THE PHOSPHORYLATION OF YEAST INORGANIC PYROPHOSPHATASE AND FORMATION OF STOICHIOMETRIC AMOUNTS OF ENZYME-BOUND PYROPHOSPHATE

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1. Introduction

Baker's yeast inorganic pyrophosphatase (EC 3.6.1.1) is able to bind orthophosphate with a dissociation constant of the order of 1 mM [1–3]. Despite the moderate strength of the binding, it results in formation of a chemical bond of an acyl phosphate type [4]. Since phosphate is the product of PP_i hydrolysis and the substrate in the reverse reaction, one could have supposed the phosphorylated enzyme to be an obligatory catalytic intermediate. However, blocking the active site of the enzyme with pyrophosphate did not eliminate P_i binding [5]. This observation necessitated further studies of the reaction of pyrophosphatase with P_i. They were partly stimulated by the finding of N. N. Vorobjeva in this laboratory that the phosphorylated enzyme can be isolated by gel filtration in the absence of Mg^{2+}.

Here, the following features of the enzyme–phosphate reaction are revealed:

(i) A stoichiometric amount of protein-bound PP_i forms from P_i in the active site of pyrophosphatase;
(ii) 0.5 mol P_i/mol of active sites is independently bound in a different centre;
(iii) The bound P_i and PP_i are rapidly exchanged for medium phosphate in the presence of Mg^{2+}.

2. Materials and methods

Inorganic pyrophosphatase with spec. act. 600 IU/mg at pH 7.2, 25°C was isolated from baker's yeast as in [6]. The molarity of the enzyme solutions was calculated from the absorbance at A_{280} nm using 1.45 for $A_{280}^{0.1\%}$ [7] and M_r = 64 000 [8]. Radioactive phosphate (carrier-free) and pyrophosphate (40 Ci/mol) were purchased from Amersham Radiochemical Centre. The traces of polyphosphates in $^{32}P_i$ were hydrolyzed by heating with 0.5 M HCl at 95°C for 2 h. Unless noted otherwise, phosphate was used as a tetramethylammonium salt which was prepared from free acid and tetramethylammonium hydroxide.

The phosphorylation of inorganic pyrophosphatase was accomplished by incubating 7.6 μM enzyme with $^{32}P_i$ (10^6–2×10^7 cpm, depending on the concentration) for several minutes in the presence of 0.1 M Tris–HCl (pH 7.2) and 1 mM $MgCl_2$ at 18°C, in 50 μl total vol. The excesses of $^{32}P_i$ and $MgCl_2$ were removed by centrifugal gel filtration [9] using 1 ml Sephadex G-50 fine equilibrated against 0.1 M Tris–HCl (pH 7.2). The original procedure of Penefsky was slightly modified in order to gain both adequate separation and reasonable recovery of the protein at a low concentration. The centrifugation was carried out at 460 × *g* at the bottom of the gel and the protein was additionally eluted from the column by application of the buffer (125 μl) and 2 min centrifugation. The volume of the combined eluate was measured and the recovery of the protein (usually 50–60%) was determined from enzymic activity. The amounts of $^{32}P_i$ in the eluate in controls lacking the enzyme were <10% of those of the enzyme-bound $^{32}P_i$ in the related experiments at all concentrations of $^{32}P_i$ in the incubation medium. E_p and E_{pp+p} used in the studies of their stability were obtained at 2 and 10 mM $^{32}P_i$, respectively.

For analysing the effect of PP_i hydrolysis in the active site on the stability of E_p, 0.2 ml 0.18 μM E_p

Abbreviations: E_p, enzyme containing bound P_i; E_{pp+p}, enzyme containing both bound P_i and PP_i

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solution in 0.1 M Tris–HCl (pH 7.2) was rapidly combined with 0.02 ml 1.7 μM non-radioactive PP_i–1.1 mM $MgCl_2$, incubated for 15 s at 4°C, and separated immediately from the released $^{32}P_i$ by centrifugal gel filtration. The quantitative recovery of the enzyme was achieved in this case by adding 1 mg bovine serum albumin/ml to the buffer for Sephadex equilibration.

The amounts of the protein-bound P_i and PP_i in the eluates were measured after adding 0.4 M $HClO_4$ and 1 μmol each of non-radioactive P_i and PP_i and extracting $^{32}P_i$ as a phospho–molybdate complex [10]. The radioactivities of the aqueous and organic phases were determined using a liquid scintillation counter.

The separation of P_i and PP_i by ion-exchange chromatography was performed on a 0.8 × 20 cm Dowex 1 × 8 column equilibrated against 0.02 M Tris–HCl (pH 8.0). The elution was carried out with a linear gradient of KCl (0.1–0.3 M) of 100 ml total vol.

The enzyme–substrate compound stabilized by fluoride was isolated as in [11], with the only exception that the centrifugal gel filtration was employed.

Enzymic activity was measured with an automatic phosphate analyzer [12].

3. Results and discussion

The incubation of pyrophosphatase with $^{32}P_i$ resulted in binding of the label in the form of P_i and PP_i. These enzyme compounds withstood gel filtration but were completely destroyed by $HClO_4$ treatment. The identity of the released P_i and PP_i was confirmed by ion-exchange chromatography. The amounts of the protein-bound P_i and PP_i depended on the P_i concentration (fig.1) and their maximal values were ~1 and 2 mol/mol enzyme dimer, respectively. P_i binding did not change when the incubation time was varied from 0.25–30 min. The binding was a readily reversible process since the equilibration of the system upon addition of carrier-free $^{32}P_i$ to the enzyme preincubated with 1.5 mM non-radioactive P_i took <0.5 min. Change in pH from 6.2 to 7.8 hardly affected binding at 2 mM P_i. Decrease in $MgCl_2$ from 1 mM to 10 μM at 10 mM P_i resulted in deceleration of the binding ($t_{1/2}$ ~ 5 min) and only protein-bound P_i was detected. It is of interest that the substitution of 1 mM $CaCl_2$ for $MgCl_2$, which causes strong inhibition of the enzymic activity, did not affect the formation of enzyme-bound P_i and PP_i at 1 and 10 mM P_i.

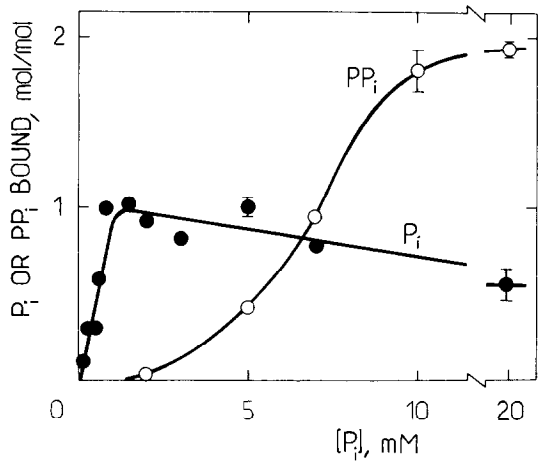

Fig.1. Dependence of the amounts of enzyme-bound P_i and PP_i on P_i concentration.

The incubaton of isolated E_p and E_{pp+p} at pH 7.2, 18°C resulted in their decomposition with $t_{1/2}$ of 250 min and 6.5 min, respectively.

The results reported above were obtained with a tetramethylammonium phosphate. With sodium or potassium salts, binding decreased at >3 mM P_i, and <0.1 mol/mol PP_i and P_i were found on the protein at 10 mM P_i. The same decrease in binding was observed when an equivalent amount of KCl or NaCl was added to the incubation medium containing the tetramethylammonium salt. Increasing $MgCl_2$ to 10 mM did not make this effect less pronounced which meant that it was hardly due to competition between Mg^{2+} and alkali metal ions.

Assuming that PP_i formed in the active site, the existence of an additional site for P_i binding was to be postulated. This conclusion was confirmed by the studies of P_i binding to the stable enzyme–PP_i compound formed in the presence of fluoride [13]. For its decay, $t_{1/2}$ of 2 h was reported [11,13]. The incubation of this compound prepared from non-radioactive PP_i with 1 or 10 mM $^{32}P_i$ resulted in the binding of P_i in equiv. 0.93 and 1.07 mol/mol, respectively, while PP_i binding decreased to 0.04 mol/mol. That P_i in E_p did not locate in the active site was also evidenced by the observation that E_p retained full enzymic activity, although 64% of P_i remained enzyme-bound when each molecule of E_p hydrolyzed ~1000 molecules of PP_i. This is also supported by the observation that bound P_i is not utilized in the synthesis of PP_i, since no radioactive protein-bound PP_i

was found after the incubation of labelled E_p for 10 s with 10 mM nonradioactive P_i and 1 mM $MgCl_2$ followed by centrifugal gel filtration. At the same time, only 38% of $^{32}P_i$ in E_p was exchanged for the medium P_i.

The ability of inorganic pyrophosphatase to shift the 2 $P_i \rightleftharpoons PP_i$ equilibrium in the active site towards PP_i may reflect a common feature of the mechanisms of polyphosphate synthesis in the cell and lends support to the hypothesis that related membrane systems require energy for the release of polyphosphates from the active sites, rather than for the formation of P–O–P bonds [14]. It should be noted that PP_i, like ATP, is an energy-rich compound and its synthesis is coupled to the work of the electron-transfer chain [15–18].

Evidence that pyrophosphatase can form and tightly hold PP_i has been obtained also [10] using a different approach. However, only 0.1 mol/mol protein-bound PP_i was observed. This low yield may be explained by the fact that potassium phosphate, which strongly inhibits the formation of PP_i, was used.

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