Quaternary Structure and Metal Ion Requirement of Family II Pyrophosphatases from *Bacillus subtilis*, *Streptococcus gordonii* and *Streptococcus mutans*

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**RUNNING TITLE: Family II Pyrophosphatases**
SUMMARY

Pyrophosphatase (PPase) from *Bacillus subtilis* has recently been found to be the first example of a family II soluble PPase with a unique requirement for Mn$^{2+}$. In the present work, we cloned and overexpressed in *Escherichia coli* putative genes for two more family II PPases (from *Streptococcus mutans* and *Streptococcus gordonii*), isolated the recombinant proteins and showed them to be highly specific and active PPases (catalytic constants of 1700-3300 s$^{-1}$ at 25 °C in comparison with 200-400 s$^{-1}$ for family I). All three family II PPases were found to be dimeric Mn-metalloenzymes, dissociating into much less active monomers upon removal of Mn$^{2+}$. The dimers were found to have one high-affinity Mn-specific site ($K_d$ of 0.2-3 nM for Mn$^{2+}$ and 10-80 µM for Mg$^{2+}$) and two or three moderate-affinity sites ($K_d$ ∼ 1 mM for both cations) per subunit. Mn$^{2+}$ binding to the high-affinity site, which occurs with a half-time of less than 10 s at 1.5 mM Mn$^{2+}$, dramatically shifts the monomer ⇔ dimer equilibrium in the direction of the dimer, further activates the dimer and allows substantial activity (60-180 s$^{-1}$) against calcium pyrophosphate, a potent inhibitor of family I PPases.
INTRODUCTION

Inorganic pyrophosphatase (EC 3.6.1.1; PPase)\(^1\) catalyzes specifically interconversion of pyrophosphate and orthophosphate. Owing to the simplicity of its substrate, PPase is a convenient system to study the mechanism of phosphoryl transfer from polyphosphates, including nucleoside triphosphates, to water, an essential but still incompletely understood biochemical transformation. Soluble PPase is essential for life (1, 2), since it provides a thermodynamic pull for biosynthetic reactions (3).

Until recently, only one family (family I) of soluble PPases had been known, of which the PPases of *Saccharomyces cerevisiae* and *Escherichia coli* are the most extensively characterized representatives (4, 5). Despite variability of subunit size and of quaternary structure (eukaryotic PPases are dimers of 30-35 kDa subunits, whereas prokaryotic PPases are hexamers of \(\sim 20\) kDa subunits), PPases of family I have a highly conserved active site structure formed by 14-16 amino acid residues and 3-4 \(\text{Mg}^{2+}\) ions and very similar catalytic properties. Catalysis by these enzymes proceeds via direct attack of water on a phosphorus atom without formation of a covalent intermediate. The metal ions are the key to catalysis and mediate the major protein-PP\(_i\) interactions, which serve to shield the charge on the electrophilic phosphorus, activate the nucleophilic water molecule and increase the acidity of the leaving phosphate group (6-8).

Recently, a long-known PPase of *Bacillus subtilis* (9) was found to have a completely different amino acid sequence and therefore to belong to a different family (family II) of soluble PPases (10, 11). *B. subtilis* PPase is similar to family I PPases in requiring divalent metal ions for activity (9) but, unlike bacterial family I PPases, it is formed by large subunits (34 kDa) and displays 10-20 times greater activity (9, 12). Another unique property of *B. subtilis* PPase is its preference for \(\text{Mn}^{2+}\) over \(\text{Mg}^{2+}\) as the activator. Search through GenBank revealed four more
putative prokaryotic members of family II (two streptococcal and two archeal), showing 40-57% identity in amino acid sequence (10, 11). One of them (from *Methanococcus jannaschii*) has been recently cloned and expressed in *E. coli* (13). The catalytic mechanism employed by family II PPases and the structural basis for their remarkable activity remain to be elucidated.

In this work, we cloned and overexpressed in *E. coli* the putative genes for family II PPases from *Streptococcus mutans*, implicated together with dietary sugars as the principal cause in the development of dental caries (14), and *Streptococcus gordonii*, another human oral bacterium. The recombinant proteins were purified and shown, along with the *B. subtilis* PPase, to be highly active dimeric PPases with a unique requirement for Mn$^{2+}$. 
MATERIALS AND METHODS

Bacterial Strains, Plasmids and Other Materials—Restriction endonucleases were purchased from Fermentas (Vilnius, Lithuania) and Dynazyme polymerase from Finnzymes (Espoo, Finland). The plasmid vector pET-15b was obtained from Novagen (Madison, USA) and primers for polymerase chain reaction (PCR) from Medprobe (Oslo, Norway). The QuikChange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, USA) and a Wizard DNA Clean-up system from Promega (Madison, USA). DEAE fast flow, Phenyl-Sepharose CL-4B and Superdex 200 prep grade columns were from Amersham Pharmacia Biotech (Uppsala, Sweden).

E. coli XL2blue (Stratagene) and E. coli C43(DE3) (15) were used as hosts in the cloning and expression, respectively. S. gordonii ATCC 10558 and S. mutans ATCC 25175 were obtained from the Culture Collection of the University of Göteborg (Sweden) and the Institute of Dentistry (University of Turku, Finland), respectively. The E. coli strains were grown in 2 x YT broth or on LA plates (16). Ampicillin (100 µg/ml) was added when required.

DNA Manipulation and Protein Expression—Genes for Sg-PPase and Sm-PPase were expressed in E. coli under the inducible phage T7 promoter by making use of the pET system. Chromosomal DNA was isolated from both streptococcal strains as described by Ushiro et al. (17). The open reading frames encoding Sg-PPase and Sm-PPase were amplified by polymerase chain reaction (PCR) using a 5’-sense oligonucleotide primer containing a restriction site for NcoI and a 3’-reverse complement primer with a BamHI site. The PCR product was purified by using the Wizard DNA Clean-up system, digested with NcoI and BamHI and ligated into the vector pET-15b. In creating the NcoI site, a T→G mutation following the initiation codon (ATG) was made both in S. gordonii and S. mutans PPase genes. After ligating the PCR products into the
vector, the mutations were reversed by using the QuikChange site-directed mutagenesis kit, and the produced DNA constructions were transformed into *E. coli* Xl2blue<sup>b</sup> and *E. coli* C43(DE3) for DNA sequencing and expression, respectively. The expression was induced for 5-6 h by 1 mM IPTG. B-PPase was expressed in *E. coli* as described by Shintani et al. (11).

**Protein Purification**—Sg-PPase and Sm-PPase were purified to homogeneity by DEAE Fast Flow ion exchange column chromatography in 20 mM Tris/HCl pH 7.3, 10 mM MgCl<sub>2</sub>, 10 mM MnCl<sub>2</sub>, followed by gel filtration on Superdex 200, prep grade in 0.15 M Tris/HCl pH 7.2, 15 mM MgCl<sub>2</sub>, 1.5 mM MnCl<sub>2</sub>. B-PPase was additionally chromatographed on a Phenyl Sepharose column, which was equilibrated with 1.7 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in the buffer used for the ion exchange chromatography and eluted with a downward gradient of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> concentration. Enzyme purity was checked by electrophoresis in 8-25% gradient polyacrylamide gels in the presence of 0.55% SDS, using the Phast System (Pharmacia, Sweden).

Metal ions were removed from the enzyme stocks (30-50 mg/ml) as follows: the enzyme solution was diluted 20-fold with 83 mM TES/KOH buffer, pH 7.2, containing 2 mM EDTA and 17 mM KCl, incubated for 2 days (B-PPase) or 4 days (Sg-PPase and Sm-PPase) at 4 °C and subjected to two 40-fold dilution/reconcentration cycles in a Centricon YM-30 centrifugal filter device (Amicon), using 83 mM TES/KOH buffer, pH 7.2, containing 50 µM EGTA and 17 mM KCl. Stock PPase solutions and other solutions used in experiments involving long incubations were sterilized by passing them through a 0.2 µm filter.

Concentrations of B-PPase, Sg-PPase and Sm-PPase were determined on the basis of extinction coefficients ε<sub>280</sub><sup>0.1%</sup> of 0.264, 0.343 and 0.307 calculated from the amino acid composition using the program ProtParam (http://expasy.pku.edu.cn/tools/protparam.html) and the subunit molecular masses of 34.0, 33.5 and 33.4 kDa, respectively, calculated from the amino
acid sequences (11). The above value of \( \varepsilon_{280}^{0.1\%} \) for B-PPase was confirmed by direct measurement of the absorbance of the solution prepared from dried and weighed enzyme. The Bradford method (18), standardized against the above method, was sometimes employed as an alternative to direct measurement of the absorbance.

**Activity**—Rates of PP\(_i\) hydrolysis were determined from continuous recordings of P\(_i\) liberation obtained using an automatic P\(_i\) analyzer (19). Reactions were initiated by adding enzyme.

**Sedimentation**—Analytical ultracentrifugation was carried out in a Spinco E instrument (Beckmann, USA), with scanning at 280 nm. Sedimentation velocity was measured at 48,000-60,000 rpm, and the sedimentation coefficient, \( s_{20,w} \), was calculated using a standard procedure (20). Sedimentation equilibrium was attained at 16,000 rpm for 16 h or at 24,000 rpm for 10 h, and the molecular mass was calculated according to Chernyak and Magretova (21). The partial specific volume at 25 °C was calculated from the amino acid composition and found to be 0.735, 0.729 and 0.730 cm\(^3\)/g for B-PPase, Sg-PPase and Sm-PPase, respectively.

**Equilibrium Dialysis**—Mg\(^{2+}\) and Mn\(^{2+}\) binding was assayed by equilibrium microdialysis in combination with atomic absorption spectroscopy to measure Mg and Mn content in the dialysis chambers (22).

Except where noted, all activity and binding measurements were performed at 25 °C in the medium containing 83 mM TES/KOH buffer, pH 7.2, 50 \( \mu \)M EGTA and 17 mM KCl. For incubations with Mg\(^{2+}\), EGTA concentrations was increased to 0.5 mM. Bovine serum albumin was added at a concentration of 1 mg/ml to all incubation media, except for those used in sedimentation and equilibrium dialysis.

**Calculations**—Equations 1 and 2, derived from Scheme I, describe the activity (\( A \)) of an equilibrium mixture of dimer (D) and monomer (M) as a function of enzyme concentration at a
zero or fixed concentration of divalent metal ion. $A_D$ and $A_M$ are the specific activities of dimer and monomer, respectively, $[E]_t$ is the total enzyme concentration, expressed in monomers, $\alpha_D$ is the fraction of dimeric enzyme, $k_d$ and $k_a$ are the rate constants and $K_d = k_d/k_a$ is the dissociation constant.

$$k_a$$

$$2M \leftrightarrow D$$

$$k_d$$

SCHEME I. Monomer-dimer equilibrium.

$$A = A_M (1 - \alpha_D) + A_D \alpha_D \quad \text{(Eq. 1)}$$

$$\frac{2(1 - \alpha_D)^2 [E]_t}{\alpha_D} = K_d \quad \text{(Eq. 2)}$$

The Mn$^{2+}$ concentration dependence of the equilibrium activity could be described by Equations 1 and 2 in combination with Equations 3-5, where $A_M$, $A'_D$ and $A''_D$ are the activities of monomer, metal-free dimer and metal-bound dimer, respectively, $K_{d,0}$ is $K_d$ at zero Mn$^{2+}$ concentration and $K_{M1}$ is the dissociation constant governing Mn$^{2+}$ binding to dimer. Equation 5 is an implicit extended mass balance equation for metal. The second and third terms on the right side of Equation 5 correspond to protein-bound and EGTA-bound metal, respectively, $[\text{EGTA}]_t$ is the total concentration of EGTA in the system (50 µM), and $K_{MEGTA}$ is the dissociation constant for its complex with Mn$^{2+}$. This treatment implies that only dimer can bind Mn$^{2+}$, which has a dual effect on activity: Mn$^{2+}$ both increases the amount of dimer (by decreasing $K_d$) and
further activates it (by increasing $A_d$). Equations 1 and 2 or 1-5 were simultaneously fit to data with the program SCIENTIST (MicroMath).

$$K_d = \frac{K_{d0}}{1 + [\text{Mn}^{2+}] / K_{M1}}$$  

(Eq. 3)

$$A_d = A_d' + \frac{A_d'' - A_d'}{1 + K_{M1} / [\text{Mn}^{2+}]}$$  

(Eq. 4)

$$[\text{Mn}^{2+}] = [\text{Mn}]_i - \frac{2\alpha_d [E]_i}{1 + K_{M1} / [\text{Mn}^{2+}]} - \frac{[\text{EGTA}]_i}{1 + K_{M\text{EGTA}} / [\text{Mn}^{2+}]}$$  

(Eq. 5)

The dissociation constants for Mn-EDTA and Mn-EGTA complexes at pH 7.2 (0.013 and 6.3 nM, respectively), used to estimate the concentrations of free Mn$^{2+}$ in solutions containing EGTA and EDTA, were calculated from the stability constants of their deprotonated Mn$^{2+}$ complexes, taking into account the $pK_a$ values of EGTA and EDTA (23).
RESULTS

Cloning and Expression of the Streptococcal PPase Genes—The putative open reading frames of Sg-PPase and Sm-PPase were amplified by PCR. By sequencing the genes we noticed Sg-PPase to have four differences from the gene-deduced amino acid sequences found from GenBank: positions 109, 135, 137 and 166 are occupied by Ser, Gly, Pro and Val (the corresponding codons are AGT, GGC, CCA and GTC), respectively, rather than by Asn, Ser, Ser and Ala, as found in GenBank. These differences were reproduced in two independent PCR amplifications, ruling out possible mutations during gene manipulations.

The PCR products expressed under phage T7 promoter in E. coli yielded transformants with about 100-fold higher PPase activity than the host strain, clearly indicating that the two open reading frames encode PPases. Because of the high expression level, the recombinant enzymes were easily purified to homogeneity by ion exchange and gel filtration chromatography, the first of these steps readily separating the chromosome-encoded E. coli PPase from the streptococcal PPases. From 50 to 100 mg of pure enzymes were obtained from 1 l of cell culture, corresponding to about 5 g of cell paste.

Quaternary Structure—The sedimentation coefficients measured for the B. subtilis and the two streptococcal PPases in the presence of Mg\(^{2+}\) or Mn\(^{2+}\) by the sedimentation velocity method were within 3.5-4.1 S (Table I). In the absence of the divalent metal ions (2 mM EDTA present), \(s_{20,w}\) decreased markedly for B-PPase and less markedly for Sg-PPase and Sm-PPase, indicating dissociation into lower molecular mass species.

The sedimentation equilibrium method gave molecular masses of 63 ± 3 kDa for B-PPase and 68 ± 5 kDa for Sg-PPase in the presence of 1.5 mM MnCl\(_2\) (Fig. 1) and 32 ± 2 kDa for B-PPase.
in the presence of 2 mM EDTA. The molecular masses of B-PPase and Sg-PPase dimers predicted from their amino acid sequences (11) are 68.0 and 67.1 kDa, respectively. These proteins are therefore clearly dimers in the presence of MnCl₂, but B-PPase dissociates into monomers in the absence of the metal ions. The assumption of a monomeric or trimeric structure in the presence of MnCl₂ resulted in a poor fit (Fig. 1).

Activity versus Enzyme Concentration Profiles—The equilibrium between different oligomeric forms of enzyme can be studied by measuring its specific activity as a function of enzyme concentration if the specific activities of the oligomeric forms differ and their interconversion is slow on the time scale of enzyme assay (24). Earlier, we used this approach in studies of *E. coli* PPase variants with weakened quaternary structure (25-28). The specific activities of the three PPases under study increased with increasing enzyme concentration in the stock solution containing 2 mM EDTA (no free divalent metal ion) or 1.5 mM Mg²⁺ and remained unchanged if the stock solution contained 1.5 mM Mn²⁺ (Fig. 2). The activity values shown, except for the Sm-PPase-EDTA curve (see below), correspond to equilibrium, as indicated by comparison with similar curves obtained after 1 and 2 days of incubation (not shown; see also Fig. 4 below). Three sources of evidence indicated that the profiles for B-PPase and Sg-PPase incubated with EDTA, and the profiles for all three PPases incubated with Mg²⁺, in Fig. 2 describe a monomer-dimer equilibrium. First, they obeyed Equations 1 and 2 derived for such an equilibrium. Second, the enzymes preincubated at low concentration could be completely reactivated upon addition of 3.5 mM MnCl₂, as illustrated in Fig. 3 for Sg-PPase. Moreover, the reactivation rate increased with increasing enzyme concentration, exhibiting half-times of 14 and 1.4 min at enzyme concentrations of 1.8 and 18 nM, respectively, as expected for the second-order reaction of dimer formation from monomers. By contrast, the half-times would be similar
for a first-order reaction, involving only one molecule of the reactant. Third, these findings agree with the sedimentation data above, showing that $s_{20,w}$ is greater in the presence of Mn$^{2+}$ or Mg$^{2+}$ than in the presence of EDTA at 20-26 µM enzyme concentration (Table I).

The activity of Sm-PPase preincubated with EDTA was only partially restored upon addition of MnCl$_2$, indicating irreversible inactivation during the incubation. Interestingly, the inactivation was significant only at [E]$_t$ > 50 µM and increased with increasing [E]$_t$, suggesting the occurrence of enzyme aggregation. This explanation is supported by the observation that Sm-PPase, but not B-PPase or Sg-PPase, could be precipitated by heating its solution above 30 °C.

Three immediate inferences can be made from the data in Figs. 2 and 3 and the parameter values listed in Table II. First, dimers are more active than monomers. Second, Mg$^{2+}$ and, especially, Mn$^{2+}$ stabilize dimer versus monomer (compare the $K_d$ values), primarily due to a change in $k_a$. Dimer stability is the lowest with B-PPase. Third, the $A_D$ values are low and similar in magnitude with Mg$^{2+}$ and EDTA (at least for B-PPase and Sg-PPase) but high with Mn$^{2+}$, indicating that Mn$^{2+}$ activates dimer, whereas Mg$^{2+}$ does not.

**Metal-Dependent Activity Modulation**—Figure 4 shows the time-courses of activity upon dilution of the EDTA-treated PPases into media containing EDTA, Mg$^{2+}$ or Mn$^{2+}$. In all cases EDTA stimulated inactivation and Mn$^{2+}$ stimulated activation, whereas the effect of Mg$^{2+}$ was variable and much smaller. The inactivation by Mg$^{2+}$ and EDTA proceeded on the time scale of hours, whereas the activation by Mn$^{2+}$ was complete in 30 s, except for a small slower phase seen with Sm-PPase. Importantly, the enzymes were predominantly dimeric at the start of incubation (Fig. 2), therefore the major activation is not due to stimulation of dimer formation, except for the slower phase seen with Sm-PPase (Fig. 4). By contrast, the inactivation clearly results from dimer dissociation into monomers because the data in Fig. 2 indicated that the PPases
equilibrated with EDTA or Mg$^{2+}$ at the concentrations used for Fig. 4 represent a mixture of
dimer and monomer. A similar pattern of inactivation by EDTA was reported for wild-type B-
PPase (12). Values for $k_d$ (Scheme I) estimated from the data in Fig. 4 are summarized in Table
II.

The Mn$^{2+}$ concentration dependence of the equilibrium activity (Fig. 5) could be described by
assuming that Mn$^{2+}$ selectively binds to dimer (with a dissociation constant of $K_{M1}$), thus shifting
the equilibrium in Scheme I in the direction of dimer, and further activates it. Estimates of $K_{M1}$
(Table III) were obtained by fitting Equations 1-5 to the data in Fig. 5. Values of $A_{D''}$ and $K_{M1}$
were treated as adjustable parameters in these fittings, and values of $A_M$, $A_D'$ and $K_{d,0}$ were
constrained to the values of $A_M$, $A_D$ and $K_d$ determined above in the presence of EDTA (Table II).
For Sm-PPase, no $K_{d,0}$ and $A_D'$ values are available (see above); therefore, $K_{d,0}$ was set to zero
and $A_D'$ was also treated as an adjustable parameter, allowing estimation of the upper limit for
$K_{M1}$. Remarkably, the values of $K_{M1}$ estimated for all three PPases are extremely low (Table III),
characteristic of metalloenzymes.

**Direct Measurements of Mn$^{2+}$ and Mg$^{2+}$ Binding**— As measured by equilibrium dialysis,
dimeric Sg-PPase has one high-affinity and two or three low-affinity sites for Mn$^{2+}$ and Mg$^{2+}$ per
subunit (Fig. 6). The binding curves for both cations exhibited saturation, not clearly seen in Fig.
6 because of the logarithmic scaling of the metal concentration axis. The high-affinity site
demonstrated a marked preference for Mn$^{2+}$ over Mg$^{2+}$ whereas the low-affinity sites bound Mn$^{2+}$
with only slightly greater strength than they bound Mg$^{2+}$. Very similar binding curves were
obtained for B-PPase and Sm-PPase (not shown). The dissociation constant $K_{M1,obs}$
characterizing metal binding to the high-affinity site was estimated from these data using only
points measured at <1 µM for Mn$^{2+}$ and < 100 µM for Mg$^{2+}$ (Table III). The values of $K_{M1,obs}$
thus obtained did not vary much between the three PPases and the values of $K_{M1,obs}$ for Mn$^{2+}$ binding to B-PPase and Sg-PPase were slightly greater than the corresponding values of $K_{M1}$ determined above. This difference appears to result from the presence of appreciable amounts of monomer, that lacks the high-affinity site possessed by the dimer, at low metal ion concentrations. For the same reason, values of $K_{M1,obs}$ for Mg$^{2+}$ shown in Table III may also exceed the corresponding $K_{M1}$ values for this cation.

In the presence of 50 $\mu$M Mn$^{2+}$, no high-affinity site was seen for Mg$^{2+}$ and the total binding stoichiometry decreased by one in Sg-PPase (Fig. 7) and B-PPase (not shown). This is consistent with Mn$^{2+}$ and Mg$^{2+}$ competing for the same M1 site, which binds Mn$^{2+}$ more tightly than Mg$^{2+}$ by four orders of magnitude (Table III). Mg$^{2+}$ binding to the low-affinity sites changed insignificantly in the presence of 50 $\mu$M Mn$^{2+}$ (Fig. 7).

**Metal Ion Requirements for Activity**—No hydrolytic activity was observed when the family II PPases were assayed in the absence of divalent metal ions. The activity was maximal when Mn$^{2+}$ was present in the preincubation or assay medium, or in both of these media (Fig. 8, curves $\text{Mn/Mn,Mg}$, $\text{Mg/Mn,Mg}$, $\text{Mn/Mn, Mn/Mg}$). However, if Mn$^{2+}$ was present only during preincubation, the activity gradually decreased during the assay, resulting in a nonlinear Pi production curve (curve $\text{Mn/Mg}$). The latter effect clearly resulted from displacement of Mn$^{2+}$ from the high-affinity site by Mg$^{2+}$. That the curve $\text{Mg/Mn,Mg}$ was linear and had the maximal slope suggested that the back substitution occurs quite rapidly during catalysis.

The Michaelis-Menten parameters for the Mg$^{2+}$-activated PP$_i$ hydrolysis were determined for dimeric and monomeric PPases obtained by preincubations identical to those used for Fig. 2. The preincubations with Mn$^{2+}$ or Mg$^{2+}$ at the enzyme concentrations indicated in Table IV yielded dimer, whereas the preincubations with EDTA (no divalent metal ion present) yielded a mixture
of monomer and dimer, with monomer being the dominant species. Fitting of rate versus substrate concentration profiles (not shown) to the sum of two Michaelis-Menten equations, one for monomer and the other for dimer, yielded $k_{\text{cat}}$ and $K_m$ values for monomers and $K_m$ values for dimers. The results presented in Table IV indicate that preincubation with Mn$^{2+}$ renders dimeric PPases better catalysts in terms of $k_{\text{cat}}$ and worse catalysts in terms of $K_m$ than preincubation with Mg$^{2+}$ or without divalent metal ions.

Ca$^{2+}$ is known to strongly inhibit family I PPases by replacing the activating Mg$^{2+}$ in its complex with enzyme and substrate (29). Typically, activity drops by 50% at $[\text{Ca}^{2+}]/[\text{Mg}^{2+}] \approx 1/50$. By contrast, no inhibition ($\leq 15\%$) was observed when the family II PPases were assayed in the presence of 2-10 mM Ca$^{2+}$ (20 mM Mg$^{2+}$, 57 µM total PPi). Moreover, Ca$^{2+}$ was able to activate family II PPases preincubated with Mn$^{2+}$ (compare curves Mn/Ca and Mn/none in Fig. 8). Although the activities measured with 0.5 mM Ca$^{2+}$ (64 s$^{-1}$ with B-PPase, 135 s$^{-1}$ with Sg-PPase and 160 s$^{-1}$ with Sm-PPase) were only about 10% of the activities conferred by Mn$^{2+}$, they compare well with the activities of family I PPases measured with their best activator, Mg$^{2+}$. However, Ca$^{2+}$ did not support PPi hydrolysis by Sg-PPase and Sm-PPase from which Mn$^{2+}$ was removed by preincubation with EDTA (activities less than 0.2 and 0.004 s$^{-1}$, respectively).

**Substrate Specificity**—Dimeric PPases preincubated with 1.5 mM Mn$^{2+}$ and assayed in the presence of 0.17-2 mM Mn$^{2+}$ or 20 mM Mg$^{2+}$ plus 0.07 mM Mn$^{2+}$ exhibited low but measurable activity against tripolyphosphate (0.03-0.06 s$^{-1}$ at 57 µM substrate) but not against ATP ($< 0.004$ s$^{-1}$) (data not shown).
DISCUSSION

Family II of Soluble PPases—A new family of soluble PPases (family II) has recently been observed by two research groups (10, 11). The first verified member of family II PPases was *B. subtilis* PPase, but in addition amino acid sequences of four putative members of this family were found in the GenBank, two streptococcal and two archeal (11). The results shown above indicate that the two streptococcal sequences do belong to highly efficient and specific PPases. Furthermore, the open reading frames encoding the putative family II PPases of *Methanococcus jannaschii* (13) and *Archaeoglobus fulgidus* have recently been expressed in *E. coli* and also shown to be PPases.

Table V summarizes the major properties distinguishing families I and II of the bacterial PPases. Kinetically, family II PPases are superior in terms of *k*\textsubscript{cat} but their full activity can hardly be manifested *in vivo* because of high *K*\textsubscript{m} values. *PPi* strongly inhibits the biosynthesis pathways which produce it as a by-product and for this reason is unlikely to be accumulated to the levels comparable with *K*\textsubscript{m}. As a result of high *K*\textsubscript{m}, hydrolysis rate will be more sensitive to *PPi* concentration, perhaps allowing for a better control of the level of this important metabolite. Knowledge of free *PPi* concentration in cells possessing family II PPases is required to address this point more specifically. B-PPase was initially reported to be insensitive to fluoride (10), a well-known inhibitor of family I PPases, but more recent studies (13) have shown both families to be fluoride-sensitive.

A search through GenBank, using *B. subtilis* PPase as a template, indicated 11 more verified and 3 unverified full-length putative family II PPase sequences. The amino acid sequences of *Thermotoga maritima*, *Clostridium difficile*, and *Geobacter sulfurreducens* putative PPases
include a specific 230-residue long insertion between residues E67 and V68 (B-PPase numbering), indicating that their subunit size is significantly larger than those of the other family II PPases (60 kDa versus 33-34 kDa; Table V) However, the conservation pattern of the amino acid residues strongly suggests that all fourteen of these sequences represent family II PPases. Interestingly, Vibrio cholerae appears to be the first example of a species having genes for PPases from both families. According to subunit size and dependence on Mn\(^{2+}\), the PPase of Bacillus megaterium, described by Tono and Kornberg (30), may also belong to family II. So far, all family II PPase sequences belong to bacteria.

Quaternary Structure and Its Role in Catalysis—Unlike the bacterial PPases of family I, which are hexamers, the three PPases studied in this work are dimers, dissociating into monomers at low enzyme concentration in the absence of Mn\(^{2+}\). The dimeric form, which is expected to be dominant under physiological conditions (Mn\(^{2+}\) present), is much more active. The latter fact is at variance with the data of Kuhn et al., who reported B-PPase (12) and M. jannaschii PPase (13) to exist as an inactive dimer and active trimer. It should be noted that their molecular mass estimates were obtained by gel filtration, a method more prone to error than the sedimentation analysis used in the present study. The dimeric structure of Sm-PPase has recently been confirmed by x-ray crystallography (31)

The metal-free dimer is more active than the monomer due to increased \(k_{\text{cat}}\) and decreased \(K_m\) values (Table IV). With family I PPase from E. coli, dissociation of the hexamer to trimers and dimers increases \(K_m\) without affecting \(k_{\text{cat}}\) (27, 28). This result was interpreted as evidence that hexamer formation and substrate binding both induce a catalytically optimal structure for the active site. With family II PPases, substrate binding is not sufficient to produce a catalytically optimal structure, as indicated by decreased \(k_{\text{cat}}\).
Role of Mn\(^{2+}\)—Family I PPases can utilize both Mg\(^{2+}\) and Mn\(^{2+}\) as cofactors; however, Mg\(^{2+}\) is more efficient in all cases. With family II PPases, the efficiency of these cations as cofactors is reversed (Table IV), and the available data indicate that this difference is due to a unique site, which binds Mn\(^{2+}\) with an affinity characteristic of metalloenzymes. This site is quite specific for Mn\(^{2+}\) versus Mg\(^{2+}\) - the ratio of the respective \(K_{M1,obs}\) values is 5,000-23,000 (Table III). Mn\(^{2+}\) binding to this site controls activity in three ways. First, it dramatically shifts the monomer \(\rightleftharpoons\) dimer equilibrium in the direction of the more active dimer (\(K_d\) changes more than 10\(^5\)-fold; Table II). A similar but smaller effect is exerted by Mg\(^{2+}\) (\(K_d\) changes 1,000- to 3,600-fold).

Kuhn and Ward (12) also observed an effect of Mn\(^{2+}\) and Co\(^{2+}\) on the quaternary structure of B-PPase, consistent with metal ion binding to the high-affinity site, but interpreted this effect in terms of a dimer-trimer equilibrium. Second, Mn\(^{2+}\) changes the kinetic parameters \(k_{cat}\) and \(K_m\) for the dimer (Table IV). Interestingly, both \(k_{cat}\) and \(K_m\) are increased in the presence of Mn\(^{2+}\), but the effect on \(k_{cat}\) is larger. This means that switching from Mg\(^{2+}\) to Mn\(^{2+}\) at the high-affinity site would always activate the enzymes, but the effect would be larger at higher substrate concentration. The high-affinity site of the dimer obtained in the absence of metal ions apparently binds Mg\(^{2+}\) from the assay medium, which explains the similarity of the kinetic parameters for this dimer and the dimer obtained in the presence of Mg\(^{2+}\) (Table IV). Finally, Mn\(^{2+}\) bound to the high-affinity site allows substantial activity with Ca\(^{2+}\) (Fig. 8). This means that CaPP\(_1\) is a reasonably good substrate for family II PPases. By contrast, CaPP\(_1\) is a strong, non-hydrolyzable inhibitor of family I PPases (29, 32, 33). At the low-affinity sites and in the PP\(_i\) complex (true substrate), Mn\(^{2+}\) and Mg\(^{2+}\) appear to be equally effective in catalysis, as indicated by similar activities of Mn\(^{2+}\)-pretreated Sg-PPase (i.e., containing Mn\(^{2+}\) at the high-affinity site) with Mn\(^{2+}\) and Mg\(^{2+}\) as activators (curves Mn/Mn and Mn/Mg in Fig. 8). Kuhn and Ward have reported that...
Co$^{2+}$ can activate B-PPase 70% as much as Mn$^{2+}$ by binding to the high-affinity site, but no activation was observed with other transition raw and alkali earth metal ions [12].

Recent x-ray crystallographic studies (31) have indicated a two-domain structure for each subunit of dimeric Sm-PPase, the C-terminal domain being quite flexible. The active site was located at the domain interface and contained two protein-bound metal ions. One had three Asp and one His, and the other had two Asp and one His sidechains as ligands. The four-ligand site apparently corresponds to the high-affinity site and the three-ligand site to one of the low-affinity sites detected in the present study. The other low-affinity site(s) appear(s) to have low occupancy in the crystals. In terms of the three-dimensional structure, the effects of Mn$^{2+}$ and Mg$^{2+}$ on dimer stability may result from decreased domain flexibility caused by metal ion binding at the domain interface.

Cells of B. subtilis (34) and S. mutans (35) accumulate large amounts of Mn$^{2+}$. Hence this cation appears to be a physiological ligand of family II PPases, at least, at the high-affinity site, and the effects of Mn$^{2+}$ reported above may well be involved in PPase activity regulation in vivo. S. mutans has been identified as the primary cause of dental caries (14) and manganese as a caries promoting element (36) due to its stimulation of S. mutans growth (37). Keeping in mind that Mn$^{2+}$ decreases the suppressive effect of fluoride both on the activity of family II PPase (13) and on the growth of S. mutans (37), it is tempting to speculate that the action of Mn$^{2+}$ on S. mutans is due to its effect on Sm-PPase. In in vitro studies of family II PPases, one should keep in mind that the high-affinity site may also bind other metal ions (13) with unpredictable consequences for quaternary structure and activity. Therefore parameters and factors, such as the concentration and nature of the added metal ion, enzyme concentration and purity of reagents, should be carefully controlled in the solutions used to store and assay these enzymes.
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REFERENCES

1. Chen, J., Brevet, A., Formant, M., Leveque, F., Schmitter, J.-M., Blanquet, S., and Plateau, P. (1990) *J. Bacteriol.* **172**, 5686-5689

2. Lundin, M., Baltscheffsky, H., and Ronne, H. (1991) *J. Biol. Chem.* **266**, 12168-12172

3. Kornberg, A. (1962) In: *Horizons in Biochemistry* (Eds. Kasha, M., Pullman, B.) Academic Press, New York, 251-264

4. Cooperman, B. S., Baykov, A. A., and Lahti, R. (1992) *Trends Biochem. Sci.* **17**, 262-266

5. Baykov, A. A., Cooperman, B. S., Goldman, A., and Lahti, R. (1999) *Progr. Mol. Subcell. Biol.* **23**, 127-150

6. Heikinheimo, P., Lehtonen, J., Baykov, A., Lahti, R., Cooperman, B., and Goldman, A. (1996) *Structure* **4**, 1491-1508

7. Harutyunyan, E. H., Oganessyan, V. Yu., Oganessyan, N. N., Avaeva, S. M., Nazarova, T. I., Vorobyeva, N. N., Kurilova, S. A., Huber, R., and Mather, T. (1997) *Biochemistry* **36**, 7754-7760

8. Heikinheimo, P., Tuominen, V., Ahonen, A.-K., Teplyakov, A., Cooperman, B. S., Baykov, A. A., Lahti, R., and Goldman, A. (2001) *Proc. Natl. Acad. Sci. USA*, in press

9. Tono, H. and Kornberg, A. (1967) *J. Biol. Chem.* **242**, 2375-2382

10. Young, T. W., Kuhn, N. J., Wadeson, A., Ward, S., Burges, D. and Cooke, G. D. (1998) *Microbiology* **144**, 2563-2571

11. Shintani, T., Uchiumi, T., Yonezawa, T., Salminen, A., Baykov, A. A., Lahti, R. and Hachimori, A. (1998) *FEBS Lett.* **439**, 263-266

12. Kuhn, N. J. and Ward, S. (1998) *Arch. Biochem. Biophys.* **354**, 47-56
13. Kuhn, N. J., Wadeson, A., Ward, S., and Young, T. W. (2000) *Arch. Biochem. Biophys.* **379**, 292-298

14. Loesche, W. J. (1986) *Microbiol. Rev.* **50**, 353-380

15. Miroux, B. and Walker, J. E. (1996) *J. Mol. Biol.* **260**, 289-298

16. Sambrook, J., Fritch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

17. Ushiro, I., Lumb, S. M., Aduse-Opoku, J., Ferretti, J. J., and Russell, R. R. B. (1991) *J. Dent. Res.* **70**, 1422-1426

18. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248-254

19. Baykov, A. A. and Avaeva, S. M. (1981) *Anal. Biochem.* **116**, 1-4

20. Chervenka, C. H. (1972) *Methods for the Analytical Ultracentrifuge*. Spinco Division of Beckman Instruments, Inc., Palo Alto

21. Chernyak, B. Ya. and Magretova, N. N. (1982) *Anal. Biochem.* **123**, 101-109

22. Käpylä, J., Hyytiä, T., Lahti, R., Goldman, A., Baykov, A. A., and Cooperman, B. S. (1995) *Biochemistry* **34**, 792-800

23. Dawson, R. M. C., Elliott, D. C., Elliott, W. H., and Jones, K. M. (1986) *Data for Biochemical Research*. Clarendon Press, Oxford

24. Kurganov, B. I. (1982) *Allosteric Enzymes. Kinetic Behavior*, Chapter 4, Wiley, New York

25. Baykov, A. A., Dudarenkov, V. Yu., Käpylä, J., Salminen, T., Hyytiä, T., Kasho, V. N., Husgafvel, S., Cooperman, B. S., Goldman, A., and Lahti, R. (1995) *J. Biol. Chem.* **270**, 30804-30812

26. Fabrichniy, I. P., Kasho, V. N., Hyytiä, T., Salminen, T., Halonen, P., Dudarenkov, V. Yu., Heikinheimo, P., Chernyak, V. Ya., Goldman, A., Lahti, R., Cooperman, B. S.,
27. Velichko, I. S., Mikalahti, K., Kasho, V. N., Dudarenkov, V. Yu., Hyytiä, T., Goldman, A., Cooperman, B. S., Lahti, R., and Baykov, A. A. (1998) *Biochemistry* **37**, 734-740

28. Salminen A., Efimova I.S., Parfenyev A.N., Magretova N.N., Mikalahti K., Goldman A., Baykov A.A., and Lahti R. (1999) *J. Biol. Chem.* **274**, 33898-33904

29. Moe, O. A., and Butler, L. G. (1972) *J. Biol. Chem.* **247**, 7315-7319

30. Tono, H. and Kornberg, A. (1967) *J. Bacteriol.* **93**, 1819-1824

31. Merckel, M., Fabrichniy, I. P., Salminen, A., Kalkinen, N., Baykov, A. A., Lahti, R., and Goldman, A. (2001) *Structure*, in press.

32. Kurilova, S. A., Bogdanova, A. V., Nazarova, T. I., and Avaeva, S. M. (1984) *Bioorg. Khim.* **10**, 1153-1160

33. Avaeva, S. M., Vorobjeva, N. N., Kurilova, S. A., Nazarova, T. I., Pol’akov, K. M., Rodina, E. V., and Samygina, V. P. (2000) *Biochemistry (Moscow)* **65**, 442-458

34. Charney, J., Fisher, W. P., and Hegarty, C. P. (1951) *J. Bacteriol.* **62**, 145-148

35. Martin, M. E., Byers, B. R., Olson, M. O. J., Salin, M. L., Arceneaux, J. E. L., and Tolbert, C. (1986) *J. Biol. Chem.* **261**, 9361-9367

36. Glass, R. L., Rothman, K. J., Espinal, F., Velex, H., and Smith, N. J. (1973) *Arch. Oral. Biol.* **18**, 1099-1004

37. Beighton, D. (1980) *Microbios* **28**, 149-156
FOOTNOTES

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1 The abbreviations used are: B-PPase, *B. subtilis* pyrophosphatase; PPase, pyrophosphatase; Sg-PPase, *S. gordonii* pyrophosphatase; Sm-PPase, *S. mutans* pyrophosphatase; TES, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid.

2 Salminen, A., unpublished.
FIGURE LEGENDS

Fig. 1. Sedimentation equilibrium distribution of Sg-PPase in the presence of 1.5 mM MnCl$_2$ at 25°C. The relative concentration of Sg-PPase measured by its absorbance at 280 nm was plotted as a function of the radial distance at 16,000 rpm. The initial enzyme concentration was 20 µM. The experimental points were fitted to a homogeneous species model with a single molecular weight of 33.5 kDa (dotted line), 67.1 kDa (solid line) or 100.6 kDa (dashed line). The residuals shown on the top are for the best fit, obtained with the 67.1 kDa molecular weight.

Fig. 2. Specific activities of family II PPase pre-equilibrated at different enzyme concentrations. The enzymes were preincubated for 1 day (B-PPase) or 3 days (Sg-PPase and Sm-PPase) at 25 °C with 2 mM EDTA (closed circles), 1.5 mM MgCl$_2$ (open circles) or 1.5 mM MnCl$_2$ (triangles), and their activities were assayed with 57 µM PPI, 20 mM MgCl$_2$, 40 µM EGTA and 0.15 M Tris/HCl, pH 7.2. The lines are drawn according to Equations 1 and 2 using the parameter values shown in Table II.

Fig. 3. Time-courses of activity during Mn$^{2+}$-stimulated dimerization of Sg-PPase at 1.8 nM (open circles) or 18 nM (closed circles) enzyme concentration. Sg-PPase pre-incubated at 0.1 µM concentrations with 2 mM EDTA, as in Fig. 2, was diluted with the buffer supplemented with 3.5 mM MnCl$_2$. Aliquots were withdrawn in time and assayed for PPase activity as in Fig. 2. The lines are drawn according to second-order kinetics using the $k_a$ values given in the text.
Fig. 4. **Time-courses of PPase activity during incubation with Mg^{2+} (open circles), Mn^{2+} (triangles) or EDTA (closed circles).** For the incubation with Mn^{2+}, stock solution of B-PPase (2.4 mM) was diluted to 26 µM using the buffer supplemented with 1.5 mM MnCl_{2} and aliquots were withdrawn over time for activity assay as in Fig. 2. In all other cases, stock solutions of B-PPase, Sg-PPase and Sm-PPase (2.4, 3.2 and 1.1 mM enzyme concentrations, respectively) were diluted to 20 µM with the buffer supplemented with 50 µM EGTA and then further diluted, within 2 min, to 0.1 µM with the buffer supplemented with 1.5 mM MgCl_{2}, 1.5 mM MnCl_{2} or 2 mM EDTA. The lines for the EDTA and Mg^{2+} incubations are drawn for a first-order reaction with the \( k_d \) values shown in Table II.

Fig. 5. **Mn^{2+} concentration dependence of PPase activation.** The enzymes (20-26 µM) were preequilibrated with Mn^{2+} for 1 day (B-PPase) or 4 days (Sg-PPase and Sm-PPase) before activity was assayed as in Fig. 2. The upper scale refers to free Mn^{2+} concentration in the medium before PPase was added and, therefore, neglects Mn^{2+} binding by PPase. The lines are drawn according to Equations 1-5 using the \( A_M \), \( K_d \) and \( K_{M1} \) values found in Tables II and III (see text).

Fig. 6. **Mn^{2+} and Mg^{2+} binding to Sg-PPase as measured by equilibrium dialysis.** \( n \) is the number of metal ions bound per subunit, the abscissa shows free metal ion concentrations. The incubation medium contained 410-800 µM PPase, 10-3000 µM Mg^{2+} or 1-3500 µM Mn^{2+} and 50 µM EGTA (total concentrations). The equilibration was performed for 4 days at 25 °C. The solid lines are drawn for a one-site model using the \( K_{M1,obs} \) value shown in Table III.
Fig. 7. Mn$^{2+}$ and Mg$^{2+}$ binding to Sg-PPase as measured by equilibrium dialysis at fixed free Mn$^{2+}$ concentration (50 μM). PPase concentration was 700-900 μM.

Fig. 8. Time courses of PP$_i$ hydrolysis by Sg-PPase. The enzyme was preequilibrated for 3 days with 1.5 mM Mn$^{2+}$ or 1.5 mM Mg$^{2+}$ and assayed with 57 μM PP$_i$ in the presence of 1 mM Mg$^{2+}$, 1 mM Mg$^{2+}$ plus 0.1 mM Mn$^{2+}$, 0.1 mM Mn$^{2+}$, 0.5 mM Ca$^{2+}$ or no added metal ion. Curve labels: metal ion in the preincubation/metal ion(s) in the assay. Enzyme concentration in the preincubation mix was 0.045 μM (Mn) or 0.9 μM (Mg) and in the assay mix was 0.015 nM (curves Mn/Mn,Mg, Mg/Mn,Mg, Mn/Mn, Mn/Mg, Mg/Mg) or 0.11 nM (curves Mn/Ca, Mn/none).
Table I

*Sedimentation coefficients*

Before each run, the enzymes were preincubated for 1-3 h (B-PPase) or 3 days (Sg-PPase and Sm-PPase) in the buffer containing the indicated additions. The $s_{20,w}$ values are precise to ± 0.2 S.

| Enzyme | Enzyme concentration (µM) | 2 mM EDTA | 1.5 mM MgCl₂ | 1.5 mM MnCl₂ | 10 mM MnCl₂ |
|--------|---------------------------|-----------|----------------|---------------|--------------|
| B-PPase| 26                        | 2.4       | 3.5            | 3.9           | 3.8          |
| Sg-PPase| 20                       | 3.1       | 3.9            | 3.8           | 4.0          |
| Sm-PPase| 23                       | 2.9       | 3.9            | 4.1           |              |
Table II

*Parameters for Monomer-Dimer Equilibrium*

Parameter values were calculated from the dependencies shown in Figs. 2-4, except for the values of $k_d$ in the presence of Mn$^{2+}$ and values of $k_a$ in the presence of EDTA and Mg$^{2+}$, which were calculated as $k_a K_d$ and $k_d/K_d$, respectively.

| Metal ion present | $A_M$  | $A_D$  | $K_d$   | $k_d$   | $k_a$  |
|-------------------|--------|--------|---------|---------|--------|
|                   | s$^{-1}$ | s$^{-1}$ | $\mu M$ | h$^{-1}$ | $\mu M h^{-1}$ |
| **B-PPase**       |        |        |         |         |        |
| None              | 2.2 ± 0.1 | 130 ± 20 | 440 ± 100 | 9.5 ± 1.0 | 0.022 |
| Mg$^{2+}$         | 2.5 ± 0.4 | 140 ± 10 | 0.46 ± 0.03 | 2.9 ± 0.4 | 6.3 |
| Mn$^{2+}$         | 900 ± 40 | < 0.001 | < 0.12 | 120 ± 10 |
| **Sg-PPase**      |        |        |         |         |        |
| None              | 3.8 ± 0.7 | 310 ± 10 | 93 ± 10 | 0.27 ± 0.01 | 0.0029 |
| Mg$^{2+}$         | < 30 | 270 ± 10 | 0.028 ± 0.004 | 0.10 ± 0.02 | 3.6 |
| Mn$^{2+}$         | 1170 ± 80 | < 0.001 | < 1 | 1210 ± 60 |
| **Sm-PPase**      |        |        |         |         |        |
| None              | 5 ± 1 |        |         | 0.22 ± 0.01 |
| Mg$^{2+}$         | < 20 | 92 ± 2 | 0.008 ± 0.002 |
| Mn$^{2+}$         | 780 ± 40 | < 0.001 | < 0.6 | 540 ± 100 |
Table III

*Metal Binding Constants for the High-Affinity Site*

| Enzyme | $K_{M1}^{a}$ | $K_{M1,obs}^{b}$ |
|--------|--------------|------------------|
|        | Mn$^{2+}$    | Mn$^{2+}$ | Mg$^{2+}$ |
|        | $nM$ | $nM$ | $\mu M$ |
| B-PPase | 0.50 ± 0.07 | 4.7 ± 2.2 | 88 ± 6 |
| Sg-PPase | 0.20 ± 0.06 | 1.7 ± 0.6 | 9 ± 2 |
| Sm-PPase | < 3 | 0.7 ± 0.2 | 16 ± 3 |

$^{a}$ Estimated from Fig. 5.

$^{b}$ Estimated from Fig. 6 for Sg-PPase and similar dependencies for other PPases using only the data measured at < 1 and < 100 $\mu$M free Mn$^{2+}$ and Mg$^{2+}$ concentrations, respectively.
Table IV

Michaelis-Menten Parameters for Dimer and Monomer in Mg\(^{2+}\)-Activated PP\(_i\) Hydrolysis

The enzymes were preincubated as for Fig. 2 and assayed in the presence of 20 mM Mg\(^{2+}\).

Values of \(k_{\text{cat}}\) for dimeric enzymes preincubated without metal ions were calculated from the \(A_D\) values given in Table II and the \(K_m\) values shown here (see text for details). Values of \(K_m\) are in terms of total PP\(_i\) concentration.

| Enzyme | Preincubation | Dimer | Monomer |
|--------|---------------|-------|---------|
|        | [E] Metal ion | \(k_{\text{cat}}\) | \(K_m\) | \(k_{\text{cat}}\) | \(K_m\) |
|        | µM | s\(^{-1}\) | µM | s\(^{-1}\) | µM |
| B-PPase | 0.011-1.1 none | 160 | 19 ± 6 | 4.4 ± 0.2 | 170 ± 20 |
|         | 22 Mg | 160 ± 10 | 29 ± 11 |
|         | 1.1 Mn | 3120 ± 70 | 160 ± 10 |
| Sg-PPase | 0.5-10 none | 400 | 17 ± 1 | 9.3 ± 0.3 | 320 ± 80 |
|         | 1 Mg | 330 ± 10 | 14 ± 1 |
|         | 0.05 Mn | 3300 ± 70 | 87 ± 4 |
| Sm-PPase | 1.3 none | 9.6 ± 1.4 | 4.7 ± 0.3 | 200 ± 40 |
|         | 1.3 Mg | 110 ± 10 | 10.2 ± 0.5 |
|         | 0.045 Mn | 1710 ± 40 | 96 ± 5 |
# Table V

**Comparison of Families I and II of Bacterial PPases**

| Criterion                        | Family I | Family II |
|---------------------------------|----------|-----------|
| Subunit size (kDa)              | 18-25    | 33-60$^a$|
| Quaternary structure            | Hexamer  | Dimer     |
| $k_{\text{cat}}$ (s$^{-1}$)     | 200-300  | 1700-3300$^b$ |
| $K_m$ for PP$_i$ (µM)            | 1-6      | 90-160$^b$ |
| Ca$^{2+}$-supported activity (s$^{-1}$) | 0        | 60-160    |
| Inhibition by Ca$^{2+}$          | Yes      | No        |

$^a$ The upper limit shown is for three putative family II PPases (see Discussion).

$^b$ In the presence of Mn$^{2+}$. 
Figure 1
Figure 2

Specific activity (s⁻¹) vs. Enzyme (M)

- B. subtilis
- S. gordonii
- S. mutans
Figure 4
Figure 5

Specific activity (s⁻¹) vs. Total Mn²⁺ added (M)
Figure 6  Metal ion concentration (M)
Quaternary structure and metal Ion requirement of Family II pyrophosphatases from Bacillus subtilis, Streptococcus gordonii and Streptococcus mutans
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