Inorganic Pyrophosphatase Activity in Cell-free Extracts of *Ureaplasma urealyticum*

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Cell-free extracts of *Ureaplasma urealyticum* strains Pi and T960 (CX8) (serovars 6 and 8, respectively) metabolized inorganic pyrophosphate (PP_i). The inorganic pyrophosphatase (PPase) activity was greatest with Mg²⁺ as cofactor, but Mn²⁺ acted as a poor substitute. The PPases of the two serovars differed electrophoretically. Although the highest PPase activity was obtained using PP_i as substrate, the enzyme could also utilize to a lesser degree both tripolyphosphate and trimetaphosphate. No activity was observed against β -glycerophosphate, naphthyl phosphates, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, thiamin pyrophosphate, phosphoribosylpyrophosphate, ADP or ATP. Acid- and alkaline-phosphatase activities were observed with naphthyl phosphates as substrates, but they did not have the same electrophoretic mobility on gels as the PPase activity. *U. urealyticum* PPase was inhibited by oxidized glutathione, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, phenylglyoxal, *p*-chloromercuribenzoic acid, Mn²⁺, Zn²⁺ and Ca²⁺. Neither reduced glutathione, L-cysteine nor Co²⁺ enhanced activity. PP_i can act as a substrate or regulator of certain metabolic reactions, and PP_i metabolism can function in bacterial bioenergetics; its role in ureaplasmas is presently unclear.

INTRODUCTION

Although thirty years have passed since the identification of *Ureaplasma urealyticum* (Shepard *et al.*, 1974), relatively little is known about its metabolism and energy generating pathways (Pollack, 1979, 1986; Shepard & Masover, 1979), mainly because of poorly defined growth requirements and consequent low cell yield in culture. These mollicutes are one of the smallest cellular forms known, surviving with an extremely small genome (Razin, 1985) and limited metabolic capacity (Pollack, 1986). As such, ureaplasmas can be useful research tools for identifying basic biochemical activities conserved in nature, and indicating the bare essentials for cell survival.

Urease, which appears to be the most abundant protein in crude cell-free extracts of *U. urealyticum*, has been partially characterized (Eng *et al.*, 1986). However, the role of urea hydrolysis in ureaplasma survival is still not clear. The most recent data suggest that ammonia diffusion subsequent to urea hydrolysis may be responsible for establishing a chemical potential across the ureaplasma membrane, thereby driving ATP formation (Romano *et al.*, 1980, 1986). While ureaplasmas are nonglycolytic organisms (Shepard & Masover, 1979), they have some enzymes of the glycolytic pathway (Cocks *et al.*, 1985), and, therefore, may have the potential to form pyruvate from other substrates. Although they have many of the enzymes for pentose

Abbreviations: ACP, acid phosphatase; ALP, alkaline phosphatase; PPase, inorganic pyrophosphatase; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; NEM, *N*-ethyl-maleimide; PCMB, *p*-chloromercuribenzoic acid; TNBS, 2,4,6-trinitrobenzenesulphonic acid.

phosphate metabolism (Cocks *et al.*, 1985), the importance of this pathway in ureaplasmas is still not clear (Pollack, 1986). *U. urealyticum* does not metabolize arginine, unlike some other nonfermentative mollicutes (Shepard & Masover, 1979). Enzymes of purine metabolism have been identified in *U. urealyticum* extracts (Cocks *et al.*, 1985; Davis *et al.*, 1984), so the possibility exists for energy conservation through the pathways of purine salvage. The presence of a complete tricarboxylic acid cycle and a cytochrome-containing electron transport system has not been shown (Pollack, 1979, 1986). We have been evaluating enzyme activities in cell-free extracts of ureaplasmas in an attempt to identify the presence of other pathways that may play an important role in the pathogen's metabolism.

Inorganic pyrophosphate (PP_i) metabolism and the roles of inorganic pyrophosphatases (PPases) (EC 3.6.1.1) in microbes have been reviewed by Lahti (1983). PP_i may be a substrate, functioning as a source of energy while donating orthophosphate in ATP-sparing reactions (Wood, 1977). This requires different PP_i-dependent (ATP-sparing) enzymes which have been identified in a small number of microbes, including the mollicute *Acholeplasma laidlawii* (Pollack & Williams, 1986; Tryon & Pollack, 1984). In addition, the PP_i in photosynthetic bacteria can serve as an energy donor in several energy-generating metabolic reactions (Keister & Minton, 1971; Keister & Raveed, 1974), and contribute to the establishment of an energy-linked proton-motive force (Moyle *et al.*, 1972). As a regulator, PP_i tends to inhibit particular reactions; PPase could therefore limit inhibition by reducing the PP_i level (Lahti, 1983).

Since PP metabolism may be linked to bacterial bioenergetics, extracts of *U. urealyticum* were tested for PPase activity. Because the 14 serovars of *U. urealyticum* can apparently be separated into two distinct clusters (Razin *et al.*, 1983; Robertson & Stemke, 1982), extracts of two serovars (6 and 8) were compared as representatives of the different clusters.

METHODS

Chemicals. Unless noted otherwise, chemicals and enzymes were obtained from Sigma.

Cultures. *Ureaplasma urealyticum* strains Pi (serovar 6) and T960 (CX8) (serovar 8) were cultured in a modification of U17-B medium (Sayed & Kenny, 1980; Shepard & Luncford, 1978). Basal medium contained (w/v): trypticase soy broth (BBL), 1.5%; yeast extract (Difco), 0.2%; NaCl, 0.5%; MgCl₂, 0.05%; MES (Research Organics), 1.76%; and phenol red (United States Biochemical), 0.001%. The basal medium was brought to pH 6.0, clarified by filtration (0.2 µm), and sterilized by autoclaving (121 °C, 15 min). Supplement for the basal medium contained γ-globulin-free horse serum (Gibco Laboratories, Life Technologies Inc.), 1.0% (v/v); CVA enrichment (Gibco), 0.25% (v/v); urea (ultra-pure, Schwarz-Mann Biotech), 30 mM; sodium sulphite, 1.0 mM; and penicillin G, 10 U ml⁻¹. Supplement was clarified by sterile filtration (0.2 µm) and then added to the cooled sterile basal medium. Cultures were inoculated (1:1000) with an exponential phase culture and incubated for 22–24 h at 37 °C. This procedure produced a colour changing unit titre (Rodwell & Whitcomb, 1983) of 10⁶–10⁷.

Preparation of cell-free extract. A modification of the procedure of O'Brien *et al.* (1983) was used. Cells in exponential growth were collected by centrifugation (20 000 g, 45 min, 4 °C), washed twice in buffered saline (0.25 M-NaCl; 0.01 M-MgCl₂; 0.02 M-Tris, pH 7.5), suspended in hypotonic extraction buffer (0.01 M-Tris, 0.001 M-EDTA, 0.1% Triton X-100, pH 7.4), subjected to freeze-thaw (–80 °C to 37 °C), and sonicated at 200 W for 10 min (ten cycles of 1 min sonication followed by 1 min cooling, all on ice). The sonicate was centrifuged (37 000 g, 45 min, 4 °C) and the supernatant extract was concentrated using an ultrafiltration system (Centricon 10, Amicon). The concentrate was diluted with an equal volume of sample buffer (0.06 M-Tris, 0.28% Triton X-100, 20% glycerol, pH 6.8). The final protein concentration was determined by the technique of Bradford (1976) using a Bio-Rad protein assay kit, with bovine serum albumin as the standard. Samples of extract were stored at –80 °C until used.

Electrophoresis of cell-free extracts. Proteins in the cell-free extract were electrophoretically separated on polyacrylamide gels to provide partial purification of the enzyme to be assayed. Sample wells were loaded with 30 µg extract protein. Electrophoresis was performed using a modification of the Laemmli discontinuous buffer system (Hames, 1981), substituting Triton X-100 (0.1%) for SDS. An LKB 2001 vertical electrophoresis unit was used with 1.5 mm thick gel slabs prepared with a 7.0% acrylamide resolving gel and a 3.75% acrylamide stacking gel. Acrylaide (FMC Corp.) was used as the crosslinker (acrylamide:Acrylaide, 32:1), and gels were cast on GelBond (FMC Corp.) to facilitate handling of the gel for the enzyme assays. Electrophoresis was carried out at 100 V per gel using an LKB 2002 power supply, with cooling to 15–18 °C. Electrophoresis was stopped when the tracking dye was 1 cm from the bottom of the gel (approx. 5 h). Gels were rinsed with reagent-grade water before use in assays.

Assays for PPase activity. After electrophoresis, gels were cut into five to seven test strips for enzyme assays. The assay system for PPase activity was a post-incubation coupling technique (Harris & Hopkinson, 1976) utilizing an assay mixture of 0.025 M-Tris, 5.0 mM-PP_i, pH 7.8; 5.0 mM-MgCl₂; and 1% Noble agar. Gel test strips were overlaid with assay mixture and incubated at 37 °C. After 45 min the overlay was removed. The test strip was rinsed with water and immersed in developer (1.25%, w/v, molybdic acid in 2 M-H₂SO₄; 5%, w/v, L-ascorbic acid added just before application) in the dark to indicate the sites of phosphate release (stained areas appeared in approx. 15 min).

Yeast PPase (0.2 U) and concentrated culture medium (30 µg protein) were run as controls. Substrates (5.0 mM) tested in the assay incubation mix included: tetrasodium pyrophosphate (PP_i, J. T. Baker Co.), pentasodium tripolyphosphate, trisodium trimetaphosphate, phosphoribosylpyrophosphate (PRPP), thiamin pyrophosphate (TPP), ATP, ADP, fructose 1,6-bisphosphate, β-glycerophosphate, α- and β-naphthyl phosphates, glucose 6-phosphate and fructose 6-phosphate.

Divalent cations (Mn²⁺, Ca²⁺, Cd²⁺, Co²⁺, Cu²⁺, Zn²⁺) (CdCl₂ and ZnCl₂ from Fisher Scientific Co.) were evaluated as cofactors for *U. urealyticum* PPase. The cations were tested individually both as a replacement for Mg²⁺ and as ions competing with Mg²⁺.

PPase activity in the crude cell-free extracts was also assayed using a pyrophosphate assay reagent (Sigma), following the manufacturer's instructions. The procedure was used to evaluate PP_i hydrolysis during an 8 min period, samples with extract (95 µg extract protein, per ml PP_i standard) being compared to others containing an equal volume of sample buffer.

Inhibition and activation of PPase activity. PPase activity was also evaluated for the effects of NH₄⁺ (5.0 mM, 50.0 mM), *N*-ethylmaleimide (NEM, 0.5 mM), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC, 10.0 mM), oxidized glutathione (1.0 mM), reduced glutathione (1.0 mM), sodium fluoride (20.0 mM, Fisher Scientific Co.), *p*-chloromercuribenzoic acid (PCMB, 0.5 mM), phenylglyoxal (10.0 mM), 2,4,6-trinitrobenzenesulphonic acid (TNBS, 0.5 mM), and L-cysteine (0.5 mM).

Assays for acid phosphatase (ACP) and alkaline phosphatase (ALP) activities. Partially purified enzyme was used in simultaneous capture techniques to assay for ACP and ALP activities (Harris & Hopkinson, 1976). The ACP assay mixture consisted of 0.05 M-citrate buffer, pH 4.5; 0.1% substrate; 0.12% MgSO₄·7H₂O; and 0.1% Fast Blue BB or RR. The ALP assay mixture contained 0.06 M-borate buffer, pH 9.7; 0.05% substrate; 0.12% MgSO₄·7H₂O; and 0.05% Fast Blue BB salt. Both enzyme activities were assayed with α-naphthyl phosphate and β-naphthyl phosphate as substrate, and with 80 µg extract protein. β-Glycerophosphate and PP_i were tested as ACP or ALP substrates by using them with the PPase post-incubation assay technique at pH 4.5 and 9.7. Potato ACP (0.36 U) and bovine ALP (0.5 U) were used as positive controls in these assays.

RESULTS

PPase activity in crude cell-free extract

Crude extracts of *U. urealyticum* actively hydrolysed PP_i. Under these assay conditions, the crude extract hydrolysed an average of 7.5×10^{-4} µmol PP_i min⁻¹ (µg extract protein)⁻¹, during the 8 min assay period.

Activity of partially purified PPase

PPase activity was found in extracts of both the *U. urealyticum* serovars tested (Fig. 1). Similar results were obtained with all batches tested (thirteen of serovar 8 and five of serovar 6). Enzyme activity did not vary detectably in the pH range 6.2 to 8.0. No PPase activity was found in the culture medium. Comparison of the activity sites on the gel indicated that the PPase of serovar 8 exhibited a relative mobility of 1.28, while that of serovar 6 had a relative mobility of 0.85. The greatest enzyme activity was obtained with Mg²⁺ as cofactor. Mn²⁺ (5.0 mM) was a poor substitute for Mg²⁺. Ca²⁺, Cd²⁺, Co²⁺, Cu²⁺, or Zn²⁺ (5.0 mM) could not replace Mg²⁺.

Tripolyphosphate could be used as substrate by extract PPase, but was hydrolysed less rapidly than PP_i. Trimetaphosphate was a poorer substrate than polyphosphate. Organic pyrophosphates, such as TPP, ATP and ADP, could not act as substrates, although extremely weak PPase activity was obtained with PRPP. The latter was most probably due to contaminating PP_i released by the relatively unstable PRPP.

Since other phosphatase activities have been reported in ureaplasmas (Black, 1973), enzymes in the gel test strips were assayed with a variety of other phosphatase substrates, including α-naphthyl phosphate, β-naphthyl phosphate, β-glycerophosphate, fructose 1,6-bisphosphate, fructose 6-phosphate and glucose 6-phosphate. Extracts of both serovars 6 and 8 possessed ACP

Fig. 1. Zymogram indicating PPase activity in extracts from *U. urealyticum*. Lane 1, yeast PPase, 0.2 U; lane 2, *U. urealyticum* strain T960 (CX8) (serovar 8); lane 3, *U. urealyticum* strain Pi (serovar 6). If the yeast PPase is assigned an electrophoretic mobility of 1.0, the relative mobilities of strains T960 and Pi are 1.28 and 0.85, respectively.

Fig. 2. Zymogram showing ACP activity in extracts from *U. urealyticum*. Lane 1, potato ACP, 0.36 U; lane 2, *U. urealyticum* strain T960 (CX8) (serovar 8); lane 3, *U. urealyticum* strain Pi (serovar 6).

activity only against the naphthyl phosphates (Fig. 2). Weak ALP activity in the extracts was observed with α -naphthyl phosphate only. ACP activity had a different electrophoretic mobility from the *U. urealyticum* PPase activity of either extract; similar results were obtained with ALP, indicating that the PPase and ACP/ALP are different. PP_i was not hydrolysed by the extract ACP or ALP.

Inhibition of PPase activity

Several different ions (5.0 mM) inhibited the *U. urealyticum* extract PPase activity when incubated with the enzyme for 15 min prior to the addition of assay mix with Mg²⁺. Mn²⁺ and Zn²⁺ resulted in almost complete inhibition of PPase activity, while the presence of Ca²⁺ resulted in only moderate inhibition. Cd²⁺, Co²⁺ and Cu²⁺ had no measurable inhibitory effect on the enzyme, while NH₄⁺ (5–50 mM) caused little or no inhibition.

The effect of known PPase inhibitors on yeast and *U. urealyticum* extract PPase activity was tested. NEM (0.5 mM) and TNBS (0.5 mM) had no effect on either PPase activity. NaF (20.0 mM) inhibited yeast PPase, but had no effect on extract activity. Phenylglyoxal (10.0 mM), EDAC (10.0 mM), PCMB (0.5 mM), and oxidized glutathione (1.0 mM) each inhibited enzyme activity of both *U. urealyticum* and yeast. These inhibitors were most effective when preincubated with the

gel strips prior to application of the assay mixture, rather than being added with the assay mixture at the time of incubation.

Activation of PPase activity

Neither L-cysteine (0.5 mM) nor reduced glutathione (1.0 mM) significantly activated *U. urealyticum* extract or yeast PPase activity, nor did Co^{2+} or Cu^{2+} enhance activity when added with the assay mix.

DISCUSSION

The electrophoretic technique and assay procedure used in this investigation overcame a significant problem in studying ureaplasma enzymes, i.e. low yield of cell-free extract from large volumes of culture (O'Brien & Barile, 1983). By separating and concentrating proteins, the electrophoretic technique permitted the study of enzyme activity in relatively small amounts of crude cell-free extract.

O'Brien *et al.* (1981, 1983) have reported the presence of PPase in each of 15 *Mycoplasma* species tested, and in three of five *Acholeplasma* species. They did not find the enzyme activity in *A. laidlawii*, *A. axanthum*, or *U. urealyticum*. Using slightly different methods we have found that extracts of *U. urealyticum* possess PPase activity, distinct from ACP or ALP activity. The extract was very active in hydrolysing PP_i in the presence of Mg^{2+} ; less activity was obtained with tripolyphosphate or trimetaphosphate as substrate, and these activities may have been due in part to PP_i contamination of the substrates. The enzyme did not hydrolyse organic pyrophosphates, indicating that MgPP_i is the probable substrate for the *U. urealyticum* PPase.

Comparison of the relative mobilities of PPases from *U. urealyticum* serovars 6 and 8 indicated that the enzymes are different. Since these two serovars are in the two different serovar clusters of *U. urealyticum*, the PPase activity could prove to be a useful marker for distinguishing the two clusters; however, this requires further study.

The optimum cofactor for the *U. urealyticum* PPase in the assay procedure (pH 7.8) is Mg^{2+} , suggesting a greater similarity to the PPases of *Streptococcus* and *Lactobacillus* (Lahti, 1983; Lahti & Niemi, 1981) than to the PPase of *Bacillus subtilis* (Tono & Kornberg, 1967) for which the optimum cofactor is Mn^{2+} , a cation that was not a cofactor for the enzyme of *Streptococcus faecalis* (Lahti & Niemi, 1981), and gave only slight activity with *U. urealyticum* PPase. Co^{2+} , which is a weak cofactor for *S. faecalis* PPase (Lahti & Niemi, 1981), did not support activity of the *U. urealyticum* enzyme. However, we evaluated the cations at a single concentration (5.0 mM), so the possibility remains that *U. urealyticum* PPase may be functional with other ions at different concentrations and/or a different pH, as Oginsky & Rumbaugh (1955) reported for Co^{2+} and the PPase of *S. faecalis*.

The inhibition of extract PPase activity by Mn^{2+} and Zn^{2+} may account in part for the growth inhibition observed when Mn^{2+} is present in culture medium (Robertson & Chen, 1984), and could explain the decrease in isolations of ureaplasmas from semen when the semen Zn^{2+} levels increase during prostatitis (Weidner *et al.*, 1985).

In his review, Lahti (1983) notes that the PPase of *Streptococcus* is reportedly activated by reduced glutathione and thiol compounds, and inactivated by oxidized glutathione and thiol inhibitors. The measured effect, however, depended upon the form of PPase studied, i.e. the high activity form associated with extracts of exponential phase cultures, or the low activity form found in older cultures. If the *U. urealyticum* PPase was similar, activators would have had little effect and thiol inhibitors a large effect on the enzyme activity, since the extract was prepared from cells in the exponential phase of growth. This is consistent with our observations that oxidized glutathione and PCMB were inhibitory, while reduced glutathione and L-cysteine produced little or no activation.

That PPase is present in organisms with such a small genome (i.e. mycoplasmas and ureaplasmas) may indicate that PP_i hydrolysis is a basic activity in the cell. Our studies do not identify the role of PP_i hydrolysis in ureaplasmas, for which there are several possibilities. The presence of PP_i -dependent phosphofructokinase in *A. laidlawii* B-PG9 suggests that PP_i

utilization in this organism is an energy-conserving (ATP-sparing) process (Pollack & Williams, 1986). Tryon & Pollack (1984) speculated that PP_i may be more available as a substrate in *A. laidlawii* B because this strain apparently lacks significant PPase activity. However, PPase was found in the majority of *Mycoplasma* and *Acholeplasma* species tested by O'Brien *et al.* (1981), and in the ureaplasmas tested here, suggesting that PPase-dependent PP_i hydrolysis in mollicutes may not function primarily to spare ATP hydrolysis.

Since Romano *et al.* (1980, 1986) have suggested that orthophosphate concentration influences urea hydrolysis which in turn affects ATP synthesis by ureaplasmas, it may be that PPase helps to regulate phosphate levels in these organisms thereby optimizing urease activity and subsequent ATP synthesis. PPase-regulated phosphate exchange across chromatophore membranes has been reported as a dark reaction of *Rhodospirillum* (De Meis *et al.*, 1986). In fact, PPase has been extensively studied with *Rhodospirillum*, an unusual prokaryote containing chromatophores in which PP_i metabolism is a significant part of the energy generating pathways. In these organisms, chromatophore PPase is involved in PP_i -driven ATP synthesis from ADP and P_i (Keister & Minton, 1971), P_i - PP_i exchange (Keister & Raveed, 1974), proton translocation across the membrane (Moyle *et al.*, 1972), and several other energy-linked reactions (cited in De Meis *et al.*, 1986; Keister & Raveed, 1974). The H^+ -translocating PPases of *Rhodospirillum* (Moyle *et al.*, 1972) and oat root tonoplast vesicles (Wang *et al.*, 1986) have been suggested as another source of proton motive force used for energy requiring processes. While energy generating mechanisms involving PP_i hydrolysis may function in *U. urealyticum*, this possibility needs confirmation.

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