JOURNAL OF BACTERIOLOGY, Oct. 1986, p. 318–321
0021-9193/86/100318-04$02.00/0

Vol. 168, No. 1

Purification and Characterization of an Inorganic Pyrophosphatase from the Extreme Thermophile *Thermus aquaticus*

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Received 6 March 1986/Accepted 11 June 1986

An inorganic pyrophosphatase was purified over 600-fold to homogeneity as judged by polyacrylamide gel electrophoresis. The enzyme is a tetramer of M_r = 84,000, has a sedimentation coefficient of 5.8S, a Stokes radius of 3.5 nm, and an isoelectric point of 5.7. Like the enzyme of *Escherichia coli*, the pyrophosphatase appears to be made constitutively. The pH and temperature optima are 8.3 and 80°C, respectively. The K_m for PP_i is 0.6 mM. A divalent cation is essential, with Mg^{2+} preferred. The enzyme uses only PP_i as a substrate.

Thermus aquaticus is an extreme bacterial thermophile (2). Since it can be grown on defined media at temperatures up to 80°C, it is widely used for studying life processes at high temperatures. To gain a better understanding of the overall physiology of *T. aquaticus*, we have been investigating some of its enzymes. We have previously reported the purification and characterization of a repressible alkaline phosphatase (19, 26) which has the ability to hydrolyze a wide variety of phosphate monoesters. However, it is inactive on PP_i, a substrate easily hydrolyzed by the *Escherichia coli* analog (6).

This communication details the purification and characterization of an enzyme from *T. aquaticus* with PP_i as a substrate. Inorganic pyrophosphatases are ubiquitous and may play an important role in shifting the overall equilibrium of many anabolic processes, suggesting a potential use as a molecular tool in vitro. Several enzymatic processes, including DNA and RNA synthesis, are inhibited by small amounts of PP_i (4, 7). Addition of the pyrophosphatase could shift the equilibrium in favor of synthesis. Moreover, in certain pathways, PP_i may replace ATP as a source of energy (12). It has been speculated that PP_i might be the evolutionary precursor for ATP (25).

MATERIALS AND METHODS

Strain. *T. aquaticus* YT-1 was supplied by Paul Ray (Burroughs Wellcome Co., Research Triangle Park, N.C.).

Culture medium. Cells were grown in a defined salts medium containing 0.3% glutamic acid (carbon and nitrogen source) and supplemented with biotin and thiamine (each at 0.1 mg/liter) and nicotinic acid (0.05 mg/liter). The salts included in 1 liter were: nitrilotriacetic acid, 100 mg; $CaSO_4 \cdot 2H_2O$, 60 mg; $MgSO_4 \cdot 7H_2O$, 100 mg; NaCl, 8 mg; KNO_3, 103 mg; $NaNO_3$, 689 mg; $ZnSO_4 \cdot 7H_2O$, 5 mg; H_3BO_3, 5 mg; $CuSO_4$, 0.16 mg; $NaMoO_4 \cdot 2H_2O$, 0.25 mg; $CoCl_2 \cdot 6H_2O$, 0.4 mg; $FeCl_3$, 0.28 mg; and $MnSO_4 \cdot H_2O$, 22 mg. Phosphate, as indicated below, was then added to the medium, and the pH was adjusted to 8.0 with NaOH.

Growth conditions. Cells were grown in four 500-ml Erlenmeyer flasks at 75°C in a water bath shaker. When the cultures reached 170 Klett units, 1 liter was transferred to a 16-liter carboy in a hot-water incubator maintained at 75°C. Sterile air was used to aerate the cultures. After 24 h, the cells were collected by centrifugation.

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Enzyme assays. Alkaline phosphatase was measured with 1 mM *p*-nitrophenyl phosphate in 500 mM Tris hydrochloride (pH 9.0) at 75°C. The reaction was stopped after 10 min with 0.5 ml of 0.4 M NaOH, and the absorbance was read at 410 nm. One unit of activity is defined as the release of 1 μmol of nitrophenol per min at 75°C.

Inorganic pyrophosphatase was assayed by the methods of Lanzetta et al. (11) and Taussky and Shorr (20). The former method was more sensitive and used for determination of K_m. The assay (1.0 ml) contained 100 mM Tris hydrochloride (pH 8.3), 1 mM PP_i, 1 mM $MgCl_2$, and enzyme. After 5 min at 80°C, the reaction was stopped on ice. A 50-μl sample was mixed with 800 μl of a 3:1 mixture of 0.045% malachite green hydrochloride and 4.2% ammonium molybdate in 4 N HCl (11). After 1 min, 100 μl of 34% sodium citrate was added. After 30 min at room temperature, the A_{660} was measured. One unit of activity is defined as the release of 1 μmol of phosphate per min at 80°C.

Protein determinations. Protein concentrations were determined by the method of Lowry et al. (14).

Gel electrophoresis. Nondenaturing tube gels (9.5 cm by 0.20 cm^2) were prepared by the method of Davis (3), with a 7% acrylamide running gel and a 2% stacking gel. Electrophoresis was carried out at 4°C in 2.5 mM Tris–1.9 mM glycine buffer (pH 9.5) for 2.0 h at 2.5 mA per gel. The gels were stained with 0.1% Coomassie brilliant blue in 7% acetic acid. Sodium dodecyl sulfate-polyacrylamide slab gels of 0.15 mm thickness, with an 11% running gel and a 3% stacking gel, were prepared and run by the method of Laemmli (8). Samples were heated for 10 min in boiling water in 60 mM Tris hydrochloride (pH 6.8)–4% sodium dodecyl sulfate–0.7 M-mercaptoethanol–12% (wt/vol) glycerol–0.001% bromophenol blue. The gels were stained with both 0.1% Coomassie brilliant blue and silver stain (16).

Glycerol gradient centrifugation. Glycerol gradients (10 to 30% [vol/vol]) were prepared in a buffer containing 20 mM Tris hydrochloride (pH 8.0), 5 mM β-mercaptoethanol, and 1 mM EDTA. Samples (100 μl) of inorganic pyrophosphatase, yeast alcohol dehydrogenase (7.6S), bovine serum albumin (4.3S), and myoglobin (2.0S) were layered on the gradient. Centrifugation was carried out for 24 h at 40,000 rpm at 2°C in a Beckman SW50.1 rotor. Fractions of 140 μl were collected from the bottom of the tube and assayed immediately. Sedimentation coefficients were determined by the method of Martin and Ames (15).

Sephacryl S-300 gel filtration. A Sephacryl S-300 column (112.5 cm by 0.20 cm^2) equilibrated in 20 mM Tris hydro-

TABLE 1. Effects of P_i and PP_i concentrations on the levels of inorganic pyrophosphatase and alkaline phosphatase in *T. aquaticus*[a]

Medium addition	Concn (M)	Sp act (U/mg)	
		Inorganic pyrophosphatase	Alkaline phosphatase
P_i	8×10^{-3}	9.0	0.1
	4×10^{-5}	7.1	13.3
PP_i	8×10^{-3}	9.0	0.1
	4×10^{-5}	7.4	70.1

[a] Cells were grown in 500-ml Erlenmeyer flasks at 75°C, and crude extracts were prepared and assayed as described in the Materials and Methods.

chloride (pH 8.0)–5 mM β-mercaptoethanol–1 mM EDTA–10% (wt/vol) glycerol–0.5 M NaCl was used for purification and to determine the Stokes radius of the enzyme (1). Native molecular weights were calculated by the method of Siegel and Monty (18).

RESULTS

Relative levels of inorganic pyrophosphatase in *T. aquaticus*. The relative levels of alkaline phosphatase and inorganic pyrophosphatase were compared in media containing high or low concentrations of P_i and PP_i. In contrast to alkaline phosphatase, which is inducible up to 700-fold, the levels of inorganic pyrophosphatase remained relatively constant under all conditions tested (Table 1). This indicates that the pyrophosphatase is constitutive and that it is not regulated by the phosphate concentration in the medium nor is it induced by its substrate.

Purification of inorganic pyrophosphatase. A typical purification scheme is summarized in Table 2. For the purification of the enzyme, 16 liters of cells were harvested and processed. After collection, the cells were suspended in 0.01 M Tris hydrochloride (pH 8.0) on ice and ruptured with a Branson 20-kc magnetostrictive ultrasonic oscillator, operated at 3.5 A for 20 s. All purification steps were carried out at 4°C. The extract was centrifuged at 27,000 × *g* for 10 min, and the supernatant fluid was removed (crude extract). This was passed over a DEAE-Sephacel column (19.8 cm^2 by 35.4 cm) equilibrated in 40 mM Tris hydrochloride (pH 8.0)–1 mM β-mercaptoethanol. The proteins were eluted with a 2,000-ml gradient of 0 to 0.7 M NaCl in starting buffer at a flow rate of 40 ml/h. The enzyme peak eluted at ~0.25 M NaCl. The fractions were pooled, dialyzed against 40 mM Tris hydrochloride (pH 8.0)–1 mM β-mercaptoethanol, and applied to a hydroxylapatite column (2.27 cm^2 by 26.4 cm) equilibrated in the same buffer. The proteins were eluted with a 1,000-ml linear gradient of 0 to 10 mM PP_i in starting buffer at a flow rate of 20 ml/h. The peak eluted at ~1.7 mM PP_i.

The enzyme was dialyzed against 5 mM PP_i–30 mM NaCl–40 mM Tris hydrochloride (pH 8.0)–1 mM β-mercaptoethanol. The enzyme was rechromatographed on a DEAE-Sephacel column (2.27 cm^2 by 26.4 cm). A double linear gradient of 500 ml was used to elute the enzyme (17). The initial buffer contained 5 mM PP_i and 30 mM NaCl, while the final buffer contained no PP_i and 1.0 M NaCl.

Active fractions were pooled, dialyzed against 20 mM Tris hydrochloride (pH 8)–0.5 M NaCl–5 mM β-mercaptoethanol–1 mM EDTA–10% (vol/vol) glycerol, and concentrated by vacuum dialysis to 250 μl. The sample was brought to a volume of 850 μl with 40% (vol/vol) glycerol and layered onto a Sephacryl S-300 column equilibrated in dialysis buffer (see above). The peak fractions were pooled, dialyzed, and concentrated as before. The enzyme was then electrophoresed on nondenaturing polyacrylamide gels. The gels were then sliced into 0.3-cm segments, and the enzyme was recovered by incubation in 40 mM Tris hydrochloride (pH 8.0)–1.0 M NaCl–1 mM β-mercaptoethanol at 70°C for 30 min.

Criteria of homogeneity. The purified inorganic pyrophosphatase was subjected to electrophoresis on both nondenaturing and denaturing gels. One of the nondenaturing gels was stained for protein; a sister gel was sliced, eluted, and assayed. A single band was seen which corresponded to the activity (Fig. 1). Upon electrophoresis on a sodium dodecyl sulfate gel, a single protein band was also seen (data not shown).

Physical properties. The physical properties of the enzyme are summarized as follows. The purified enzyme gave a single protein band on denaturing polyacrylamide gels with an M_r = 21,000. Using the method of Siegel and Monty (18) to calculate native molecular weight from the Stokes radius (3.5 nm) and sedimentation value (5.8S), a native M_r = 84,000 was obtained. Since the denatured molecular weight of the enzyme is 21,000, this indicates that the enzyme is a tetramer of four equal-sized (and most likely identical) subunits. No active monomers could be obtained. The isoelectric point of the enzyme is 5.7.

Temperature optimum. Since *T. aquaticus* can be grown at temperatures up to 80°C, the temperature optimum of the enzyme was determined and found to be ~80°C (Fig. 2). This corresponds to the optima of other enzymes previously studied in this organism.

Effect of pH. The activity of the enzyme was assayed at various pH values at 80°C, using Tris hydrochloride or Tricine-NaOH. The Tris system provided better activity at a pH optimum of 8.3 (Fig. 3).

Divalent cation requirement. The effect of various divalent cations on the catalytic activity of the enzyme was investigated. The enzyme has an absolute requirement for divalent cations. Magnesium provided the best activity at an optimum of 3 mM. Cobalt, manganese, and zinc could be substituted, resulting in 21.6, 19.3, and 12.8%, respectively, of the activity observed with magnesium.

Michaelis-Menten constant. The K_m of the enzyme for PP_i was 0.6 mM. This is similar to the K_m of the alkaline phosphatase from *T. aquaticus*. The use of the more sensitive method of Lanzetta et al. (11), along with the necessity of keeping the magnesium/PP_i ratio constant at 3/1, was critical to the determination of this value.

Substrate specificity. Other phosphate esters such as AMP,

TABLE 2. Purification of inorganic pyrophosphatase from *T. aquaticus*

Fraction	Total U[a] (10^3)	Total protein (mg)	Sp act (U/mg)	Purification (fold)	Yield (%)
Crude extract	157.0	5,250	30	1	100
DEAE-Sephacel I	89.4	358	250	8	57
Hydroxylapatite	63.3	23.5	2,690	90	40
DEAE-Sephacel II	47.4	7.1	6,680	223	30
Sephacryl S-300	18.0	1.5	12,000	400	11
Polyacrylamide gel electrophoresis	12.2	0.6	20,300	677	8

[a] One unit of inorganic pyrophosphatase activity is 1 μmol of P_i released per min at 80°C.

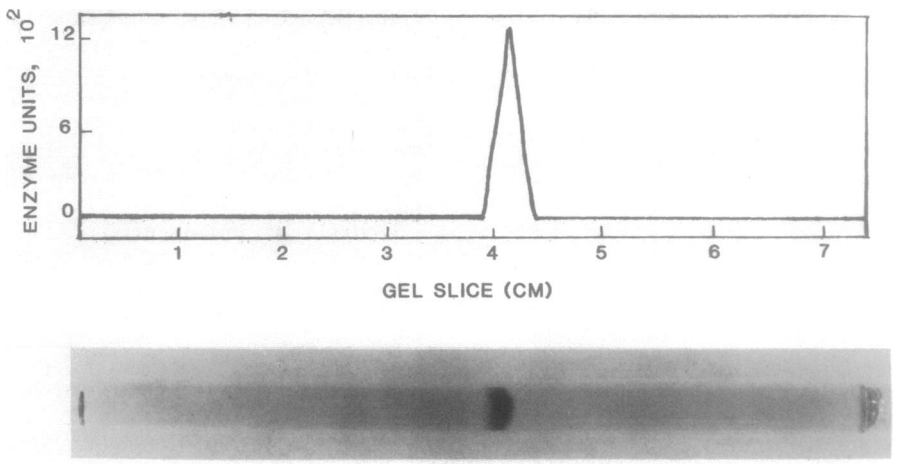

FIG. 1. Nondenaturing polyacrylamide gel electrophoresis of purified inorganic pyrophosphatase from *T. aquaticus*. Purified enzyme (5 μg) was applied to tube gels and subjected to electrophoresis at 4°C in 2.5 mM Tris–1.19 mM glycine buffer, pH 9.5. One gel was stained for protein. A sister gel was sliced and electroeluted, and the resulting eluant was tested for enzymatic activity. One unit of activity is the release of 1 μmol of P_i per min at 80°C.

D-glucose-6-phosphate, and *p*-nitrophenyl phosphate were tested as potential substrates for the purified enzyme. Only PP_i could serve as a substrate. This is in contrast to the alkaline phosphatase from *T. aquaticus* which can hydrolyze a wide variety of phosphorylated compounds but not PP_i (26).

DISCUSSION

Several studies have indicated that enzymes and other proteins of thermophiles are very similar to their corresponding counterparts from mesophiles, except for having a much higher thermostability and temperature optimum (13). The present report indicates that this is also true of the inorganic pyrophosphatase from *T. aquaticus*.

The level of the inorganic pyrophosphatase was unaffected by the concentration of P_i or PP_i in the medium, which has been found with several other bacterial species (9), indicating that this class of enzymes is constitutive and noninducible. Based on specific activity, the inorganic pyrophosphatase accounts for approximately 0.15% of the soluble protein of the bacterial cell. The enzyme has a pH optimum of 8.3 and requires a divalent cation for enzymatic activity. The native molecular weight of the enzyme is approximately 84,000. This value is considerably lower than that of inorganic pyrophosphatases from *E. coli* (24), *Bacillus stearothermophilus* (5), and *Streptococcus faecalis* (10) which have a molecular weight of about 120,000. In contrast, the *T. aquaticus* enzyme is similar in size and subunit composition to the inorganic pyrophosphate from *Thiobacillus thiooxidans*, a chemolithotropic bacterium (21) with a pyrophosphatase of about M_r = 88,000.

The pyrophosphatase, like most other thermophilic en-

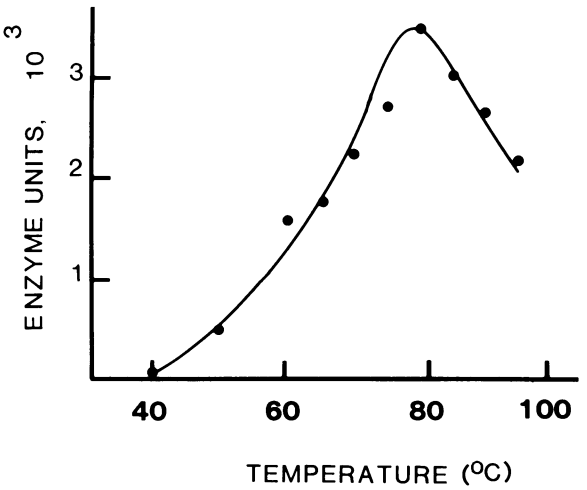

FIG. 2. Effect of temperature on the activity of purified inorganic pyrophosphatase. The enzyme (0.05 μg per assay tube) was incubated for 5 min at various temperatures as indicated in the figure. The reactions contained 100 mM Tris buffer (pH 8.3), 1 mM PP_i, and 3 mM $MgCl_2$. The assay of Taussky and Schorr (20) was used. The pH of the buffer was adjusted at each temperature, as Tris has a high temperature coefficient. One unit of activity is the release of 1 μmol of P_i per min.

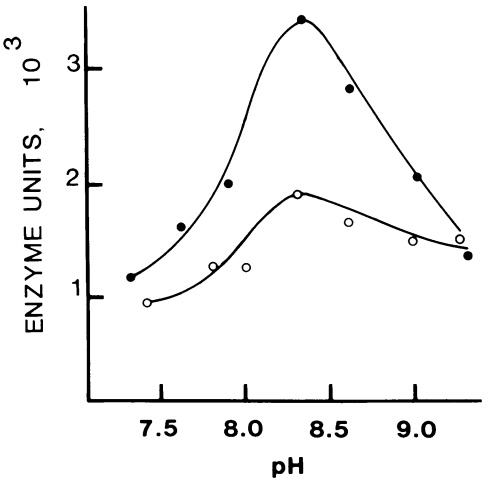

FIG. 3. Effect of pH on the activity of purified inorganic pyrophosphatase. The enzyme (0.05 μg per assay tube) was incubated at 80°C for 5 min at various pHs as given in the figure. The reaction mixture contained 1 mM PP_i, 3 mM $MgCl_2$, and 100 mM of either Tris buffer (●) or Tricine-NaOH buffer (○). One unit of enzyme activity is the release of 1 μmol of P_i per min at 80°C.

for multiple enzyme reactions, especially those that are immobilized such as the system used to generate NAD from nicotinamide mononucleotide (22). In this study, NAD pyrophosphorylase was immobilized by absorption on hydroxylapatite, and inorganic pyrophosphatase was immobilized by ionic binding to DEAE-cellulose. The addition of the inorganic pyrophosphatase increased the conversion of nicotinamide mononucleotide to NAD from 50% to 80%. We immobilized the *T. aquaticus* pyrophosphatase by drying onto nitrocellulose filters and maintained catalytic activity.

The pyrophosphatase, in contrast to the alkaline phosphatase of *T. aquaticus* (26), probably does not play a significant role in providing phosphate to the cell. The latter enzyme responds to growth in low phosphate with a 700-fold induction of the enzyme (26). For *E. coli*, Wilkins (23) has shown that regulation of alkaline phosphatase by phosphate is linked to intracellular nucleotide pool size. The pyrophosphatase probably plays an important role in preventing end product inhibition of other enzymes utilizing nucleoside triphosphates and producing PP_i as a product. Enzymes involved in DNA and RNA biosynthesis, as well as aminoacyl-tRNA synthetases, appear to be strongly inhibited by PP_i. The pyrophosphatase would ensure maintenance of a high rate of reaction for these enzymes. In this respect, *T. aquaticus* pyrophosphatase, with its high stability, may prove useful in vitro. Indeed, this enzyme can be used to increase the rate and extent of reaction of terminal deoxyribonucleotidyl transferase in the biosynthesis of synthetic homopolymers (R. R. Meyer, unpublished data).

ACKNOWLEDGMENTS

We thank Douglas Winget for his help and many useful suggestions.

This work was supported in part by a Grant-in-Aid of Research from the Society of Sigma Xi.

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