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Tightly bound pyrophosphate in *Escherichia coli* inorganic pyrophosphatase

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Hexameric inorganic pyrophosphatase of *Escherichia coli* contains about 1 mol/mol of 'structural' pyrophosphate, which survives gel filtration and prolonged incubation with Mg^{2+}, does not exchange with medium phosphate and pyrophosphate but is removed with 0.8 M perchloric acid. The site of pyrophosphate binding seems to be another than the active site. An additional 0.9 mol of enzyme-bound pyrophosphate is formed in the presence of phosphate and Mg^{2+} but this pyrophosphate is in fast equilibrium with medium phosphate and appears to be bound to the active site.

Inorganic pyrophosphatase; Pyrophosphate synthesis; Active site; Enzyme-substrate interaction; *(Escherichia coli)*

1. INTRODUCTION

Inorganic pyrophosphatase (EC 3.6.1.1) is the simplest enzyme which transfers phosphoryl from a polyphosphate to water. Pyrophosphatase is present in virtually any cell and is mainly localized in cytosol. All cytosolic pyrophosphatases isolated so far have similar catalytic properties but their quaternary structures differ considerably and depend on the origin of the enzyme. Procaryotic pyrophosphatases contain 4 or 6 identical subunits of about 20 kDa while eucaryotic ones contain 2 identical subunits of 30–35 kDa per molecule. *E. coli* [1] and baker's yeast [2] pyrophosphatases are the best-known examples of the two groups. Their genes have been recently cloned, and experiments involving site-directed mutagenesis are now under way [3,4].

The present study revealed another structural peculiarity of procaryotic pyrophosphatases, which consists in the presence of tightly-bound PP_i.

2. MATERIALS AND METHODS

E. coli strain MRE 600 [5], baker's yeast [6] and rat liver [7] pyrophosphatases were isolated as described. Polyacrylamide gel electrophoresis [8] indicated that the preparations were at least 95% pure. Stock solutions of the enzymes contained 0.05–0.1 M Tris-HCl (pH 7.2) and, in the case of the rat liver enzyme, 1 mM $MgCl_2$ and 5 mM dithiothreitol.

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PP_i was extracted from the pyrophosphatases by addition of 10 μl of 5 M $HClO_4$ to 50 μl of 2–8 μM enzyme. Tubes were transferred to boiling water for 1 min and cooled under tap water. The solutions were neutralized with KOH and the $KClO_4$ formed was removed by centrifugation. The PP_i concentration was determined by a sensitive enzymatic method utilizing ATP-sulfurylase and luciferase [9]. Corrections were made for a loss of 20% PP_i during the heating step.

The same procedure was used to measure the synthesis of enzyme-bound PP_i from medium P_i. The assay mixture containing 2–8 μM enzyme, P_i, $MgCl_2$ and 0.1 M Tris-HCl (pH 7.2) was equilibrated for 10 min at 25°C, the solution was quenched with $HClO_4$ and processed as described above. Controls with 0.02 μM enzyme to measure PP_i free in solution were run in parallel under identical conditions. The concentration of free PP_i was 5–30% of that of the enzyme-bound PP_i, depending on the medium P_i level. The concentration of free Mg^{2+} ion in the incubation medium was maintained at 20 mM using a dissociation constant of 8.5 mM for magnesium phosphate [10].

3. RESULTS

Perchloric acid extracts of several preparations of *E. coli* pyrophosphatase were found to contain 1.1 ± 0.1 mol of PP_i per mol of hexameric enzyme. This PP_i could not be separated from the enzyme by Sephadex G-50 chromatography. PP_i contents of freshly isolated enzyme and of the enzyme which had been stored frozen in solution for 1 year were virtually the same. The amount of protein-bound PP_i present in partially purified preparations of pyrophosphatase correlated with their specific activities.

Although PP_i is a substrate for pyrophosphatase in the presence of Mg^{2+} ions, incubation of the enzyme with 1 mM $MgCl_2$ at pH 7.2 for 1 day at 25°C or 20 days at 4°C did not affect PP_i content. At the same time, added PP_i (10 μM) was completely hydrolyzed by

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3 μM pyrophosphatase in less than 1 min under these conditions. Likewise, incubation of pyrophosphatase at pH 5 or 9.5 in the presence of 1 mM $MgCl_2$ for 30 min at 22°C followed by readjustment of pH to 7.2 did not affect the amount of the bound PP_i. These results indicated that the *E. coli* enzyme contains tightly bound PP_i which cannot serve as its substrate.

The enzymatic method used in this work is highly specific for PP_i [9]. However, additional experiments were performed to confirm the identity of protein-bound PP_i. If the neutralized acid extract of the enzyme was supplemented with 1 mM $MgCl_2$ and 0.2 μg of baker's yeast pyrophosphatase, which displays absolute specificity towards PP_i in the presence of Mg^{2+}, no luminescence attributable to PP_i was observed. The same result was obtained if ATP-sulfurylase was omitted from the assay mixture, ruling out the possibility that the compound extracted from the *E. coli* enzyme is ATP.

No enzyme-bound PP_i (<0.02 mol/mol) was found in baker's yeast and rat liver pyrophosphatases.

The neutralized acid extracts of *E. coli* pyrophosphatase were also analyzed for phosphate using a sensitive malachite green procedure [11]. No phosphate (<0.05 mol/mol) other than that derived from PP_i because of its partial decomposition was found.

The amount of the enzyme-bound PP_i increased considerably when *E. coli* pyrophosphatase was incubated with P_i in the presence of Mg^{2+} (fig.1). This additional PP_i dissociated from the protein during gel filtration or simple dilution with the buffer containing no P_i and Mg^{2+} and could be only detected if perchloric acid was added to the complete incubation mixture. The synthesis of PP_i was absolutely Mg^{2+}-dependent. That this additional PP_i was actually enzyme-bound was evidenced by a linear dependence of its amount on pyrophosphatase concentration. The characteristics of PP_i-synthesis by the *E. coli* enzyme were thus very close to those reported for the baker's yeast pyrophosphatase [12,13], except for its extent. The maximal levels of PP_i incorporation for the *E. coli* and yeast enzymes were 0.9 and 0.35 mol/mol, respectively (fig.1). The latter value is in accord with the data of others [12,13], but some preparations of yeast pyrophosphatase obtained in this laboratory could synthesize much higher amounts of bound PP_i [14].

The exchange, if any, of the endogeneous PP_i with medium P_i under conditions favoring the synthesis of additional PP_i on the enzyme was very slow. In these experiments, *E. coli* pyrophosphatase (26 μM) was incubated with 10 mM $^{32}P_i$ and 10 mM $MgCl_2$ for 1 h at pH 7.2 and then passed through a Sephadex G-50 column equilibrated with 0.1 M Tris-HCl (pH 7.2). The eluted protein contained the same amount of PP_i, as measured by the coupled enzymatic procedure, of which <0.5% was $^{32}PP_i$, as measured by the isobutanol/benzene extraction procedure after converting P_i into phosphomolybdate [13].

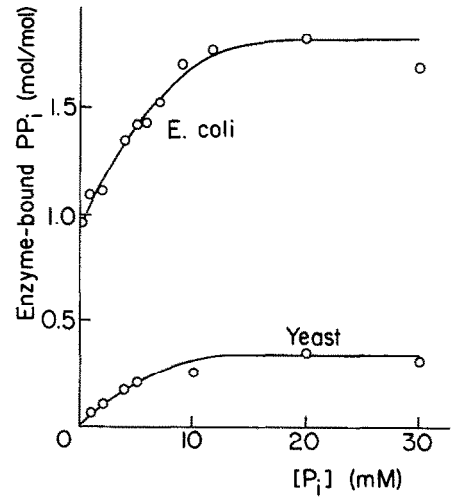

Fig.1. Dependence of the amount of PP_i bound to *E. coli* and yeast pyrophosphatases on medium P_i concentration in the presence of 20 mM free Mg^{2+}.

4. DISCUSSION

The experiments reported in this paper demonstrate the presence of two types of PP_i-binding sites on *E. coli* pyrophosphatase. The affinity of one of them is so high that removal of PP_i from it requires denaturation of the enzyme. To our knowledge, this is the first pyrophosphatase which has been reported to contain 'structural' PP_i. Preliminary experiments showed that such PP_i may be also present in *Thermus thermophilus* pyrophosphatase provided by Dr I.P. Kuranova of the Moscow Institute of Crystallography. No PP_i was found, on the contrary, in two eucaryotic pyrophosphatases. One can speculate that the presence of 'structural' PP_i is a general property of procaryotic pyrophosphatases.

The lack of exchange between endogenous PP_i and medium P_i makes it unlikely that this PP_i is bound at the active site of *E. coli* pyrophosphatase. It should be noted that the incubation medium used in these experiments contained about 1 μM PP_i free in solution, because of onset of the equilibrium between P_i and PP_i [15]. Since the Michaelis constant for PP_i in the presence of 20 mM Mg^{2+} is as low as 0.3 μM (Shestakov, A.A., unpublished), 1 μM PP_i would have displaced bound PP_i from the active site.

The role of the 'structural' PP_i in procaryotic pyrophosphatases can only be guessed at. Recent studies of baker's yeast pyrophosphatase have revealed the presence of a non-catalytic site, which binds PP_i in a readily reversible manner, although quite tightly (K_d ~ 0.1 μM) [16]. The occupancy of this site leads to increased affinity of the enzyme for the activating Mg^{2+} ion. It is tempting to speculate that the 'structural' PP_i

of the procaryotic pyrophosphatases has the same role as the PP_i bound at the regulatory site of the more evolved yeast enzyme.

The additional PP_i bound in the presence of P_i seems to be located at the active site and is therefore a catalytic intermediate, as has been shown previously for the yeast enzyme [12,13]. *E. coli* pyrophosphatase provides thus another example of an enzyme which can dramatically shift the equilibrium of polyphosphate synthesis in its active site. This property of the enzyme along with the presence of tightly-bound substrate makes it similar to H^+-ATPase [17,18], which catalyzes ATP synthesis coupled to transmembrane proton movement. *E. coli* pyrophosphatase may be thus used to derive data relevant to the mechanism of both PP_i and ATP synthesis in biological systems.

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