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# A fluoride-insensitive inorganic pyrophosphatase isolated from *Methanothrix soehngenii*

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**Abstract.** An inorganic pyrophosphatase [E.C. 3.6.1.1] was isolated from *Methanothrix soehngenii*. In three steps the enzyme was purified 400-fold to apparent homogeneity. The molecular mass estimated by gelfiltration was 139 ± 7 kDa. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis indicated that the enzyme is composed of subunits with molecular masses of 35 and 33 kDa in an $\alpha_2\beta_2$ oligomeric structure. The enzyme catalyzed the hydrolysis of inorganic pyrophosphate, tri- and tetrapolyphosphate, but no activity was observed with a variety of other phosphate esters. The cation $Mg^{2+}$ was required for activity. The pH optimum was 8 at 1 mM $PP_i$ and 5 mM $Mg^{2+}$. The enzyme was heat-stable, insensitive to molecular oxygen and not inhibited by fluoride. Analysis of the kinetic properties revealed an apparent $K_m$ for $PP_i$ of 0.1 mM in the presence of 5 mM $Mg^{2+}$. The $V_{max}$ was 590 µmol of pyrophosphate hydrolyzed per min per mg protein, which corresponds to a $K_{cat}$ of 1400 per second.

The enzyme was found in the soluble enzyme fraction after ultracentrifugation, when cells were disrupted by French Press. Upto 5% of the pyrophosphatase was associated with the membrane fraction, when gentle lysis procedure were applied.

**Key words:** *Methanothrix soehngenii* – Acetate degradation – Energetics – Inorganic pyrophosphatase – Fluoride inhibition

*Methanothrix soehngenii (M. soehngenii)* uses solely acetate as carbon and energy source (Zehnder et al. 1980; Huser et al. 1982). The growth yield (1.4 g/mol acetate) and the growth rate (average doubling time of 7 days) are low, but the affinity ($K_s$ = 0.5 mM) for acetate of *M. soehngenii* is high (Huser et al. 1982; Jetten et al. 1990b). In *M. soehngenii* acetate is activated to acetyl-CoA by acetyl-CoA synthetase [1] (Jetten et al. 1989b).

| | | |
|---|---|---|
| Ac + ATP + HSCoA | → Ac-SCoA + AMP + $PP_i$ | [1] |
| AMP + ATP | → 2 ADP | [2] |
| $PP_i$ + $H_2O$ | → 2 $P_i$ | [3] |
| Ac + 2 ATP + HSCoA | → AcSCoA + 2 ADP + 2 $P_i$ | [4] |

The formed AMP is converted to ADP by adenylate kinase [2] and pyrophosphate is hydrolyzed by pyrophosphatase [3] (Jetten et al. 1989b). The sum of these reactions [4] indicates that acetate in *M. soehngenii* is activated to acetyl-CoA at the expense of two energy rich phosphate-bonds (Jetten et al. 1989b). Because the conversion of acetyl-CoA to methane is supposed to be coupled to the synthesis of 1 < ATP < 2 (Thauer et al. 1989), it is not likely that *M. soehngenii* will just hydrolyze pyrophosphate. For a favourable energy balance the energy from the pyrophosphate bond has to be conserved. Therefore, the inorganic pyrophosphatase of *M. soehngenii* was investigated.

Enzymes relatively specific for the hydrolysis of inorganic pyrophosphate are widely distributed in nature. Inorganic pyrophosphatases have been purified from heterotrophic (Josse 1966a; Wong et al. 1970; Josse and Wong 1971; Hachimori et al. 1975; Lathi and Niemi 1981; Lathi 1983), chemolithoautotrophic (Tominaga and Mori 1977), sulfate reducing (Ware and Postgate 1971) and phototrophic bacteria (Klemme et al. 1971; Randahl 1979) and from yeasts (Kunitz and Robbins 1961). The isolation and characterization of this enzyme from an archae is not yet reported. The purification and some of the properties of inorganic pyrophosphatase from *M. soehngenii* are described here.

## Materials and methods

### *Gases, chemicals, and biochemicals*

All chemicals were at least of analytical grade. Triton X-305, tri- and tetrapolyphosphate were purchased from Sigma (Amsterdam, The

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*Abbrevation:* PMSF, phenylmethylsulfonyl fluoride

Netherlands). Tetrasodium pyrophosphate was from Janssen (Beerse, Belgium). All other chemicals were from Merck (Darmstadt, FRG). Biochemicals were obtained from Boehringer Mannheim (Almere, The Netherlands). Sodium dodecylsulfate, acrylamide and hydroxylapatite were from Biorad (Utrecht, The Netherlands). Q-Sepharose, Phenyl Superose HR 5/5, Superose 6 HR 10/30 and molecular mass standards for gelfiltration and sodium dodecylsulfate polyacrylamide gelelectrophoresis were obtained from Pharmacia Fine Chemicals (Woerden, The Netherlands). Ti(III)citrate (100 mM) was prepared by adding 5 ml 1 M Ti(III)chloride to 40 ml of anaerobic 0.2 M sodium citrate solution, pH was adjusted with 2 M Tris to 7.5.

### *Growth of Methanothrix soehngenii*

*Methanothrix soehngenii* strain Opfikon, (Huser et al. 1982; Zehnder et al. 1989), was mass cultured on 80 mM sodium acetate in 25 l carboys with a sterile gas outlet, containing 20 l of the medium as described previously (Huser et al. 1982). Cultures were incubated without stirring at 35 °C in the dark under a $N_2/CO_2$ (80/20%) gas phase. Cells were harvested after 6 weeks at the late log phase by continuous centrifugation (Heraeus Sepatech, Osterode, FRG), washed in 50 mM anaerobic Tris/HCl pH 8.0, frozen in liquid nitrogen and stored at −20 °C under $N_2/H_2$ (96/4%). The yield was about 1 g wet weight per liter.

### *Enzyme purification*

The purification of the pyrophosphatase was regularly performed within one day. Unless indicated otherwise all procedures were carried out aerobically at room temperature (±19 °C). A frozen cell paste was thawed, diluted with 50 mM Tris/HCl pH 8.0 (Buffer A) in a 1:3 ratio, sonified 5 times 30 s and disrupted by passing twice through a French pressure cell at 135 MPa and centrifuged 10 min at 10,000 × *g*. In this way 90–95% of the cells were disrupted. The supernatant was centrifuged 150 min at 110,000 × *g*. The supernatant after ultracentrifugation contained about 10 mg of protein per ml and is referred to as crude extract. Protein was estimated with Coomassie brillant blue G250 as described by Bradford (1976).

When membrane association was studied, cells were lyzed more gentle. One gram of freshly haversted cells were resuspended in 10 ml 20 mM Tricine/KOH pH 7.5, 10mM $MgSO_4$ and 0.4 M sucrose (buffer B). The suspension was sonified 20 times one second to break the long filaments. The homogeneous suspension was centrifuged 20 min 15,000 × *g* and the pellet was resuspended in 20 ml of buffer B, containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The cells were lyzed by passage through a French pressure cell at 65 MPa and thereafter centrifuged 15 min at 12,000 × *g* to remove unbroken cells. This gentle method disrupted 40–50% of the cells. The supernatant was ultracentrifuged one hour at 300,000 × *g* and the pellet was washed three times with buffer B, containing 0.5 mM PMSF. The obtained membrane fraction was assayed immediately for pyrophosphatase, acetyl-coenzyme A synthetase and ATP-ase activity in the standard assays.

The crude extract (20 ml) was applied to a column (3.3 by 10 cm) of Q-Sepharose (fast flow) equilibrated with buffer A. The column was washed with 150 ml of buffer A. The inorganic pyrophosphatase was collected just after the void volume. The rest of the adsorbed protein was washed from the column in a 200 ml linear gradient of 1 M NaCl in buffer A. The flow rate was 3 ml/min. Fractions (12 ml) were collected and analysed for pyrophosphatase activity. Fractions with pyrophosphatase activity were pooled and concentrated in an Amicon ultrafiltration cell (Grace, Rotterdam, The Netherlands) with a PM 30 filter. The concentrated enzyme solution was applied to a hydroxylapatite column (1 by 6 cm) equilibrated with buffer A. The adsorbed protein was eluted from the column in a 120 ml linear gradient of 1.7 M $(NH_4)_2SO_4$ in buffer A. Fractions of 6 ml were collected at a flow rate of 2 ml/min. The inorganic pyrophosphatase was eluted at 0.7 M $(NH_4)_2SO_4$. Fractions with pyrophosphatase activity were pooled and concentrated in an Amicon ultrafiltration cell with a PM 30 filter. The remaining step in the purification was performed with a FPLC system (Pharmacia/LKB, Woerden, The Netherlands). The enzyme solution was mixed in a 1:1 ratio with 4 M $(NH_4)_2SO_4$ in buffer A and applied to a Phenyl Superose HR (5 mm × 5 cm) column. The column was washed with 5 ml of 2 M $(NH_4)_2SO_4$ in buffer A at a flow rate of 0.5 ml/min and then a 15 ml gradient from 2 M to 0 M $(NH_4)_2SO_4$ in buffer A was applied. Pyrophosphatase was collected at 0.05 M $(NH_4)_2SO_4$. Fractions with pyrophosphatase activity were concentrated to 0.3 ml in a Centricon PM 30 and frozen in liquid nitrogen and stored at −80 °C until use.

### *Enzyme assays*

Inorganic pyrophosphatase (E.C. 3.6.1.1) was measured by following the formation of inorganic phosphate. The reaction mixture contained (in µmol per ml): Tris/HCl (pH 8.0), 40; $MgCl_2$, 5 and sodium pyrophosphate, 1. Samples of 100 µl were taken in 0.5 min time intervals and added to 2 ml of stop reagent (4 mM ammoniumheptamolybdate, 0.7 mM potassium antimony(III) oxide tartrate and 27 mM L-ascorbic acid in 1 M $H_2SO_4$). Phosphate was determined as a reduced molybdenium blue complex at 690 nm after the addition of 8 ml of distilled water to the stop reagent.

ATP-ase (E.C. 3.6.1.3) was measured by following the formation of inorganic phosphate from ATP. The standard reaction mixture was the same as for the pyrophosphatase assay, except that $PP_i$ was replaced by 2 mM of ATP. Acetyl-coenzyme A synthetase was assayed as described (Jetten et al. 1989b).

In all assays an appropriate amount of enzyme was used. The reactions were started by the addition of enzyme or substrate. The assays were performed under both strict anaerobic (flushed assay mixtures, 0.1 mM Ti(III)citrate, $N_2$ as gas phase) and aerobic conditions. Since no difference in activity was found with the purified enzyme under either condition, assays were routinely performed aerobically. The heat stability was investigated by heating samples of 1 ml pyrophosphatase (10 µg protein) in 50 mM Tris/HCl pH 8.0 and 10mM $MgCl_2$ in Eppendorf cups for 10 min at several temperatures. After cooling to 35 °C, 1 mM of pyrophosphate was added and the activity determined with the standard assay. The substrate specificity of the enzyme was determined by replacing 1 mM pyrophosphate in the standard assay for 1 mM of various other phosphate-esters. Several compounds were tested for their ability to inhibit pyrophosphatase. The enzyme was preincubated 30 min at 35 °C with 5 mM inhibitor in 50 mM Tri/HCl pH 8.0, 5 mM $Mg^{2+}$. Thereafter 1 mM $PP_i$ was added and the activity determined with the standard assay.

## Results

### *Localization*

When the bacterial cells were rigorously disrupted by prolonged sonification and two passages through a French pressure cell at 135 MPa, the pyrophosphatase was found in the soluble fraction after ultracentrifugation. However, when gentle lysis conditions were applied in the presence of 0.5 mM of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF), upto 5% of the pyrophosphatase activity was associated with the membrane fraction, while the acetyl-coenzyme A synthetase was exclusively found in the cytoplasmic fraction. The control enzyme ATPase was completely recovered in the membrane fraction.

**Table 1.** Purification of inorganic pyrophosphatase from *Methanothrix soehngenii*. The inorganic pyrophosphatase was purified by liquid chromotography as described in 'Materials and methods'. Enzyme activity was determined by following the formation of inorganic phosphate. One unit is the amount catalyzing the hydrolysis of 1 μmol of pyrophosphate per min at 35 °C in 40 mM Tris/HCl containing 5 mM magnesium chloride and 1 mM sodium pyrophosphate, final pH 8.0

| Fraction | Protein (mg) | Activity (U) | Specific activity (U/mg) | Purification -fold | Yield (%) |
|---|---|---|---|---|---|
| Crude extract (20 ml) | 224 | 291 | 1.3 | 1 | 100 |
| Q-sepharose (3.3 cm × 10 cm) | 2.5 | 292 | 117 | 90 | 100 |
| Hydroxylapatite (1 cm × 6 cm) | 0.8 | 180 | 225 | 173 | 62 |
| Phenyl superose HR (5 mm × 5 cm) | 0.3 | 155 | 515 | 396 | 53 |

*Enzyme purification*

The purification of pyrophosphatase required no strict anaerobic conditions. The purification was carried out at room temperature. In three steps a 400 fold purified enzyme was obtained (Table 1). Q-Sepharose chromatography proved to be an effective first step in the purification of pyrophosphatase, since the enzyme was one of the first proteins to elute from the column after the void volume. Hydroxylapatite was used to separate pyrophosphatase from interfering acetyl-CoA-synthetase. Phenylsuperose removed the last contaminating proteins from the pyrophosphatase.

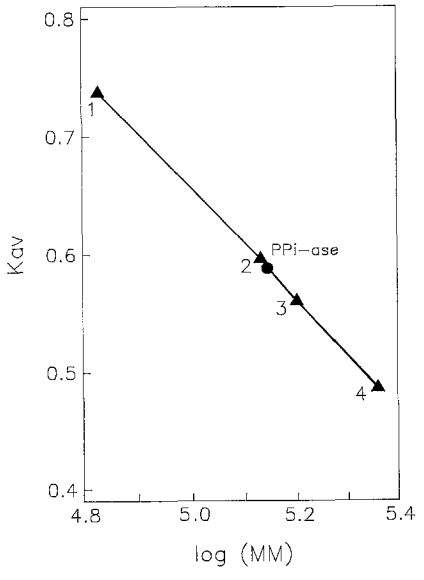


**Fig. 1.** Molecular mass determination of purified pyrophosphatase on superose 6 HR 10/30. Superose 6 HR (10 mm × 30 cm) was equilibrated with 50 mM Tris/HCl pH 8.0 containing 150 mM sodium chloride. The column was developed at a flow rate of 0.3 ml/min. Standards (▲) were bovine serum albumine (Monomer, 67 kDa, [1] and Dimer, 134 kDa, [2]), aldolase (152 kDa, [3]) and catalase (232 kDa,[4]). The purified pyrophosphatase is marked by ● MM = molecular mass; $K_{av}$ = distribution coefficient

*Characterization of the purified enzyme*

The molecular mass of the native pyrophosphatase was estimated by gelfiltration on Superose 6 HR 10/30 and appeared to be 139 ± 7 kDa, when compared with standards of known molecular mass (Fig. 1). Native polyacrylamide gel electrophoresis revealed one band when stained with coomassie brillant blue R 250. When the native gels were incubated 1–2 min in pyrophosphatase assay mixture and stained with ammonium-heptamolybdate and methylgreen in 1 M $H_2SO_4$, this band showed a strong accumulation of released phosphate (Fig. 2). Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of the purified enzyme revealed two subunits with relative molecular mass equal to 35 and 33 kDa of equal intensity, which suggests an $\alpha_2\beta_2$ subunit stoichiometry for the native enzyme (Fig. 3).

*Stability*

The activity of the purified enzyme did not decrease significantly in the presence of air. The enzyme could be stored at +4 °C for at least one week without appreciable loss of activity. When the enzyme was kept at −20 °C, aerobically or anaerobically, 50% of the activity was lost within 24 h. However, when the enzyme was frozen in liquid nitrogen, it could be stored for at least two months at −80 °C without loss of activity. The enzyme was relatively thermostable in the presence of 0.01 M $Mg^{2+}$. Heating for 10 min at 60 °C resulted only in a loss of activity of 15%. Complete inactivation occured after heating at 90 °C (Fig. 4).

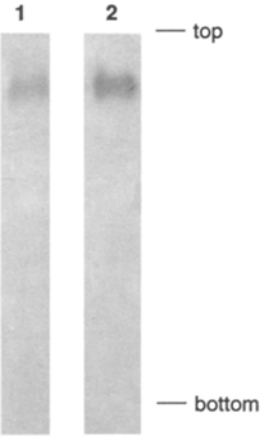


**Fig. 2.** Native polyacrylamide gel electrophoresis of the purified pyrophosphatase from *Methanothrix soehngenii*. Protein was separated on 7% polyacrylamide slab gels, which were subsequently used either for protein staining with Coomassie brillant blue R 250 (Laemmli 1970) or for activity staining. In the latter case, the gels were submerged in 50 mM Tris/HCl containing 5 mM magnesium chloride and 1 mM sodium pyrophosphatase, final pH 8, for 1–2 min. Gels were washed with distilled water and incubated 30 min in 1 M $H_2SO_4$ containing 15 mM ammonium heptamolybdate, 1.5 g Triton X-305/l and 40 mg methylgreen/l (Baykov and Volk 1985). The accumulated phosphate became visible as a dark green band. *Lane 1*, 10 μg pyrophosphatase stained with coomassie R 250; *Lane 2*, 0.5 μg pyrophosphatase stained for accumulated phosphate after 1 min incubation in the standard assay mixture

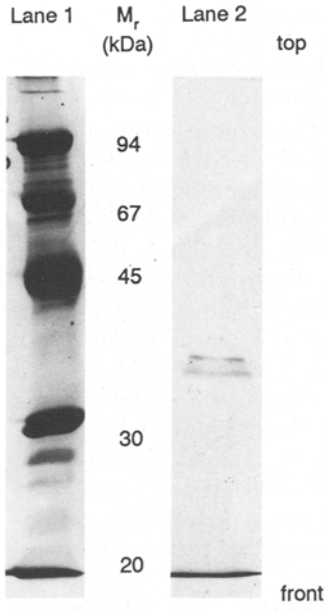


**Fig. 3.** Sodium dodecylsulfate/polyacrylamide gel electrophoresis of purified inorganic pyrophosphatase from *Methanothrix soehngenii*. Protein was separated on 12% polyacrylamide slab gels, which were subsequently stained with silver (Wray et al. 1981). *Lane 1*, molecular mass standards: trypsin inhibitor, 20.1 kDa; carbonic anhydrase, 30 kDa; ovalbumin, 43 kDa; bovine serum albumine, 67 kDa; and phosphorylase b, 94 kDa. *Lane 2:* 5 µg purified enzyme

*Substrate specificity*

In addition to pyrophosphate, a $Mg^{2+}$-dependent hydrolysis of tri- and tetrapolyphosphates was catalysed by the purified enzyme at 44% and 8% of the $PP_i$ hydrolysis rate, respectively. Organic phosphates at 5 mM (ATP, ADP, AMP, phosphoenol pyruvate, thiamine pyrophosphate, acetylphosphate, glucose-1-phosphate, glucose-6-phosphate and paranitrophenyl phosphate) were not hydrolyzed by the enzyme. $Mn^{2+}$ and $Co^{2+}$ could replace $Mg^{2+}$ as cation. With these cations 160% and 24% of the activity of $Mg^{2+}$ at 1 mM was found, respectively. At concentrations higher than 1 mM $Mn^{2+}$ and $Co^{2+}$ precipitation of pyrophosphate occured. $Ca^{2+}$ did not promote the pyrophosphatase activity.

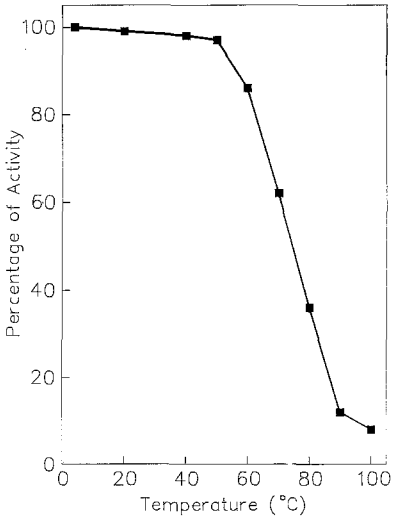


**Fig. 4.** Heat stability of the inorganic pyrophosphatase from *Methanothrix soehngenii*. Samples of 1 ml pyrophosphatase (10 µg protein) were heated for 10 min at the temperatures indicated. After cooling to 35 °C, the activity was determined as described in 'Materials and methods'

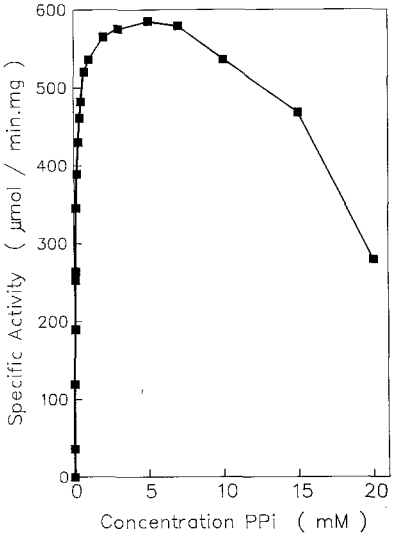


**Fig. 5.** Relation between pyrophosphate concentration and specific activity of inorganic pyrophosphatase from *Methanothrix soehngenii*. Samples of 1 ml pyrophosphate (5 µg protein) in 50 mM Tris/HCl, pH 8.0, and 5 mM $MgCl_2$ were incubated in Eppendorf cups for 10 min 35 °C. Then the indicated amounts of pyrophosphate were added and the activity determined as described in 'Materials and methods'

*Kinetic properties*

The reaction rate at different pyrophosphate and $Mg^{2+}$ concentrations did not follow Michaelis-Menten kinetics. Half maximal rates were obtained at 0.1 mM $PP_i$ at 5 mM $Mg^{2+}$. High concentrations of pyrophosphate inhibited the pyrophosphatase (Fig. 5). The optimal $Mg^{2+}$ to pyrophosphate ratio was 5. The $V_{max}$ at the optimal pH of 8 (50 mM Tris/HCl) and at 35 °C, was 590 µmol of pyrophosphate hydrolyzed per min per mg protein, which corresponded to a $K_{cat}$ of 1400 per second.

**Table 2.** Inhibition of inorganic pyrophosphatase from *Methanothrix soehngenii*. Inorganic pyrophosphatase (10 µg) was preincubated 30 min at 35 °C in 50 mM Tris/HCl containing 10 mM magnesium chloride and 5 mM inhibitor, final pH 8.0. Thereafter 1 mM sodium pyrophosphate was added and the activity determined as described in'Material and methods'; 100% activity corresponds to 278 µmol $PP_i$ hydrolyzed per min per mg protein

| Inhibitor | Relative activity |
|---|---|
| None | 100 |
| KF | 82 |
| *p*-Cl-Hg-benzoate | 82 |
| Titanium citrate | 78 |
| $NaN_3$ | 74 |
| Sodium dithionite | 71 |
| Iodoacetamide | 70 |
| KCN | 60 |
| ATP | 38 |
| ATP + 10 mM $Mg^{2+}$ | 87 |
| EDTA | 35 |
| EDTA + 10 mM $Mg^{2+}$ | 83 |

*Inhibition studies*

Pyrophosphatase was inhibited for more than 40% by KCN, ATP and EDTA (Table 2). ATP and EDTA inhibited the enzyme, probably by competition for magnesium. The inhibition could be reversed by addition of extra (10 mM) $Mg^{2+}$. The enzyme was not inhibited by fluoride, which is an potent inhibitor of the pyrophosphatases of *Escherichia coli* (90%, 1 mM) (Josse and Wong 1971), yeast (50%, 6.7 μM) (Smirnova and Baykov 1983) and *Thiobacillus* (89%, 0.1 mM) (Tominaga and Mori 1977). Reducing agents (titanium citrate, sodium dithionite) stimulated the activity of pyrophosphatase in aerobically or anaerobically prepared crude extracts 2 to 5 fold (data not shown). The purified enzyme, however, was not stimulated by reducing agents, it was slightly inhibited (Table 2).

## Discussion

Together with the acetyl-CoA synthetase and adenylate kinase, the inorganic pyrophosphatase plays an important role in the acetate activation of *Methanothrix soehngenii* (Jetten et al. 1989b). From the increase in specific activity upon purification and from the 50% recovery, it can be calculated that about 0.2% of the soluble cell protein of *M. soehngenii* is pyrophosphatase. This is somewhat lower than the value of other important proteins involved in the acetate metabolism of *M. soehngenii* (Jetten et al. 1989a, b; 1990 a).

The purified pyrophosphatase of the methanogenic archae *Methanothrix soehngenii* shows some marked differences with enzymes isolated from bacteria and eukaryotes. The apparent molecular mass of the enzyme was estimated to be 139 kDa. This value is different from molecular masses reported for pyrophosphatases from *E. coli* (120 kDa) (Wong et al. 1970; Jose and Wong 1971), *Thiobacillus* (80 kDa) (Tominaga and Mori 1977), *Rhodospirillum* (100 kDa) (Randahl 1979), *Streptococcus* (128 kDa) (Lathi and Niemi 1981), *Bacillus* (122 kDa) (Hachimori et al. 1975), *Desulfovibrio* (42 kDa) (Ware and Postgate 1971) and yeast (60 kDa) (Kunitz and Robbins 1961). Polyacrylamide gel electrophoresis indicated that inorganic pyrophosphatase from *M. soehngenii* is composed of subunits with molecular masses of 35 and 33 kDa arranged in $\alpha_2\beta_2$ oligomeric structure. This subunit structure is different from those described for other microorganisms. The yeast enzyme consists of two identical subunits of 30 kDa (Kunitz and Robbins 1961), the *Ballius* enzyme of two subunits of 70 kDa (Hachimori et al. 1975), while the *Desulfovibrio* enzyme consisted of only one subunit of 42 kDa (Ware and Postgate 1971). The enzyme from *Thiobacillus* was composed of 4 identical subunits of 20 kDa (Tominaga and Mori 1977), and the *Streptococcus* enzyme of four subunits of 32.5 kDa (Lathi and Niemi 1981). The enzyme of *E. coli* has six identical subunits with molecular mass of 20 kDa (Wong et al. 1970; Josse and Wong 1971).

The enzyme required magnesium for full activity; manganese and cobalt could replace magnesium. The effectiveness of manganese and cobalt as cation for the hydrolysis of pyrophosphate is limited to a narrow range of concentration of these ions. Manganese- or cobalt-pyrophosphate salts are poorly soluble as compared to magnesium pyrophosphate (Kunitz and Robbins 1961). A similar cation specificity is reported for other pyrophosphatases, although the stimulation by manganese is relatively high for the enzyme in *Methanothrix* (Josse and Wong 1971; Tominaga and Mori 1977; Lathi 1983). The relationship of magnesium and pyrophosphate concentration relative to the pyrophosphatase activity was studied extensively with the *E. coli* (Josse 1966b; Lathi 1983; Baykov et al. 1990) and yeast enzyme (Smirnova et al. 1989). As the actual substrate a stoichiometric complex, $MgPP_i$, is proposed (Josse 1966b; Lathi 1983). For the *Methanothrix* enzyme a magnesium to pyrophosphate ratio of 5 was found to be optimal. As observed for the enzymes of *E. coli* and yeast, high concentrations of free pyrophosphate inhibited the pyrophosphatase of *Methanothrix* (Lathi 1983).

The enzyme of *Methanothrix* was not inhibited by fluoride, which is an potent inhibitor of the pyrophosphatases of *E. coli* (90%, 1 mM) (Josse and Wong 1971), yeast (50%, 6.7 μM) (Smirnova and Baykov 1983) and *Thiobacillus* (89%, 0.1 mM) (Tominaga and Mori 1977). Sofar only the enzyme of *Streptococcus* is reported not to be inhibited by fluoride (Lathi and Niemi 1981). However, this enzyme exists in two confirmations, with different activity and stability. The pyrophosphatase found in cell extract of *Ureoplasma* was also not inhibited by fluoride (Davis et al. 1987). The inhibition by fluoride is extensively studied with the yeast enzyme (Smirnova and Baykov 1983; Smirnova et al. 1989). It was proposed that fluoride strengthens the binding of the enzyme to the magnesium pyrophosphate complex. The fluoride insensitivity of the *Methanothrix* enzyme could indicate that this enzyme has an altered active site in which fluoride is not able to covalently bind the magnesium pyrophosphate to the magnesium enzyme complex. As described for several other pyrophosphatases, the enzyme of *Methanothrix* was also not inhibited by sulfhydryl-reagents, which indicates that cysteine residue do not play an important role in the $PP_i$ hydrolysis (Josse and Wong 1971; Tominaga and Mori 1977; Lathi and Niemi 1981; Lahti 1983).

The inorganic pyrophosphatase activity in both aerobically or anaerobically prepared crude extract of *Methanothrix* could be stimulated by addition of reducing agents. The aerobically purified enzyme was not stimulated by reducing agents. This could indicate that a second, oxygen-sensitive pyrophosphatase was lost during the aerobic purification procedure. However, when crude extract was separated on native polyacrylamide gels and stained for accumulated phosphate after incubation in aerobic or anaerobic assay-mixtures, only one activity band could be detected in the crude extracts. The activation of pyrophosphatase by reducing agents has been observed for several sulfate reducing bacteria (Ware and Postgate 1971). They proposed that the reductant activation might act as a control mechanism for obligate anaerobes to survive short periods of oxygen-exposure.

As reported for many pyrophosphatases of Gram-negative bacteria, the enzyme of *Methanothrix* is also remarkably heat-resistent in the presence of magnesium (Blumenthal et al. 1967; Tominaga and Mori 1977; Lathi 1983). The enzyme was highly specific for pyrophosphate, although it hydrolyzed tri- and tetrapolyphosphate to some extent. No other phosphate esters were hydrolyzed by the enzyme. This reflects the high specificity reported for other pyrophosphatases (Josse 1966a; Josse and Wong 1971; Tominaga and Mori 1977; Lathi and Niemi 1981).

When rather rigorous methods were used to disrupt the cells, 99% of the enzyme activity of the cell extract was present in the 110,000-*g* supernatant, which suggests that this enzyme is present in the cell cytoplasm, like enzymes of *E. coli* (Josse and Wong 1971), *Bacillus* (Hachimori et al. 1975), *Streptococcus* (Lathi and Niemi 1981), *Thiobacillus* (Tominaga and Mori 1977) and yeast (Kunitz and Robbins 1961). However, when gentle lysis procedures in the presence of the serine protease inhibitor, PMSF, were applied up to 5% of the pyrophosphatase activity was associated with the membrane fraction. This membrane association could indicate that hydrolysis of the pyrophosphate, which is formed during acetate activation, is not solely used to displace the equilibrium of the acetate activation. In the chromatophores of photothrophic bacteria a proton translocating pyrophosphatase has been observed (Klemme et al. 1971; Randahl 1979; Nyren and Strid 1991). The main task of this integralmembrane enzyme is to maintain a substantial protonmotive force under circumstances of low energy (Nyren and Strid 1991). A similar function of the membrane associated pyrophosphatase of *Methanothrix* could be envisaged. Future research therefore will deal with the study of the energy in- and output of *Methanothrix* during acetate fermentation.

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