KINETIC CHARACTERIZATION OF INORGANIC PYROPHOSPHATASE FROM *BACILLUS STEAROTHERMOPHILUS*

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Received 10 April 1978

1. Introduction

Among the pyrophosphatases from mesophilic microorganisms (e.g., *Escherichia coli* [1,2], *Desulfovibro desulfuricans* [3], *Thiobacillus thiooxidans* [4]), and baker's yeast [5] the enzyme isolated from yeast has been investigated most thoroughly with respect to its kinetic mechanism and binding properties [6–10]. The isolation of inorganic pyrophosphatase from a thermophile, *Bacillus stearothermophilus*, has been reported [11]. The pH and metal ion dependence of the enzyme activity and the substrate specificity are also described. As to be expected, the pyrophosphatase from this biological source is thermostable.

The molecular properties of the inorganic pyrophosphatases from microorganisms which are scarcely related to each other are largely different. In the present paper a kinetic model for pyrophosphate hydrolysis catalyzed by inorganic pyrophosphatase (pyrophosphate phosphohydrolase EC 3.6.1.1) from *B. stearothermophilus* in the presence of magnesium ions has been established by computer model fitting. From this it follows that magnesium ions act as activators of the enzyme whereas the complex of Mg^{2+} and pyrophosphate is the true substrate.

This is consistent with kinetic models described in the literature for inorganic pyrophosphatases from other microorganisms, baker's yeast and *E. coli* [6], and therefore seems to be a general principle for the kinetics of this group of enzymes.

2. Materials and methods

The *B. stearothermophilus* strain ATCC 12980, Washington, DC, was kept at 4°C as a stock culture. Cells were cultivated under continuous aerobic conditions at 65°C, separated by centrifugation and stored frozen at −20°C until use. For enzyme preparation the cells were disrupted by ultrasonication using a Branson Sonifier 12-B (Danbury, CT). The inorganic pyrophosphatase was than isolated according to a modified procedure [12] of that in [13]. The enzyme was pure to more than 90% as judged by disc electrophoresis. It had spec. act. 185 IU.

The enzyme activity was measured at 25°C in a standard activity assay containing 0.2 mM sodium pyrophosphate and 2 mM magnesium chloride in 0.1 M Tris–HCl buffer, pH 8.4, or in assays with various concentrations of pyrophosphate and Mg^{2+} for kinetic modelling. The liberation of orthophosphate was measured following the method in [14] with molybdate reagent and 2% ferrous sulphate as a reducing agent.

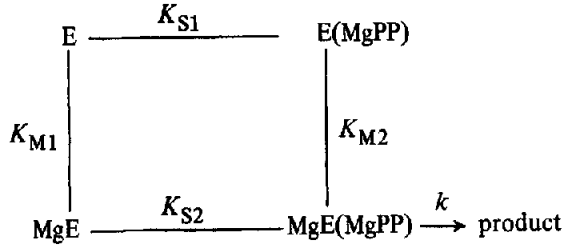
The stock solution of magnesium chloride was titrated with EDTA for determination of exact concentration.

Tris base was purchased from Boehringer, Mannheim. All other chemicals originated from VEB Laborchemie, Apolda, and were reagent grade.

Calculations of the species concentrations in assays containing pyrophosphate and magnesium ions were carried out on a Hewlett-Packard Desk Calculator model 9810. Calculated curves resulted from the kinetic model for comparison with the experimental data, and linear regression analysis as well were performed on this calculator. Computer model fitting was done by means of a BESM 6 computer using the program in [15]. The calculation of the species concentrations at pH 8.4 was performed as in [6].

3. Results

A set of kinetic data (relative enzyme reaction velocities in dependence on the concentration of the complex of magnesium ions and pyrophosphate at several constant free Mg^{2+} concentrations) has been fitted to potential equilibrium models. The same models were chosen as described in the computer model fitting calculations for inorganic pyrophosphatase from baker's yeast [6]. Out of 21 models with 3 or 4 enzyme species the best fit gave the model shown in scheme I.



Scheme 1

This model is described by the following rate equation:

$$v = \frac{V_{max} \cdot (MgPP)}{\frac{K_{S1} \cdot K_{M2}}{Mg_{free}} + K_{S2} + (\frac{K_{M2}}{Mg_{free}} + 1)(MgPP)} \quad (1)$$

(V_{max}, maximum velocity; MgPP, complex of magnesium ions and pyrophosphate; Mg_{free}, free magnesium ion concentration.) From the reciprocal form:

$$\frac{1}{v} = (\frac{K_{S1} \cdot K_{M2}}{V_{max} \cdot Mg_{free}} + \frac{K_{S2}}{V_{max}}) \frac{1}{MgPP} + \frac{\frac{K_{M2}}{Mg} + 1}{V_{max}} \quad (2)$$

it follows that the dependence of $1/v$ versus $1/(MgPP)$ at constant free magnesium concentration must be linear, and, furthermore, that the slope as well as the intercept of the curves is dependent on the free magnesium concentration.

For the set of experimental data the reciprocal representation is shown in fig.1. Straight lines are obtained at constant free magnesium concentrations without systematic deviations in any concentration

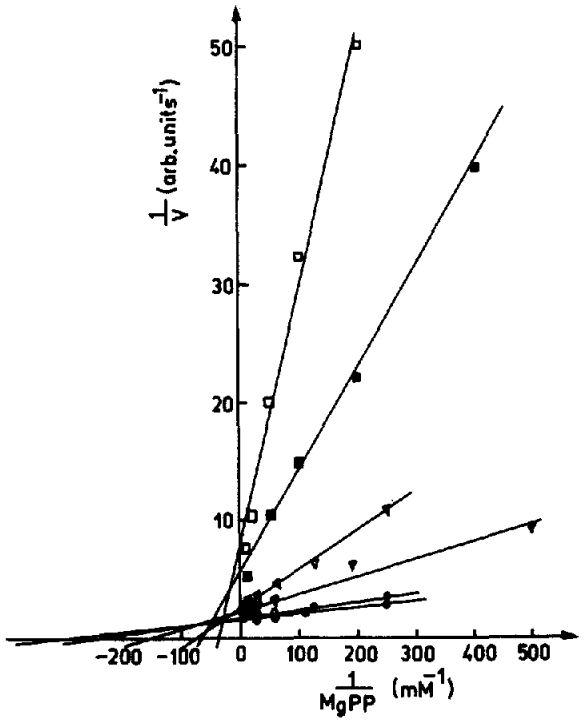


Fig.1. Plot of $1/v$ versus 1/MgPP at various concentrations of free Mg^{2+}. Mg_{free} (mM): (●) 3.96; (○) 1.84; (▲) 0.87; (△) 0.49; (■) 0.203; (□) 0.106.

range of Mg_{free} and MgPP. The lines are calculated by linear regression analysis. The correlation coefficients are within the range of 0.985–0.999. The parameters which best fit the experimental data to the model scheme 1 are represented in table 1. K_{S2} is rather low and out of the range of MgPP concentrations used in the kinetic experiments. Therefore, its value cannot

Table 1
Parameters estimated for the kinetic model scheme 1

Parameter	Value estimated by computer model fitting
K_{S1}	3.25×10^{-5} M
K_{S2}	3.8×10^{-6} M
K_{M1}	3.59×10^{-3} M
K_{M2}	4.2×10^{-4} M
V_{max}	0.8[a]

[a] The value of V_{max} is given in arbitrary units corresponding to the presentation of the experimental data in fig.2

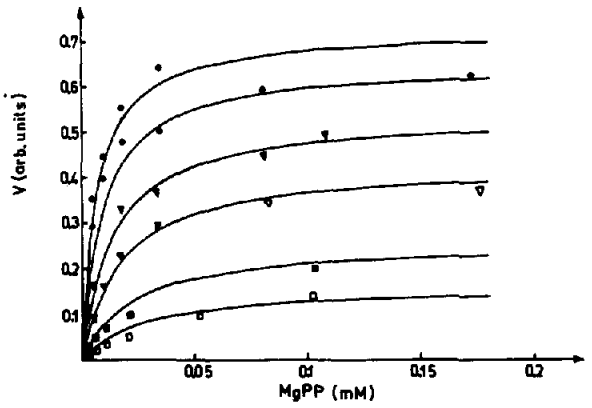


Fig.2. Comparison of the experimental data points with theoretical curves calculated according to model scheme 1. Mg_{free} (mM): (●) 3.96; (○) 1.84; (▲) 0.87; (△) 0.49; (■) 0.203; (□) 0.106.

be estimated with certainty. In fig.2 a comparison is shown of the complete set of experimental data for 6 constant free magnesium ion concentrations with the theoretical curves calculated by means of eq. (1) and by use of the parameters of table 1.

4. Discussion

There are large differences in the molecular characteristics of inorganic pyrophosphatases from various biological sources. Table 2 summarizes the heat resistance, molecular weights, subunit composition, and isoelectric points of several pyrophosphatases. The heat resistance of the inorganic pyrophosphatase from *B. stearothermophilus* up to temperatures of 80°C for at least 10 min (in the presence of Mg^{2+}) shows clearly that it belongs to the thermostable enzymes [12], but also the pyrophosphatases from *E. coli* [1] and *Th. thiooxidans* [4] are exceptionally thermostable. The temperature indicated in the heat resistance column are, however, only roughly comparable because they depend on several factors such as protein concentration, pH value, ionic strength, and effector concentrations.

As to the differences in the molecular characteristics, it is of great interest that the basic kinetic mechanism of neutral and alkaline inorganic pyrophosphatases seems to be very similar. They all need divalent metal ions for activity. With few exceptions magnesium ions are the most effective among them. Kinetic experiments lead to the conclusion that the complex of magnesium and pyrophosphate acts as the true substrate of the enzymes. An additional activation by binding of free magnesium ions to an activator site of the enzyme follows from computer model fitting performed in [6] for inorganic pyrophosphatase from baker's yeast. This model has been confirmed for this enzyme by kinetic measurements [9] and also by

Table 2
Selected properties of inorganic pyrophosphatases from thermophilic and mesophilic microorganisms

Microbial source	Thermophilic microorganism	Molecular weight	Subunit composition	IP	Heat resistance (°C)[a]	Ref.
Bacillus stearothermophilus ATCC 12980	+	140 000 ±15 000		4.0	80	[12]
Bacillus stearothermophilus NCA 2184	+	122 000 –140 000	2 (70 000) 4		75	[11]
Thiobacillus thiooxidans	–	88 000	(20 000)	5.05	80	[4]
Desulfovibrio desulfuricans	–	43 000 ±7000	6	6.55	labile	[3]
Escherichia coli	–	120 000	(20 000) 2		80	[1]
Baker's yeast	–	64 000	(32 042)		50	[16,17,20]

[a] The values given as 'heat resistance' are the temperatures at which the activity of the enzymes decreases not more than 5% within 10 min

direct binding studies [7]. A similar kinetic behaviour could be shown by computer model fitting or graphical evaluation for inorganic pyrophosphatases from *E. coli* [6], from *Rhodospirillum rubrum* [18], and from *B. stearothermophilus*, as shown in the present paper (scheme 1).

Obviously, this model expresses a general kinetic principle for neutral and alkaline inorganic pyrophosphatases. Nevertheless, the parameters in the kinetic models (e.g., binding constants for Mg^{2+} and substrate) may differ largely for these enzymes in dependence on the specific conditions and regulatory requirements in the corresponding microorganisms.

The strong noncompetitive ATP-inhibition and the much weaker AMP-inhibition of the *B. stearothermophilus* pyrophosphatase activity described [11] may be explained by the kinetic model presented in this paper as simply a competition of the adenine nucleotides with the activator site of the enzyme for free magnesium ions (especially ATP forms strong complexes with Mg^{2+} [19]). The concentration of ATP in the inhibition assay [11] is high enough to bind nearly completely the free Mg^{2+} ions.

Acknowledgements

The authors gratefully acknowledge Dr T. Rapoport and Dr M. Otto for their help in performing the computer model fitting. We also wish to thank Professor S. Rapoport for helpful discussions and critical reading of the manuscript.

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