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Purification and properties of pyrophosphatase of *Acinetobacter johnsonii* 210A and its involvement in the degradation of polyphosphate

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Abstract

Inorganic pyrophosphatase (E.C. 3.6.1.1) of *Acinetobacter johnsonii* 210A was purified 200-fold to apparent homogeneity. The enzyme catalyzed the hydrolysis of inorganic pyrophosphate and triphosphate to orthophosphate. No activity was observed with other polyphosphates and a wide variety of organic phosphate esters. The molecular mass of the enzyme was estimated to be 141 kDa by gelfiltration. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated a subunit composition of six identical polypeptides with a molecular mass of 23 kDa. The cation Mg^{2+} was required for activity, the activity with Mn^{2+}, Co^{2+} and Zn^{2+} was 48, 48 and 182% of the activity observed with Mg^{2+}, respectively. The enzyme was heat-stable and inhibited by fluoride and iodoacetamide. The analysis of the kinetic properties of the enzyme revealed an apparent K_m for pyrophosphate of 0.26 mM. In *A. johnsonii* 210A, pyrophosphatase may be involved in the degradation of high-molecular polyphosphates under anaerobic conditions: (i) it catalyses the further hydrolysis of pyrophosphate and triphosphate formed from high-molecular weight polyphosphates by the action of exopolyphosphatase, and (ii) it abolishes the inhibition of polyphosphate: AMP phosphotransferase-mediated degradation by pyrophosphate and triphosphate.

Introduction

Acinetobacter johnsonii 210A displays a special phosphate metabolism. During growth in phosphate-sufficient media, excess phosphate is taken up, polymerized to high-molecular weight polyphosphate and intracellularly deposited in polyphosphate granules (Deinema et al. 1985; Van Groenestijn 1988; Bonting et al. 1993a). Cells grown in high-$MgHPO_4$ medium contained polyphosphate granules in which Mg^{2+} and K^+ were the counterions. Ca^{2+} was found as an additional counterion in granules of cells grown in high-$CaHPO_4$ medium, and Mn^{2+} prevailed in the polyphosphate granules during growth in high-$MnHPO_4$ medium (Bonting et al. 1993a; Van Veen et al. 1994). The biosynthesis of polyphosphate is most likely catalyzed by polyphosphate kinase (Van Niel et al. 1999) and the degradation by polyphosphate: AMP phosphotransferase and polyphosphatase (Bonting et al. 1991; Bonting et al. 1993b). Both enzymes involved in polyphosphate degradation are only able to use long chain polyphosphates as a substrate upon incubation for a few minutes. Upon incubation of polyphosphatase with long chain polyphosphates for a few hours, formation of small amounts of pentaphosphate, tetraphosphate, triphosphate and pyrophosphate was observed (Bonting et al. 1993b). Besides being formed from long chain polyphosphates by polyphosphatase activity in *A. johnsonii* 210A, pyrophosphate may be formed by a variety of nucleoside triphosphate-dependent reactions such as deoxyribo- and ribonucleic acid polymerizations, coenzyme synthesis, and amino acid or fatty acid activation. Pyrophosphate formation can also be formed by photophosphorylation, oxidative phosphorylation and glycolysis (Kukko-Kalske & Heinonen 1985). Since pyrophosphate inhibits various enzymes as demonstrated for *Escherichia coli* (Kukko-Kalske & Heinonen 1985),

cells need to utilize or cleave pyrophosphate rapidly to prevent growth inhibition. One way of lowering the intracellular pyrophosphate concentration is by using it in pyrophosphate-dependent phosphotransferase reactions, such as the phosphorylation of serine and acetate in *Propionibacterium shermanii* and *Entamoeba histolytica*, respectively (Wood et al. 1977). A second way of lowering the cellular pyrophosphate concentration is by pyrophosphatase activity. This paper describes the purification and properties of pyrophosphatase of the polyphosphate-accumulating *A. johnsonii* 210A and the role of pyrophosphatase in the polyphosphate metabolism of this organism.

Materials and methods

Microorganism and cultivation

Acinetobacter strain 210A was previously identified as *Acinetobacter johnsonii* 210A and deposited in the Netherlands Culture Collection of Microorganisms under the access number LMAU A130 (Bonting et al. 1992b). The organism was grown in two 10-L carboys each containing 8 L of medium as described by Bonting et al. (1991). Cells were harvested in the log-phase at an OD_{660} of about 1.5 by continuous centrifugation and washed two times in 50 mM trishydroxymethylaminomethane-HCl (Tris-HCl), pH 7.6.

Purification of pyrophosphatase (E.C. 3.6.1.1)

Unless stated otherwise, all purification steps were performed at 4 °C. Washed cells were resuspended in 150 ml 50-mM Tris-HCl (pH 7.6), disrupted at 0 °C by sonication (10 times, 30 s each; interval pauses of 30 s; 40 W; Branson Sonic Sonifier, Danbury, Conn., USA) and centrifuged for 30 min at 30,000 × *g*. The supernatant contained about 10 mg of protein per ml and is referred to as crude extract.

After addition of $MgCl_2$ to a final concentration of 17 mM, the crude extract was placed for 10 min in a water bath with a temperature of 70 °C and subsequently rapidly cooled on ice. Denatured proteins were removed by centrifugation at 100,000 × *g* for 60 min. Under vigorous stirring, a saturated $(NH_4)_2SO_4$-solution was added to the supernatant. The 50–80% precipitate was collected by centrifugation at 20,000 × *g* for 10 min and dissolved in 50-mM Tris-HCl, pH 7.6.

The following steps were performed with a high-resolution fast protein liquid chromatography system (Pharmacia/LKB, Woerden, The Netherlands) at room temperature. All columns were equilibrated with the starting buffers of the gradients. Linear gradients were throughout. The fraction (50 ml) obtained after $(NH_4)_2SO_4$ precipitation was loaded on a hydroxylapatite column (2.2 × 20 cm). A 200-ml gradient from 0.2–1 M in 50-mM Tris-HCl, pH 7.6, was applied at a flow rate of 2 ml/min. Fractions with pyrophosphatase activity were pooled and $(NH_4)_2SO_4$ (2 M) was added to a final concentration of 1 M. To prevent overloading, two aliquots of this fraction were injected on a Phenyl Superose HR 5/5 column. Elution was with a 10-ml gradient ($0 - 1$ M $[NH_4]_2SO_4$ in 50 mM Tris-HCl buffer, pH 7.6) at a flow rate of 0.45 ml/min. Fractions with the highest activity were pooled and passed in six separate runs through a Superose 6 HR 10/30 column equilibrated with 150-mM NaCl in 50-mM Tris-HCl (pH 7.6) at a flow rate of 0.2 ml/min. The most active fractions were pooled and mixed in a 1 : 1 ratio with 50-mM TrisHCI, pH 7.6. The diluted fraction was applied to a Mono-Q HR 5/5 anion exchange column. Purified pyrophosphatase was eluted at 0.27 M NaCl in a 43-ml gradient from 0 to 0.4 M NaCl in 50-mM Tris-HCl, pH 7.6, at a flow rate of 1.1 ml/min.

Enzyme assays

Pyrophosphatase was assayed by following the release of phosphate from pyrophosphate at 30 °C. The reaction mixture contained: 50 mM Tris-HCl, pH 7.6; 10 mM $MgCl_2$; 2 mM pyrophosphate. The assay mixture (0.5 ml) contained 5 microliters of a solution of the purified pyrophosphatase or larger volumes of the less purified enzyme. The reaction was started by the addition of the enzyme.

Alkaline phosphatase (E.C. 3.1.3.1) was measured according to Bonting et al. (1992a) with 2 mM *p*-nitrophenylphosphate as the substrate.

Analytical methods

Protein was estimated according to Bradford (1976) with bovine serum albumin as the standard. The purity of pyrophosphatase was determined by sodium dodecyl sulphatepolyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli (1970). Native PAGE and activity staining of the pyrophosphatase were performed as described by Baykov & Volk (1985). Superose 6 HR 10/30 was used to

estimate the molecular size of the pyrophosphatase. Orthophosphate was determined spectrometrically according to Standard Methods (1976).

Chemicals

Tetrasodium pyrophosphate was purchased from Janssen (Beerse, Belgium). Tri- and tetraphosphate were obtained from Sigma Chemical Company (Amsterdam, The Netherlands). Acrylamide, N,N-methylenebisacrylamide, sodium dodecyl sulfate and hydroxylapatite were purchased from Bio-Rad Laboratories (Utrecht, The Netherlands). All other columns and the molecular mass standards for gel filtration and SDS-PAGE were obtained from Pharmacia Fine Chemicals (Woerden, The Netherlands). All other biochemicals were purchased from Boehringer Mannheim (Almere, The Netherlands).

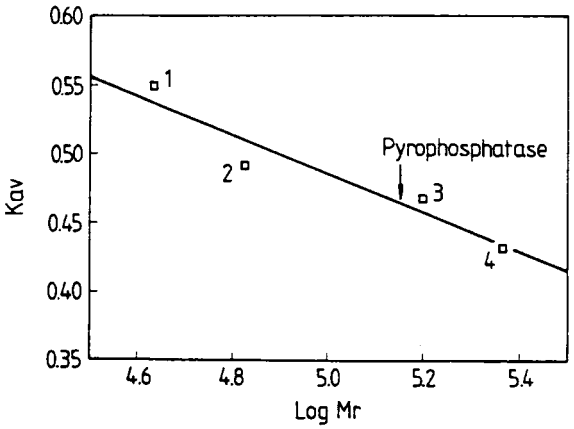

Figure 1. Molecular mass determination of the native pyrophosphatase on Superose HR 10/30. Standards were ovalbumin (45 kDa, 1), bovine serum albumin (67 kDa, 2), aldolase (158 kDa, 3) and catalase (232 kDa, 4). The position of the pyrophosphatase of *Acinetobacter johnsonii* 210A is indicated by the arrow.

Results

Purification of pyrophosphatase

The purification of the enzyme from a crude cell-free extract is summarized in Table 1. The specific pyrophosphatase activity was increased 200-fold by the purification process. Like many pyrophosphatases of Gram-negative bacteria (Blumenthal et al. 1967), the enzyme from *A. johnsonii* 210A appeared to be remarkably heat-resistant. This property was used in the purification procedure by heating the crude extract for 10 min at 70 °C. Heat-labile proteins coagulated whereas pyrophosphatase remained in the supernatant.

Characterization

The molecular mass of the native pyrophosphatase was estimated to be approximately 141 kDa by gelfiltration (Figure 1). The subunit molecular mass of the enzyme was determined by SDS-PAGE. The pyrophosphatase appeared to be composed of only one polypeptide with a molecular mass of 23 kDa (Figure 2). This suggests that pyrophosphatase of *A. johnsonii* 210A is a hexameric protein with six identical subunits.

When native gels were incubated for 1 mm with the pyrophosphatase assay mixture and subsequently stained with ammonium heptamolybdate and methylgreen in 1 M H_2SO_4, both crude extract and purified protein showed only one band with the same mobility. This points to the presence of only one pyrophosphatase in *A. johnsonii* 210A.

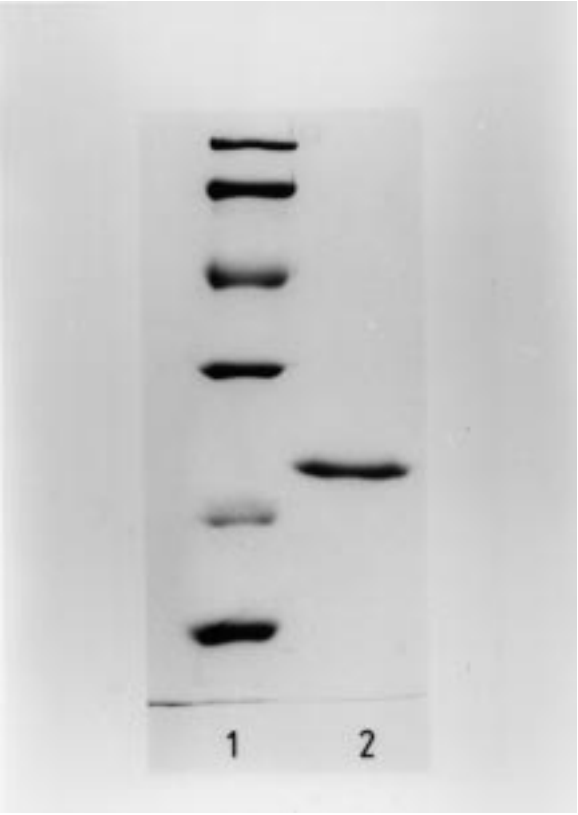

Figure 2. Analysis of purity by SDS-PAGE. Lane 1: molecular mass markers (94, 43, 30, 20.1, 14.4 kDa); lane 2: purified pyrophosphatase of *Acinetobacter johnsonii* 210A.

Table 1. Purification of pyrophosphatase of *Acinetobacter johnsonii* 210A

Step	Protein (mg)	Activity (U)[a]	Spec. act. (U/mg)	Purification (fold)	Yield (%)
Crude extract	1241	4219	3.4	1	100
Heat(70 °C, 10 min)	694	4093	5.9	1.7	97
Ammonium sulfate	105	2604	24.8	7.3	62
Hydroxylapatite	13	1394	108	31.8	33
Phenyl Superose	3.0	662	218	64.1	16
Superose HR 10/30	1.0	486	472	139	12
Mono-Q	0.37	257	695	204	6

[a]One unit equals 1 μmol of phosphate per min.

Purified pyrophosphatase kept for several weeks at −20 °C showed two bands on native gel after activity staining. By the use of gel filtration, molecular masses of approximately 140 and 70 kDa were found. This indicates a partial cleavage of pyrophosphatase into two active parts each consisting of three subunits during storage at −20 °C.

Heat stability

Pyrophosphatase of *A. johnsonii* 210A appeared to be quite resistant to heat treatment in the presence of magnesium salts. As much as 60% of the activity remained after 10 min at 80 °C in the presence of 17 mM Mg^{2+}. The enzyme lost 90% of its activity when heated for 10 min at 90 °C in the presence of 17 mM Mg^{2+}. In the absence of added Mg^{2+}, the enzyme was much less heat stable: only 10 to 25% of the activity was retained after treatment for 10 min at 70 °C.

Substrate specificity

The purified pyrophosphatase was able to hydrolyze pyrophosphate, and triphosphate at a 4 times reduced rate. Other polyphosphates such as tetraphosphate, pentaphosphate and long chain polyphosphate with 35 P-groups (P35) and organic phosphate esters could not be cleaved by the enzyme (Table 2). The commercial triphosphate and tetraphosphate applied in this experiment were purified by ion-exchange chromatography prior to use, P35 was not purified before use because contamination with pyrophosphate and triphosphate could not be detected by ion-exchange chromatography.

The hydrolysis of pyrophosphate and triphosphate by the enzyme was dependant on the presence of divalent cations. Of the cations tested at a concentration of 10 mM and pyrophosphate as substrate, Mg^{2+} (100%), Zn^{2+} (182%), Mn^{2+} (48%) and Co^{2+} (48%) were effective. Their relative activities were 100, 182, 48 and 48, respectively. Ba^{2+} and Ca^{2+} did not support pyrophosphatase activity. P35 applied at an concentration of 0.2 mg per ml, did not reduce the cleavage rate of pyrophosphate by pyrophosphatase.

Table 2. Substrate specificity of pyrophosphatase of *Acinetobacter johnsonii* 210A

Substrate	Concentration	Relative rate of hydrolysis
Pyrophosphate	2 mM	100
Triphosphate	2 mM	27
Tetraphosphate	2 mM	0
Polyphosphate (n = 35)	0.2 mg/mi	0
ATP	2 mM	0
ADP	2 mM	0
AMP	2 mM	0
Glucose-6-phosphate	2 mM	0
Phosphoenolpyruvate	4 mM	0
p-Nitrophenylphosphate	2 mM	0

Kinetic properties

The initial reaction rates at different pyrophosphate concentrations were determined to estimate the affinity of the enzyme for its substrate. Half maximal reaction rates were obtained at 0.26 ± 0.02 mM pyrophosphate, at a molar Mg^{2+}/pyrophosphate ratio of 3. The enzyme activity depended on this ratio. At ratios below 1, the cleavage of pyrophosphate by the purified enzyme was reduced by approximately 50%.

Inhibition studies

Similar to the situation in *E. coli* (Josse & Wong 1971), *Ferrobacillus ferrooxidans* (Howard & Lundgren 1970), *Thiobacillus thiooxidans* (Tominaga & Mori 1977) and *Bacillus stearothermophilus* (Hachimori et al. 1975), pyrophosphatase of *A. johnsonii* 210A was completely (100%) inhibited by sodium fluoride (2 mM). Sodium azide and potassium cyanide, both tested at a concentration of 2 mM, did not affect enzyme activity. Pyrophosphatase was also completely inhibited by 2 mM iodoacetamide, suggesting an involvement of sulfhydryl groups in the active site of the enzyme.

Discussion

With respect to heat stability, metal requirement and substrate specificity, pyrophosphatase of *Acinetobacter johnsonii* 210A appears to be similar to other bacterial pyrophosphatases. The enzyme of strain 210A clearly belongs to the thermostabile enzymes since it could withstand a temperature of 80 °C for at least 10 min. Enzymes isolated from other Gram-negative bacteria showed a somewhat similar heat resistance (Josse 1966; Blumenthal et al. 1970; Howard & Lundgren 1970; Tominaga & Mori 1977). Although the pyrophosphatase of *A. johnsonii* 210A is relatively thermostabile, storage at −20 °C resulted in partial degradation of the enzyme in two identical subunits that had retained their activity. As far as metal requirement is concerned, the divalent cations Mg^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+} all supported the pyrophosphatase activity of strain 210A, in particular Zn^{2+}. These cations were also found to be effective for other bacterial pyrophosphatases (Josse 1966; Howard & Lundgren 1970), including the enzyme of *E. coli*.

Pyrophosphatase of *A. johnsonii* 210A could only use pyrophosphate and triphosphate as substrate. Other substrates such as higher molecular weight polyphosphates and phosphate esters, almost all of them tested at a concentration of 2 mM, were not cleaved. A similar high substrate specificity has been observed for other microbial pyrophosphatases (Josse 1966; Hachimori et al. 1975; Tominaga & Mori 1977; Lahti & Niemi 1981).

In bacteria which do not accumulate significant amounts of polyphosphate such as *E. coli* (Josse 1966) and *Methanotrix soehngenii* (Jetten et al. 1992) 0.2% of the soluble protein consists of pyrophosphatase. In *A. johnsonii* 210A, 0.5% of the soluble protein consists of pyrophosphatase. This is significantly more than in the former two bacteria and this difference is possibly due to a difference in phosphate metabolism between these two organisms and *A. johnsonii* 210A which accumulates significant amounts of high-molecular weight polyphosphates that are degraded during anaerobiosis (Van Groenestijn 1988). Pyrophosphatase of *A. johnsonii* 210A may be involved in the biodegradation of high-molecular polyphophate in two ways. First, on long-term incubation with polyphosphatase, low-molecular polyphosphates such as tetraphosphate, triphosphate and pyrophosphate are formed (Bonting et al. 1993b). The present paper shows that the thus formed triphosphate and pyrophosphate can be further hydrolysed to orthophosphate by pyrophosphatase. Second, it was shown previously (Bonting et al. 1991) that the hydrolysis of high-molecular polyphosphate by polyphosphate: AMP phosphotransferase was inhibited by tetra-, tri- and pyrophosphate. Since the cellular concentration of pyrophosphate and triphosphate depends on the intracellular pyrophosphatase activity, this enzyme activity also enhances polyphosphate degradation by the above phosphotransferase via a removal of the inhibiting pyrophosphate and triphosphate.

As far as formation of polyphosphate is concerned, in *Rhodospirillum rubrum* pyrophosphate might serve as a primer in polyphosphate formation (Oh & Lee 1987). Kulaev postulated a reaction in which activated phosphate is transferred from pyrophosphate to polyphosphate (Kulaev 1979). In cell-free extracts of *A. johnsonii* 210A, pyrophosphate could not be shown to be a precursor in polyphosphate formation, the same was true of glucose-6-phosphate, acetylphosphate, phosphoglycerate and phosphoenolpyruvate. In this organism polyphosphate formation is most likely mediated by polyphosphate kinase (Van Niel et al. 1999) and pyrophosphatase acts only hydrolytically.

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