl buffer and those with Zn^{2+} and Mn^{2+} in 70 mM cholamine chloride–HCl buffer. The ionic strength ($I = 0.1$) was maintained by adding NaCl. In the study of the "ternary" complex formation, the total concentrations of calcium and pyrophosphate were calculated using the values for $K_0 = [CaPP_i]/[Ca^{2+}]\,[PP_i]$ equal to $0.16 \times 10^4\ M^{-1}$ at pH 6.5 and to $0.44 \times 10^4\ M^{-1}$ at pH 7.2 according to [2, 9]. The spectral data were treated by a linear least squares program.

Equilibrium dialysis of 0.1–0.5 ml of the enzyme solution was made against a 10–100-fold volume of 5 mM Pipes-NaOH buffer at 4° for 12–60 hr. The content of calcium and magnesium was estimated in a Hitachi-207 absorption spectrophotometer.

3. Results and discussion

Addition to inorganic pyrophosphatase of Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+} and inorganic pyrophosphate results in the formation of complexes and the appearance of difference spectra, some of which are demonstrated in fig. 1. The maxima and minima in these spectra are shown in table 1. The molar extinction differences and the association constants for the metals and substrate (tables 1 and 2) were estimated by spectral titration of the enzyme with the Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+} ions (fig. 2), and inorganic pyrophosphate (fig. 3). These constants were used to show that the metal binding is competitive. To this end, to the enzyme–metal complex, EM_1, the ions of M_2 were added in excess, so as to meet the inequality: $K_{M_2}[M_2] \gg K_{M_1}[M_1]$. The absence of spectral changes or the replacement of the EM_1 spectrum by EM_2 (see for example fig. 1, curves

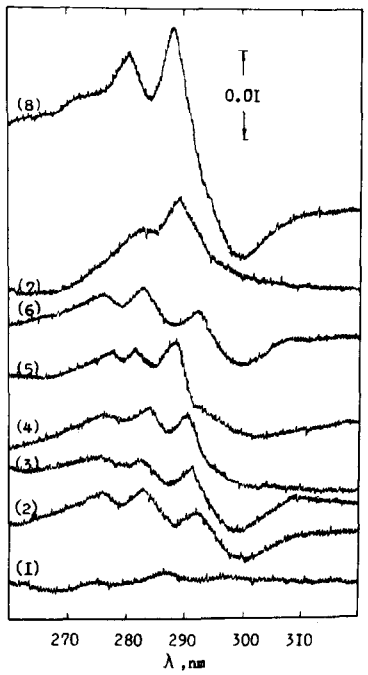


Fig. 1. UV difference spectra of the complexes of the enzyme with bivalent cations and pyrophosphate. Spectrum (1) is a typical base line. Spectra (2–8) were taken at pH 6.5 for the sample solutions containing the following concentrations of the reagents: (2) 3 mM Mg^{2+}; (3) 0.44 mM Mn^{2+}; (4) 1 mM Ca^{2+}; (5) 0.05 mM Zn^{2+}; (6) 0.1 mM Zn^{2+} and 2 mM Mg^{2+}; (7) 5.3 mM PP_i; (8) 1.04 mM Ca^{2+} and 0.01 mM PP_i. The concentration of the enzyme in sample and reference solutions was 5–9 μM.

5, 6) allowed the conclusion to be made that Zn^{2+}, Mn^{2+}, Ca^{2+} and Mg^{2+} bind with the same sites of the enzyme. On the other hand, the spectra of the various enzyme–metal complexes differ from one another in the presence of a minimum at 300 nm, in the position of their maxima (for example, λ_3) and in the amplitude of the peaks (fig. 1, curves 2–5; table 1). These facts could be due to a metal-specific difference in the structure of the various enzyme–metal complexes.

It is significant that the spectrum of the "ternary" enzyme–pyrophosphate–calcium complex is not the sum of the spectra of the enzyme–calcium and enzyme–pyrophosphate complexes. Indeed, i) in the "ternary" complex spectrum there is a minimum at 300 nm which is not present in the "binary" complex spectra (fig. 1, curves 4, 7, 8; table 1); ii) the changes in the molar extinction upon PP_i or Ca^{2+} binding to the corresponding "binary" complexes, exceed by far the changes caused by adding them to the free enzyme (table 1).

The spectral titration of the "binary" complexes by pyrophosphate and calcium (figs. 4, 5) were used to obtain data on the stoichiometry and dissociation constants of the "ternary" complex. The molar extinction differences were estimated according to equation 1, using high ligand (PP_i or Ca^{2+}) concentration points on the titration curves (figs. 4 and 5 – the inset).

Table 1
The spectral changes due to the metal and substrate binding with the enzyme (pH 6.5, I = 0.1, 20°).

	Maxima (a)			Minima (a)	$\Delta\epsilon_{max}^{\lambda_2-\lambda_4}$ (b)	$\Delta\epsilon_{max}^{\lambda_3-\lambda_4}$ (b)
Interaction	λ_1, nm	λ_2, nm	λ_3, nm	λ_4, nm	M^{-1} cm^{-1}	M^{-1} cm^{-1}
$E + Mg^{2+}$	275	282.5	292	300	1200	800
$E + Mn^{2+}$	275	282.5	291	299	1020	850
$E + Ca^{2+}$	275	283.5	290.5	–	850	700
$E + Zn^{2+}$	276	282	288	–	1000	1200
$E + PP_i$	–	281	288	–	620	910
$EPP_i + Ca^{2+}$	–	282	288.5	300	1570	1720
$ECa + PP_i$	–	281.5	288.5	300	1800	2300

(a) The values refer to the spectra of the complexes versus the "free" enzyme. (b) The values refer to the interactions pointed here.

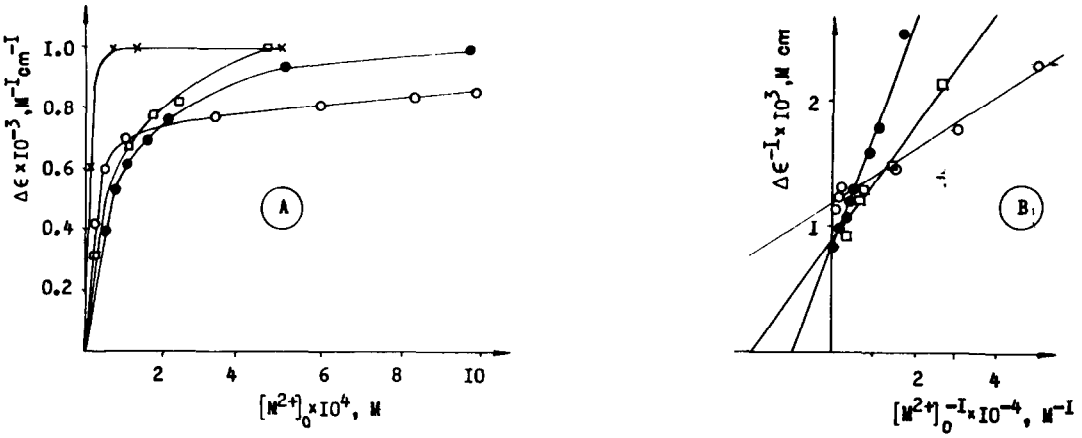


Fig. 2. Spectral titration of inorganic pyrophosphate with bivalent cations: (o—o—o) Ca^{2+}, (•—•—•) Mg^{2+}, (□—□—□) Mn^{2+}, (X—X—X) Zn^{2+}. A) Change in molar extinction difference at 283 and 300 nm upon addition of various metal ions; pH 6.5, $[E]_0$ = 5–9 μM. B) A double reciprocal plot of the data of fig. 2A.

Table 2
The association constants for various enzyme–metal complexes and the "ternary" complex in accordance with Scheme 1 (pH 6.5, I = 0.1, 20°).

Constant	Value, M^{-1}
K_{Mg}	0.93×10^4
K_{Mn}	2.2×10^4
K_{Zn}	$\geqslant 5 \times 10^4$
K_{Ca}	5.4×10^4
K_{PP}	1.1×10^3
K'_{Ca}	1.06×10^6
K^*_{Ca}	1.1×10^4
K'_{PP}	2.3×10^6
K'_{CaPP}	1.3×10^7

$$\Delta\epsilon/\Delta\epsilon_{max} = K_L[L]/(1 + K_L[L]) \qquad (1)$$

where $\Delta\epsilon_{max}$ is the molar extinction difference arising on the ligand binding with all sites to form a final complex. The affinity constant (K_L) and the number of the binding sites of the ligand (n) were determined according to equation (2)* using the values of $\Delta\epsilon_{max}$ (table 1) as is hown in fig. 4B and 5B.

$$\Delta\epsilon/(\Delta\epsilon_{max} - \Delta\epsilon)[L]_0 = K_L\{1 - n[E]_0 \Delta\epsilon/\Delta\epsilon_{max}[L]_0\} \qquad (2)$$

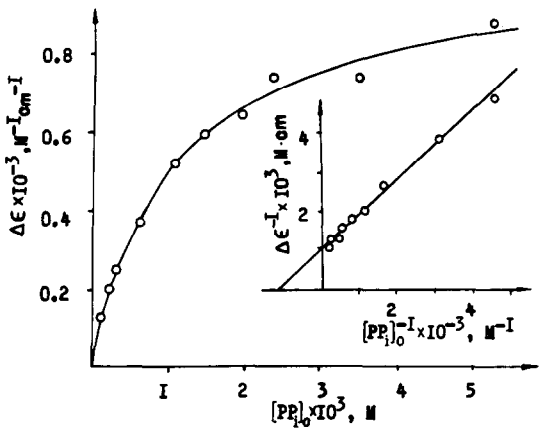


Fig. 3. Spectral titration of the enzyme with inorganic pyrophosphate. Change in molar extinction difference at 288 and 300 nm as a function of total PP_i concentration; pH 6.5, $[E]_0$ = 7 μM. Inset, a double reciprocal plot of the data.

* Equations (1) and (2) were proved by us to be valid for the case when there are n independent binding sites with equal affinity and random values of extinction changes for the ligand binding with different sites. The assumption that the pyrophosphate binding sites, should there be several, are independent and identical, follows from the kinetic data [2–6].

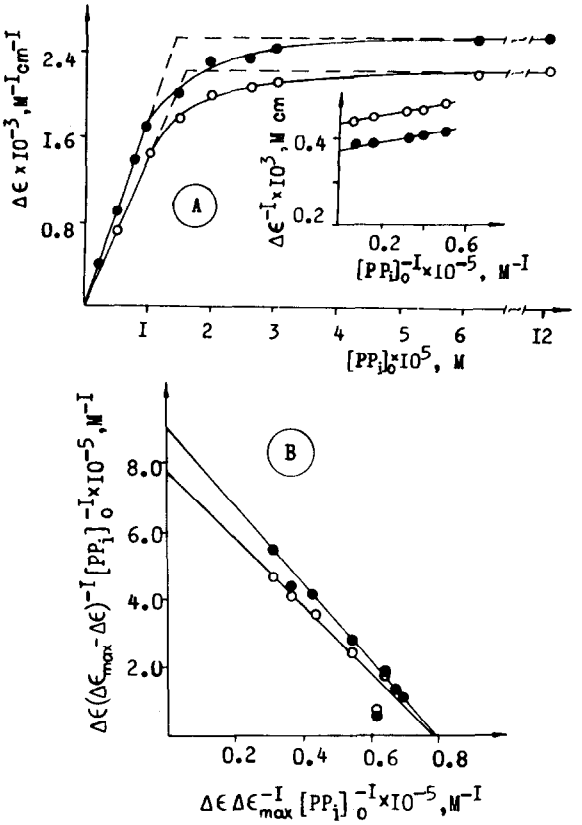
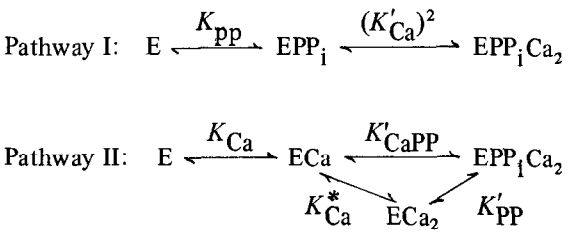


Fig. 4. Spectral titration of the enzyme with inorganic pyrophosphate at saturating Ca^{2+} concentrations. A) Change in molar extinction difference at 288 and 300 nm as a function of total PP_i concentration; (○—○—○) at pH 6.5, $[E]_0 = 9\ \mu M$, $[Ca^{2+}] = 1.0$ mM; (●—●—●) at pH 7.2, $[E]_0 = 11\ \mu M$, $[Ca^{2+}] = 1.1$ mM. Inset, a double reciprocal plot of the data of fig. 4A. B) Linear transformation of the titration curve in accordance with equation (2).

The values estimated for n equal 1.3 and 2.1 for PP_i and Ca^{2+}, respectively. The calculated apparent association constants for free forms of Ca^{2+}, PP_i and for $CaPP_i$ are equal to: $K'_{Ca\ (app)} = 1.06 \times 10^6\ M^{-1}$, $K'_{PP\ (app)} = 2.08 \times 10^6\ M^{-1}$ and $K'_{CaPP\ (app)} = 1.3 \times 10^6\ M^{-1}$. The resulting values for the number of binding sites for PP_i and Ca^{2+} and the ratio of their apparent association constants make it possible to suggest the following scheme of the "ternary" complex formation:



Scheme 1

The values of the equilibrium constants calculated according to this scheme are listed in table 2†.

It follows from table 2 that the PP_i binding constant increases by three orders of magnitude as a result of two calcium atoms being bound. The effect of the metal on the substrate affinity is apparently due to the conformational change accompanying the interaction of the enzyme with the metal.

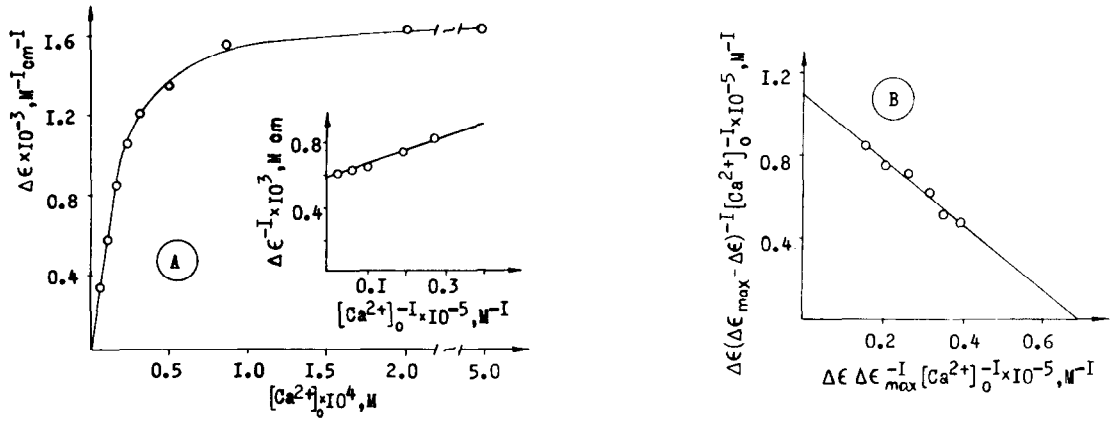


Fig. 5. Spectral titration of the enzyme with calcium ions in the presence of saturating pyrophosphate. A) change in molar extinction difference at 288 and 300 nm as a function of total Ca^{2+} concentration; pH 6.5, $[E]_0 = 7\ \mu M$, $[PP_i] = 5.3$ mM. Inset, a double reciprocal plot of the data of fig. 5A. B) Linear transformation of the titration curve in accordance with equation (2).

Table 3
The results of equilibrium dialysis.

Conditions	Free concentration of metal ion		Metal content		
	Ca	Mg	Ca	Mg	Ca + Mg
	(μM)		(g atoms/mole)		
pH 6.5, 4°					
$[E]_0$ = 10–60 μM	2–3	1	2.5 ± 0.25	0.3 ± 0.1	2.8 ± 0.3
pH 8.0, 4°	25–52	3–2	4.7 ± 0.4	1.2 ± 0.6	5.9 ± 0.7
$[E]_0$ = 50–200 μM	100–400	4–10	5.5 ± 0.7	0.2 ± 0.05	5.7 ± 0.7

The binding of the Ca^{2+} ions with the enzyme has also been demonstrated by the method of equilibrium dialysis. It follows from the data summarized in table 3 that the enzyme contains at least two strongly bound calcium atoms (with an affinity constant of not less than 0.4×10^7 M^{-1}, pH 6.5, 4°), the content of Mg being 0.3 g atoms per mole of active enzyme. The experiments with variable Ca^{2+} concentrations at pH 8.0 have shown that the maximum number of binding sites for calcium is about six. The tighter bound atoms of the metal seem to carry out some structural functions, for, as has been demonstrated by Butler et al. [4], the enzyme is inactivated by incubation with EDTA.

The information obtained from direct binding measurements correlates with the kinetic findings [5].

When this manuscript was in preparation a paper by Rapoport et al. [3] came out reporting some preliminary data on the spectral titration of the enzyme with metals, which agree with our results.

† When suggesting Scheme 1 it was taken into account that the products of the equilibrium constants involved in different pathways should be equal. In fact, the structure of the final complex does not depend on the pathway of its formation, as the total values of the extinction difference due to the "ternary" complex formation are equal (table 1). The following ratio was also used in calculating the constants: $K'_{CaPP} = K'_{CaPP\ (app)} (1 + K^*_{Ca} [Ca^{2+}])$.

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