Yeast exopolyphosphatase (scPPX) processively splits off the terminal phosphate group from linear polyphosphates longer than pyrophosphate. scPPX belongs to the DHH phosphoesterase superfamily and is evolutionarily close to the well characterized family II pyrophosphatase (PPase). Here, we used steady-state kinetic and binding measurements to elucidate the metal cofactor requirement for scPPX catalysis over the pH range 4.2–9.5. A single tight binding site for Mg\(^{2+}\) (K\(_D\) of 24 \(\mu\)M) was detected by equilibrium dialysis. Steady-state kinetic analysis of tripolyphosphate hydrolysis revealed a second site that binds Mg\(^{2+}\) in the millimolar range and modulates substrate binding. This step requires two protonated and two deprotonated enzyme groups with pK\(_A\) values of 5.0–5.3 and 7.6–8.2, respectively. The catalytic step requiring two deprotonated groups (pK\(_A\) of 4.6 and 5.6) is modulated by ionization of a third group (pK\(_A\) of 8.7). Conservative mutations of Asp\(^{127}\), His\(^{148}\), His\(^{149}\) (conserved in PPase and PPase), and Asn\(^{35}\) (His in PPase) reduced activity by a factor of 100–300. The affinity of the tight binding site by 25–60-fold. Contrary to expectations, the N35H and D127E substitutions reduced the Mg\(^{2+}\) affinity of the tight binding site by 25–60-fold. Contrary to expectations, the N35H variant was unable to hydrolyze pyrophosphate, but markedly altered metal cofactor specificity, displaying higher catalytic activity with Co\(^{2+}\) bound to the weak binding site versus the Mg\(^{2+}\)- or Mn\(^{2+}\)-bound enzyme. These results provide an initial step toward understanding the dynamics of scPPX catalysis and reveal significant functional differences between structurally similar scPPX and family II PPase.

Linear inorganic polyphosphates (polyP)\(^3\) comprising chains of tens to hundreds of phosphate units are conserved in all cells, and thus are possible agents of evolution from prebiotic times (1, 2). In eukaryotes, they account for up to 20% of dry cell weight (1, 2). Recent evidence indicates that rather than simply being a store of phosphate and energy, polyP are required for bacterial responses to a variety of stress and stringency conditions, as well as for virulence of some pathogens (3–5). PolyP are additionally involved in blood clotting (6) and proliferation of mammalian cancer cells (7).

PolyP are synthesized by polyphosphate kinase, and hydrolyzed by exo- and endopolyphosphatases. Exopolyphosphatase (PPX) processively releases the terminal phosphate groups from polyP formed by \(>\)3 phosphate residues. Based on the primary structure, PPXs are classified into two types, whose prototypes are PPXs from yeast (Saccharomyces cerevisiae) cytosol and Escherichia coli. Yeast-type PPX, reported in fungi and protozoa, belongs to the superfamily of DHH phosphoesterases (named after the conserved Asp-His-His motif) (8), whereas E. coli-type PPX is present in Eubacteria and Archaea and belongs to a sugar kinase/actin/hsp-70 superfamily (9). Genes of both yeast-type PPXs (from S. cerevisiae and Leishmania major) and E. coli type PPXs (from E. coli, Aquifex aeolicus and Sulfolobus solfataricus) have been expressed in E. coli (10–14), and the structures of S. cerevisiae (15), E. coli (16), and A. aeolicus (E. coli type) (18) PPXs have been determined. The overall folds of the two PPX families are dissimilar. Specifically, the subunits comprise two domains in yeast-type PPX and four domains in E. coli-type PPX. However, in both families, the active site is located between the domains connected by a flexible linker. Interestingly, yeast and A. aeolicus PPXs are monomeric proteins (15, 18–20), whereas E. coli PPX is dimeric (10, 16). Interestingly, the yeast cell contains up to five different exopolyphosphatases in different compartments (22).

The overall structure of yeast cytosolic PPX (scPPX) bears a striking similarity to that of the well characterized family II pyrophosphatase (PPase) (23, 24), a DHH phosphoesterase that catalyzes a similar reaction with pyrophosphate, the shortest polyphosphate. Despite only 12–17% sequence identity, 11 of the total 14 polar residues in the active site of family II PPase are conserved in all yeast-type PPXs, and two more are conserved in most of the enzymes (Fig. 1A), implying an evolutionary relationship. Moreover, the active site structures of these enzymes acid; TAPS, 3-[3-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)-1-propanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

\(^{4}\) M. S. Hasson, J. Alvarado, and D. A. Sanders, Protein Data Bank entry 1U6Z.
are very similar, particularly parts of the N-terminal domains (Fig. 1B). Despite these structural and functional similarities, the substrate specificities of scPPX and family II PPase are opposite. scPPX is highly active against linear polyphosphates formed by three or more phosphate residues but inactive against pyrophosphate (19, 20), whereas family II PPase is active against pyrophosphate but displays low activity against longer polyphosphates (27). This difference in specificity may arise from scPPX having an extended substrate binding site. Another important difference is that scPPX contains Asn35 (conserved in all other yeast-type PPXs) in lieu of His9 (Streptococcus gordonii PPase numbering) (15) (Fig. 1). In PPase, this conserved His is a ligand for one of the essential divalent metal ions activating the nucleophilic water molecule (28). Functionally, yeast (and other) PPXs have only been preliminarily characterized (19, 20, 29), and their mechanisms of action are unknown at present.

In the present study, steady-state kinetic and binding measurements were applied to determine the stoichiometry of Mg\(^{2+}\) participation at different steps of catalysis by scPPX within the pH range 4.2–9.5. These experiments also yielded information on the ionizing groups involved in metal binding and catalysis. Furthermore, we performed site-directed mutagenesis experiments to clarify the roles of several protein residues implicated in catalysis from the x-ray crystallographic data (15). The results provide an initial step toward understanding the dynamics of scPPX catalysis.

**EXPERIMENTAL PROCEDURES**

**Cloning of Yeast PPX**—The PPX1 gene encoding yeast cytosolic exopolyphosphatase was cloned from the genome of yeast AH22 strain. Genomic DNA was extracted from AH22 cells with a yeast genomic Y1 kit (Helena Biosciences). The PPX1 gene was amplified using “Ready-to-go” PCR beads (Amersham Biosciences). The forward and reverse primers used for amplifying the gene were 5′-GTCTAGACATATGTCGCTTTGAAAGACGG-3′ and 5′-GAATTCGGATCCTCACTCTTC-CAGGTGGTAGT-3′, respectively. Ndel (forward) and BamHI (reverse) restriction endonuclease sites were introduced in the primers. The following PCR program was applied: initial denaturation for 5 min at 95 °C, followed by 25 cycles of denaturation for 60 s at 95 °C, annealing for 60 s at 60 °C, and extension for 1 min at 72 °C. A final extension was carried out for 5 min at 72 °C. The PCR product was purified with the QIAquick PCR Purification kit (Qiagen). The amplified PPX1 gene and pET11c vector were digested using the two restriction endonucleases, Ndel and BamHI, and the cohesive ends were ligated with T4 DNA ligase (Amersham Biosciences). Mutations were introduced into PPX1 using the QuikChange site-directed mutagen-
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Expression and Purification of Yeast PPX—The scPPX-pET11c plasmid was transformed into Rosetta BL21 (DE3) cells (Novagen) for protein expression. A single colony was used to inoculate 50 ml of Terrific Broth (TB) medium (30) containing 100 μg/ml ampicillin and 30 μg/ml chloramphenicol, and grown overnight with shaking at 30 °C. On the following day, the overnight culture was used to inoculate 5 liters of TB medium with 100 μg/ml ampicillin and 30 μg/ml chloramphenicol, and grown at 37 °C with shaking until A₆₀₀ of 0.6. The culture temperature was decreased to 30 °C, and protein production induced with 0.5 mM isopropyl-β-d-thiogalactopyranoside. After 5 h, cells were harvested by centrifugation, and pellets stored at −20 °C.

After thawing, 6 g of cell paste was resuspended in 30 ml of ice-cold Tris buffer (25 mM Tris·HCl, 5 mM MgCl₂, pH 7.2), and homogenized twice using a French press (SLM Instruments) at 800 psi. Cell debris was removed by centrifugation at 48,000 × g for 30 min at 4 °C. The supernatant was loaded onto a Fractogel (Merck) strong anion exchange column, and the enzyme eluted using the Tris-buffer supplemented with 2 M NaCl. Based on the A₂₈₀ nm chart profile, SDS-PAGE gels, and enzyme activity, fractions containing the scPPX protein were pooled and concentrated to <1.5 ml. The sample was mixed with 15 ml of ice-cold MES buffer (25 mM MES-NaOH, 5 mM MgCl₂, pH 5.5), and precipitated proteins were removed by centrifuging at 15,800 × g for 15 min at 4 °C. The supernatant was loaded onto a SP-Sepharose (Amersham Biosciences) strong cation exchange column, and scPPX was eluted as a single peak using the MES buffer supplemented with 1 M NaCl. The fractions containing scPPX were re-pooled and concentrated to ~10 ml. Enzyme purity was further enhanced by loading the sample onto a Superdex 200 gel filtration column and eluting with 150 mM Tris·HCl buffer, pH 7.2, containing 5 mM MgCl₂.

To remove metal ions from the enzyme stocks, the solution was diluted with 20 mM Tris·HCl buffer, pH 7.2, containing 10 mM EDTA, incubated for 1 day on ice, and subjected to three 40-fold concentration/dilution cycles in a Centricon YM-30 centrifugal filter device (Amicon) using 20 mM Tris·HCl buffer, pH 7.2, containing 50 μM EGTA for dilution. Finally, enzyme solutions were concentrated to ~20 mg/ml and frozen at −70 °C.

Enzyme purity was assessed by electrophoresis on 8–25% gradient polyacrylamide gels in the presence of 0.55% sodium dodecyl sulfate using the Phast System (Amersham Biosciences). Concentrations of scPPX solutions were determined on the basis of a subunit molecular mass of 45 kDa and an extinction coefficient ε₁₃₆ of 8.3 (as estimated from the amino acid composition using ProtParam).

Equilibrium Dialysis—Mg²⁺ binding was assayed at 25 °C by equilibrium microdialysis with more than 100-fold excess of dialysis buffer, compared with enzyme solution (31). The Mg²⁺ concentrations in the two chambers were measured by atomic absorption spectroscopy. To avoid enzyme aggregation, the initial enzyme concentration was limited to 0.2 mM, and low ionic strength buffer with 10% glycerol was applied (20 mM Tris/HCl, pH 7.2, 50 μM EGTA).

Activity Measurements—Tripolyphosphate hydrolysis rates were determined from continuous recording of Pᵢ liberation using an automatic Pᵢ analyzer (32). Reactions were initiated by adding a suitable aliquot of enzyme solution, and performed for 3–4 min at 25 °C. The sensitivity of the analyzer was set in the range of 40–100 μM Pᵢ per recorder scale. Triopolyphosphate (Fluka) was added as a pentasodium salt. Phosphate contamination (~1 mol %) in tripolyphosphate had no effect on enzyme activity, as scPPX binds phosphate fairly weakly (19, 20).

The following pH buffers were used in Pᵢ hydrolysis studies at 0.09 M ionic strength, except as noted: 50 mM acetic acid + 80 mM Tris/HCl, 200 mM EGTA (pH 4.2); 20 mM acetic acid + 80 mM Tris/HCl, 200 mM EGTA (pH 4.7); 90 mM MES/Tris, 200 mM EGTA (pH 5.2); 110 mM imidazol/HCl, 100 μM EGTA (pH 6.2); 100 mM Tris/HCl, 50 mM EGTA (pH 7.2); 130 mM Tris/HCl, 50 μM EGTA (pH 7.9); 110 mM 2-amino-2-methyl-1-propyl alcohol/HCl, 5 μM EGTA (pH 9.0); 150 mM 2-amino-2-methyl-1-propyl alcohol/HCl, 1 μM EGTA (pH 9.5). At pH 4.2 and 4.7, Mg²⁺ induced acidification of the assay medium caused by binding to the acetate buffers, whereas tripolyphosphate alkalinized the medium. The combined effect was always acidification, and therefore appropriate volumes of 1 M Tris solution (carefully determined from trial experiments using a pH meter) were added to maintain the pH at the desired levels.

Calculations and Data Analysis—Both 1:1 and 2:1 complexes were formed between Mg²⁺ and Pᵢ in aqueous solution. Scheme 1 shows all existing Pᵢ species in the pH range of interest (33–36). The dissociation constant values presented at the corresponding equilibria as pH values (36).
The dependence on $[\text{Mg}^{2+}]$ and pH of the catalytic constant ($k_{\text{cat}}$) and its ratio to the Michaelis constant ($k_{\text{cat}}/K_m$) for $P_3$ hydrolysis is described in Scheme 2. In this scheme, enzyme species lacking substrate are in equilibrium with one another, similar to all enzyme-substrate species. The fully deprotonated $\text{MgP}_3\text{O}_{10}^{3-}$ is a true substrate (see “Results”). The substrate-binding step (rate constants $k_{1A}$ and $k_{1B}$ in Scheme 2A) is assumed to be essentially irreversible, as scPPX is a highly processive enzyme (19), which means that the rate of substrate release from the active site is slow, compared with the rate of hydrolysis. Under these circumstances, $k_{\text{cat}}/K_m$ approaches the value of the apparent second-order rate constant for substrate binding. The very sharp decrease in $k_{\text{cat}}/K_m$ at high $\text{Mg}^{2+}$ concentrations as pH decreases below 5 or increases above 9 is attributed to the involvement of the conjugate bases of two basic groups and the conjugate acids of two acidic groups, respectively. For simplicity, protons are assumed to bind pairwise cooperatively, i.e. no mono- and triprotonated complexes exist. Similarly, two basic groups control $k_{\text{cat}}/K_m$ at low pH and $\text{Mg}^{2+}$ concentrations. The corresponding acid dissociation constants, $K_{a1}$ in metal-free enzyme and $K_{a3}$ and $K_{a4}$ in metal-bound enzyme, are in fact a geometric mean of the two constants (one very high and the other very low) governing successive proton binding.

Ionization of the enzyme-substrate complex is described by three acid dissociation constants: $K_{a3}$, $K_{a4}$, and $K_{a7}$ (Scheme 2B). The $k_{\text{cat}}$ values were fitted to Equation 2, where $k_{\text{cat}1}$ and $k_{\text{cat}2}$ are the catalytic constants for protonated and deprotonated enzyme-substrate complexes, and $K_A$ is the dissociation constant governing metal binding.

$$k_{\text{cat}} = (k_{\text{cat}1} + k_{\text{cat}2}K_{M}/[\text{H}^+])/(1 + K_{a3}/[\text{H}^+] + [\text{H}^+]K_{a7}/K_{a6}) + ([\text{H}^+]K_{a6}/[\text{H}^+]K_{a7}/K_{a6})$$

(Eq. 2)

The dependence of $P_3$ hydrolysis rate on substrate ($\text{MgP}_3$) and metal cofactor concentrations at the fixed pH is described by Scheme 3, which is a simplified version of Scheme 2 except that the second catalytic route with the rate constant $k_{\text{cat}'}$ is added for the H148N mutant. The asterisk signifies that parameters are pH-dependent, unlike Scheme 2 parameters. Rate values were fitted to the Michaelis-Menten equation with $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ values given by Equations 3 and 4.

$$k_{\text{cat}} = (k_{\text{cat}1} + k_{\text{cat}2}K_{M}/[\text{H}^+])/(1 + K_{a3}/[\text{H}^+] + [\text{H}^+]K_{a7}/K_{a6})$$

(Eq. 3)

$$k_{\text{cat}}/K_m = (k_{\text{cat}1} + k_{\text{cat}2}K_{M}/[\text{H}^+])/(1 + K_{a3}/[\text{H}^+]K_{a7}/K_{a6})$$

(Eq. 4)

The pH dependence of $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$, measured at fixed $\text{Mg}^{2+}$ concentrations, were fitted to Equations 5 and 6, derived from Scheme 4, where $k_{\text{cat},\text{ind}}$ and $k_{\text{cat},\text{ind}}/K_{M,\text{ind}}$ are pH-independent catalytic constants, $E_{1A}$ and $E_{2A}$ are the ionization constants for the essential basic group(s), $E_{1B}$ and $E_{2B}$ are ionization constants for the essential acidic group(s), and $m$ and $n$ are the numbers of the essential basic and acidic groups, respectively, in substrate-free enzyme. The term $0.16K_{E_{2A}}/[\text{H}^+]$ was included to account for the partial activity of the deprotonated enzyme-substrate complex (see “Results”). Other assumptions

**TABLE 1**

| pH  | $K_{P1}$ | $K_{P2}$ |
|-----|---------|---------|
| 4.2 | 634     | 445     |
| 4.7 | 321     | 92.3    |
| 5.2 | 134     | 25.4    |
| 6.2 | 23.9    | 3.86    |
| 7.2 | 3.63    | 1.91    |
| 7.9 | 0.864   | 1.74    |
| 9.0 | 0.180   | 1.70    |
| 9.5 | 0.139   | 1.70    |

$^a K_{P1} = [\text{MgP}_3]/[\text{MgP}_3]$. The subscript $i$ refers to total $P_3$ concentration, i.e., all species displaying the stoichiometry are shown, regardless of protonation state.

$^b K_{P2} = [\text{Mg}^2\text{P}_3]/[\text{MgP}_3]$. The subscript $i$ refers to total $P_3$ concentration, i.e., all species displaying the stoichiometry are shown, regardless of protonation state.

**SCHEME 3.** $\text{Mg}^{2+}$ dependence of scPPX catalysis at fixed pH.
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\[ \begin{align*}
\text{H}_n\text{mE} & \rightleftharpoons K_{E1} \text{H}_n\text{E} & \rightleftharpoons K_{E2} \text{E} \\
\text{H}_2\text{EMgP}_3 & \rightleftharpoons \text{HEMgP}_3 & \rightleftharpoons \text{EMgP}_3 \\
\text{HEMgP}_3 & \rightleftharpoons K_{E1} \text{[MgP}_3\text{O}_{10}^3^-] & \rightleftharpoons K_{E2} \text{[MgP}_3\text{O}_{10}^3^-] \\
\text{EMgP}_3 & \downarrow 0.16k_{\text{cat,ind}} & \\
\text{products} & & \text{products}
\end{align*} \]

\[ \text{SCHEME 1. To calculate } k_{\text{cat}}/K_m \text{ as substrate. As the true substrate complex (i.e. the sum of MgP}_3\text{O}_{10}^3^- \text{, Mg}_2\text{P}_3\text{O}_{10}^3^- \text{, and MgH}_2\text{P}_3\text{O}_{10}^- \text{ (Scheme 1)) as substrate. As the true substrate appears to be MgP}_3\text{O}_{10}^3^- \text{, and thus exclude substrate protonation from consideration, the } k_{\text{cat}}/K_m \text{ values measured in terms of the total MgP}_3\text{ complex were multiplied by } 1 + 10^{6.3-3pH} + 10^{10.53-2pH} \text{ where necessary.}
\]

\[ k_{\text{cat}} = k_{\text{cat,ind}}(1 + 0.16K_{E2}/[H^+])(1 + [H^+]K_{E1} + K_{E2}/[H^+]) \]

\[ k_{\text{cat}}/K_m = (k_{\text{cat,ind}}/K_m)/(1 + [H^+]K_{E1} + K_{E2}/[H^+]) \]

Aside from where otherwise noted, \( K_m \) (and accordingly, \( k_{\text{cat}}/K_m \) and \( k_j \)) values were estimated in terms of the total MgP\(_3\) complex (i.e. the sum of MgP\(_3\)O\(_{10}^-\), MgHP\(_3\)O\(_{10}^-\), and MgH\(_2\)P\(_3\)O\(_{10}^-\) (Scheme 1)) as substrate. As the true substrate appears to be MgP\(_3\)O\(_{10}^-\), part of the \( k_{\text{cat}}/K_m \) dependence on pH is attributable to substrate protonation according to Scheme 1. To calculate \( k_{\text{cat}}/K_m \) (\( k_j \)) values in terms of MgP\(_3\)O\(_{10}^-\) and thus exclude substrate protonation from consideration, the \( k_{\text{cat}}/K_m \) values measured in terms of the total MgP\(_3\) complex were multiplied by \( 1 + 10^{6.3-3pH} + 10^{10.53-2pH} \) where necessary.

RESULTS

Expression and Purification of scPPX—SDS-PAGE analysis of crude extracts obtained from recombinant E. coli cells revealed an intense 45-kDa band corresponding to yeast PPX subunit (12) and a very faint band of 58 kDa representing E. coli PPX (10). Furthermore, highly purified protein samples run on SDS-PAGE gels exhibited only the 45-kDa band, indicating an absence of contaminating protein (data not shown). Accordingly, we propose that PPX activities of the purified protein is competent in substrate (MP\(_3\)) binding. Scheme 2 includes the minimal number of enzyme species to account for the dependence shown in Fig. 3, C–F. In total, four essential protons and one metal ion bind to the enzyme. Each pair of protons controlling the alkaline and acidic pH sides is assumed to bind cooperatively so that no HEMg, H\(_3\)EMg, or H\(_3\)E, species are present. This assumption was made to reduce the number of estimated parameters and was justified by the observation that the quality of the fit did not increase significantly when such species were included. Besides, the computed values for the corresponding acid dissociation constants are highly correlated in pairs for the extended scheme and thus could not be estimated separately; whereas, the values of the other parameters were not significantly affected.

The \( k_{\text{cat}}/K_m \) value, which equals the rate constant for substrate binding (see “Experimental Procedures”) estimated in terms of the total MgP\(_3\) complex decreases sharply in alkaline, and particularly, acidic media (Fig. 3, C and D) (note that the ordinate is scaled logarithmically in Fig. 3, C–F). According to Scheme 1, part of the pH dependence in the acidic medium is...
attributable to substrate MgP$_3$ protonation. However, the $k_{cat}/K_m$ values recalculated in terms of the MgP$_3$O$_{10}$$^{3-}$ complex still decrease ~10-fold when the pH is decreased by 0.5 (Fig. 3, E and F), implying the involvement of two ionizable basic groups on the enzyme.

Direct fitting of the equation describing the $k_{cat}/K_m$ dependence on both pH and [Mg$^{2+}$] (Fig. 3, E and F) and derivable from Scheme 2A resulted in a less satisfactory fit than with the $k_{cat}$ values above. A similar finding was reported for PPase catalysis (37). One possible explanation is that binding of the charged substrate ($k_{cat}/K_m$) is more prone to buffer effects than $k_{cat}$, which is measured under saturating conditions. Therefore, a different fitting procedure was employed. Equation 4 was fitted to the $k_{cat}/K_m$ values at each fixed pH, and the resulting $k_{1A^*}$, $k_{1B^*}$, and $K_m^*$ values were separately fitted to the equations, analogous to Equation 6 with $m = 2$, $n = 2$. The final parameter values obtained in this way are summarized in Table 3.

### Effects of Active Site Substitutions on Mg$^{2+}$ Binding and Kinetic Parameters—Based on the three-dimensioinal structure of scPPX (15) and comparison with the closely related family II PPase (23, 24), four important active site residues were chosen for site-directed mutagenesis. Asn$^{35}$ replaces His of family II PPase. Asp$^{127}$ is the principal ligand of metal cofactor, while His$^{148}$ and His$^{149}$ are part of the DHH motif, which participates in metal binding and, possibly, substrate activation. H148N and H149N did not affect Mg$^{2+}$ binding significantly, whereas N35H and D127N increased the binding constant 25–60-fold (Fig. 2 and Table 2).

At pH 7.2, $k_{cat}$ and $k_{cat}/K_m$ values were much lower for the mutant enzymes versus the wild type, but the dependence on [Mg$^{2+}$] was similar (Fig. 4), except for the $k_{cat}$ curve for the H148N variant. This curve was markedly shifted to higher Mg$^{2+}$ concentrations, i.e. the variant exhibited a dramatic increase in $K_{A^*}$, the metal binding constant for the enzyme-substrate complex (Table 2). A more detailed analysis revealed two more qualitative effects. Specifically, the D127N substitution eliminated substrate binding to free enzyme ($k_{1A^*}/k_{1B^*} < 0.01$), while the H148N mutation permitted catalysis in the EMP$_3$ complex ($k_{cat} > 0$). Metal binding to substrate-free enzyme ($K_{A^*}$) was hardly affected.

In the presence of 3 mM Mg$^{2+}$ as the cofactor, all mutant scPPXs exhibited at least 450-fold lower $k_{cat}$ and $k_{cat}/K_m$ values (Fig. 5 and Table 4). The acid dissociation constants for the ionizable groups were not uniformly affected. Two of the substitutions (D127N and H149N) decreased and one substitution (H148N) appreciably increased $pK_{ES1}$, the acid dissociation constant for the group responsible for the decline in $k_{cat}$ in acidic medium. For simplicity, protonation in the acidic medium was described by a single dissociation constant. Additionally, the catalytic constant for the fully deprotonated enzyme-substrate complex was assumed to be 0.16 that for the monoprotonated complex, as deduced above for wild-type scPPX (Table 2). From the effects on $k_{cat}/K_m$, a change in $pK_{E2}$ was deduced in H149N ScPPX. Additionally, the N35H, H148N, and H149N substitutions decreased $m$ or $n$, the number of the basic or acidic groups controlling $k_{cat}/K_m$, respectively.

### Cofactor Specificity of N35H scPPX—Whereas the N35H scPPX mutant enzyme did not hydrolyze PP$_3$, it displayed sign-

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**TABLE 2**

| Parameter | WT | N35H | D127N | H148N | H149N |
|-----------|----|------|-------|-------|-------|
| | Value | | | | | |
| $k_{cat}$ (s$^{-1}$) | 1150 ± 20 | 2.2 ± 0.1 | 0.14 ± 0.01 | 0.13 ± 0.01$^*$ | 0.62 ± 0.03 |
| $k_{cat}$ (s$^{-1}$) | 70 ± 10 | 0.012 ± 0.001 | <0.001 | 0.004 ± 0.0002 | 0.0048 ± 0.0006 |
| $K_{A^*}$ (μM$^{-1}$ s$^{-1}$) | 370 ± 50 | 0.06 ± 0.01 | 0.11 ± 0.05 | 0.048 ± 0.007 | 0.24 ± 0.03 |
| $K_{A^*}$ (μM) | 5 ± 2 | 9 ± 4 | 12 ± 6 | 7.5 ± 2 | 6 ± 1 |
| $K_{A^*}$ (mM) | ≤0.01 | ≤0.01 | ≤0.1 | 2.6 ± 0.8 | ≤0.01 |
| $K_{cat}$ (μM) | 24 ± 3 | 600 ± 80 | 1400 ± 100 | 35 ± 8 | 32 ± 7 |

$^*$ Values showing appreciable deviations from WT are in bold.

---

**FIGURE 3. Dependence of $k_{cat}$ (A and B) and $k_{cat}/K_m$ (C–F) of wild-type scPPX on free Mg$^{2+}$ concentration at fixed pH values (shown on the curves).** $k_{cat}/K_m$ values are expressed in terms of either the total MgP$_3$ complex (C and D) or the MgP$_3$O$_{10}$$^{3-}$ complex (E and F). Total concentrations of MgCl$_2$ and P$_3$ used in experiments measuring $k_{cat}$ and $k_{cat}/K_m$ were calculated for each data point from the concentrations of free Mg$^{2+}$ and the P$_3$ complexes using the binding constants given in Table 1. Enzyme concentration was varied in the range of 0.4–2 mM to achieve measurable reaction rates. The lines for $k_{cat}$ are drawn to Equation 2 using parameter values specified in Table 2. The lines for $k_{cat}/K_m$ are drawn to Equation 4 for each pH.
significant structural similarities to family II PPase with metal-bound His in the corresponding position. Accordingly, this variant was characterized in greater detail.

The cofactor specificity of scPPX was characterized by measuring the rate of P₃ hydrolysis in the presence of different metal ions. Enzymes were preincubated with the corresponding metal ions for 1 h to ensure binding to all the available sites. No hydrolytic activity was observed with either wild-type or N35H scPPX assayed in the absence of divalent metal ions. Wild-type scPPX activity decreased 4-fold in the order Mg²⁺ > Co²⁺ > Mn²⁺ (Fig. 6A). In contrast, the N35H mutant was 26 times more active with Co²⁺ than with Mg²⁺ and 15 times more active than with Mn²⁺ (Fig. 6B). Thus, the N35H substitution markedly increases the potency of Co²⁺ as a cofactor, compared with Mg²⁺ and Mn²⁺.

DISCUSSION

As specified earlier, the scPPX structure, particularly its active site, is very similar to that of family II PPase, which has been characterized in detail (17, 27, 28, 38, 39). In the following discussion, we interpret the data obtained for scPPX in terms of its structure, using PPase as a template.

Metal Cofactor Requirement—X-ray crystallography data show that one activating metal ion that binds to the active site of scPPX in the absence of substrate is coordinated to Asp⁴¹, Asp¹²⁷, and His¹⁴⁸ (15). This binding site corresponds to M2 of
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The kinetic data shown in Fig. 3 indicate a requirement of four ionizable groups for substrate binding (\(k_{\text{cat}}/K_m\)) and three more for substrate conversion (\(k_{\text{cat}}/k_{\text{cat}}\)). The groups governing substrate binding clearly refer to enzyme (Scheme 2A), because the substrate is assumed to be MgP3O10 \(^{3-}\), i.e. the fully deprotonated form. In contrast, groups with \(pK_a\) and \(pK_{\text{cat}}\) in the enzyme-substrate complex may, in principle, refer to both the enzyme and its bound substrate. The third group with \(pK_a\) 8.7 (Table 3) cannot belong to substrate, as its \(pK_a\) is smaller in solution (6.3, Scheme 1) and is expected to further decrease upon substrate binding to enzyme. Protonation of the group with \(pK_a\) allows the release of one bound metal ion, which in turn allows protonation of another group with \(pK_{\text{cat}}\). Interestingly, \(pK_{\text{cat}}\) is greater than \(pK_a\) by one unit (Table 3). Thus, metal removal from the enzyme-substrate complex triggers its further protonation. This behavior is consistent with the groups corresponding to \(pK_a\) and \(pK_{\text{cat}}\) being metal ligands. If \(K_a\) refers to the M1 site, consistent with the above theory, the ionizing groups may be Asp355 and Asp127.

Alternatively, one ionizing group may belong to \(P_3\).

The two basic groups controlling substrate binding in Scheme 2A are not directly involved in metal binding, as their mean \(pK_a\) values do not change significantly upon metal binding (\(pK_a\) compared with \(pK_{\text{cat}}\) in Table 3). The means by which these groups control substrate binding remain to be elucidated. Interestingly, metal binding to the HE species in Scheme 2A gives rise to EMg, i.e. release of a proton into solution. This behavior is consistent with direct competition between H\(^+\) and Mg\(^{2+}\), which presumably binds to the M1 site. However, the \(pK_a\) value for proton release from HE is greater than 9, and therefore does not belong to any of the M1 ligands (Fig. 1B). It is possible that release of the proton from the corresponding enzyme group triggers a conformational change that dramatically facilitates metal binding to the M1 site.

Interpretation of the Effects of the Mutations—The four residues substituted in this investigation are present in all 12 yeast-type PPX sequences in GenBank\(^\text{TM}\), and three of these residues

5 R. Lahti and P. Pohjanjoki, unpublished data.

in the P-O bond breaking step is unknown at present. In PPase, the M1 and M2 metals work in concert to activate the bridging nucleophilic water molecule. If a similar mechanism is operative in scPPX, the binding constant, \(K_A\) (Scheme 2B), that governs the dependence of \(k_{\text{cat}}\) on [Mg\(^{2+}\)] in the acidic medium (Fig. 2, A and B), refers to the M1 site. Alternatively, this constant may refer to the substrate-bound metal whose release is stimulated by the protonation of bound \(P_3\). The M2 metal is the least likely candidate, because the \(k_{\text{cat}}/K_m\) parameter exhibits weaker dependence on Mg\(^{2+}\) concentration in the acidic medium compared with \(k_{\text{cat}}\) (Fig. 3, A and E), whereas the substrate should tighten metal binding at M2 by donating additional phosphate oxygen ligands.

Schemes 2 and 3 assume that the monomagnesium complex of \(P_3\) is the true substrate for scPPX. However, binding of MP\(^3\) to EM is kinetically indistinguishable from that of M1P3 to E, as both pathways lead to EM\(_2\)P3. On the other hand, a scheme assuming M1P3 binding to EM is not consistent with our data. Thus both MP\(^3\) and M2P3, which are formed in comparable amounts under physiological conditions, may be substrates for scPPX, but bind to different enzyme species.

The Ionizing Groups—The kinetic data shown in Fig. 3 indicate a requirement of four ionizable groups for substrate binding (\(k_{\text{cat}}/K_m\)) and three more for substrate conversion (\(k_{\text{cat}}/k_{\text{cat}}\)). The groups governing substrate binding clearly refer to enzyme (Scheme 2A), because the substrate is assumed to be MgP3O10 \(^{3-}\), i.e. the fully deprotonated form. In contrast, groups with \(pK_a\) and \(pK_{\text{cat}}\) in the enzyme-substrate complex may, in principle, refer to both the enzyme and its bound substrate. The third group with \(pK_a\) 8.7 (Table 3) cannot belong to substrate, as its \(pK_a\) is smaller in solution (6.3, Scheme 1) and is expected to further decrease upon substrate binding to enzyme. Protonation of the group with \(pK_a\) allows the release of one bound metal ion, which in turn allows protonation of another group with \(pK_{\text{cat}}\). Interestingly, \(pK_{\text{cat}}\) is greater than \(pK_a\) by one unit (Table 3). Thus, metal removal from the enzyme-substrate complex triggers its further protonation. This behavior is consistent with the groups corresponding to \(pK_a\) and \(pK_{\text{cat}}\) being metal ligands. If \(K_a\) refers to the M1 site, consistent with the above theory, the ionizing groups may be Asp355 and Asp127.

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Characterization of Yeast Exopolyphosphatase

TABLE 4
Kinetic parameters describing pH dependence of wild-type and variant scPPXs at 3 mM Mg\(^{2+}\) according to Scheme 4

| Parameter | Value |
|-----------|-------|
| \(k_{cat,\text{rel}}\) (s\(^{-1}\)) | WT 1050 ± 90 | N35H 1.30 ± 0.05\(^a\) | D127N 0.143 ± 0.006 | H148N 0.30 ± 0.01 | H149N 0.66 ± 0.03 |
| \(pK_{\text{ES1}}\) | 5.6 ± 0.1 | 5.71 ± 0.04 | <5 | 6.52 ± 0.06 | 4.9 ± 0.1 |
| \(pK_{\text{ES2}}\) | 8.9 ± 0.2 | 9.12 ± 0.07 | >8.5 | 9.00 ± 0.06 | 9.2 ± 0.1 |
| \(k_{cat}\) (M\(^{-1}\) s\(^{-1}\)) | 86 ± 11 | 0.0335 ± 0.0004 | 0.0105 ± 0.002 | 0.0173 ± 0.0002 | 0.186 ± 0.015 |
| \(m\) | 2 | 2 | ND\(^b\) | ND | 2 |
| \(n\) | 2 | 1 | ND | ND | 1 |

\(^a\) Values showing appreciable deviations from WT are depicted in bold.

\(^b\) Not determined.

are conserved in family II PPase sequences (Fig. 1A). Our data provide functional evidence that all these groups are important for scPPX activity. Despite the conservative nature of the substitutions, \(K_{A}\), values measured at saturated Mg\(^{2+}\) concentrations were decreased at least 500-fold at pH 7.2 (Table 2). These effects are not caused by changes in the \(K_{A}\) values for essential groups, as pH 7.2 remains nearly optimal in all variant scPPXs (Fig. 5A). The rate constants for substrate binding (\(k_{cat}/K_{m}\)) were significantly decreased upon replacement (Table 2). Again, this is not caused by pH profile shifts (Fig. 5B).

The residues corresponding to Asn\(^{35}\), Asp\(^{127}\), His\(^{148}\), and His\(^{149}\) of scPPX are similarly important for activity of other DHH enzymes as disclosed by site-directed mutagenesis. Published examples include Bacillus subtilis PPase (39) (His\(^{9}\), Asp\(^{75}\), His\(^{97}\), and His\(^{98}\)) and RecJ exonuclease (21) (Asp\(^{97}\), Asp\(^{137}\), His\(^{161}\), and His\(^{162}\)). While Asn\(^{35}\) of scPPX is not conserved in these enzymes (His in PPase and Asp in RecJ exonuclease), its mutation dramatically decreased their catalytic activities. It is tempting to speculate that the nature of the variable residue at this position is related to the substrate specificity of DHH enzymes.

The roles of these residues in substrate binding and conversion remain to be established. Asp\(^{127}\) and His\(^{148}\) are ligands for the M2 metal, whose exact positioning is crucial for catalysis. His\(^{149}\) forms a salt bridge with phosphate in the M2 metal, whose exact positioning is crucial for catalysis. Unexpectedly, the N35H substitution increased possibly because only one carboxylate oxygen is replaced.

The effects of the substitutions on metal binding are not easily interpretable in terms of wild-type structure. As discussed above, \(K_{M}\) refers to the M1 site and \(K_{M2}\) refers to the M2 site, whereas the identity of \(K_{A}\) is not known. Whereas Asp\(^{127}\) belongs to both sites (Fig. 1B), its substitution affects only \(K_{M2}\) possibly because only one carboxylate oxygen is replaced. Unexpectedly, the N35H substitution increased \(K_{M2}\) but did not affect \(K_{M}\) significantly, and the H148N substitution affected neither \(K_{M2}\) nor \(K_{M}\). Similar odd effects of residue substitutions on metal binding observed in family II PPase (17) signify a highly integrated nature of the N-domain portion of the active site bearing the metal cluster. The distance between the M1 and M2 metals is quite short, allowing only a water molecule in between. As a result, changes in one binding site are easily propagated to the other site. In the N35H mutant, the substitution may alter the relative affinities of the M1 and M2 sites, so that M2 binding is weaker and is characterized by \(K_{M}\).

The above mutations induced only modest changes in the pH profiles of the catalytic parameters (Fig. 5). Among the \(pK_{a}\) values derived from the pH profiles (Table 4), \(pK_{ES1}\) is the most significantly affected. The changes in \(pK_{ES1}\) may arise from the effects of substitutions on either \(pK_{A}\) or \(pK_{M}\) in Scheme 2B. The increase in \(pK_{ES1}\) observed in the H148N variant is certainly due to \(K_{A}\) which is markedly increased in this variant (Table 2), whereas the decrease in \(pK_{ES1}\) in the D127N and H149N mutants is more likely caused by decreased \(pK_{A}\). Thus, \(pK_{A}\) may refer either to Asp\(^{127}\) or His\(^{149}\). The decrease in \(pK_{ES2}\), which corresponds to \(pK_{A}\) in Scheme 2A upon the H149N substitution, arises from indirect effects, because the \(pK_{A}\) of Asn is greater than that of His. The effects of the N35H and H149N substitutions on \(n\), the number of the acidic groups controlling \(k_{cat}/K_{m}\), is more difficult to explain, and may reflect a change in the catalytic mechanism.

In summary, our data indicate that the metal center of scPPX has a highly integrated structure that is very sensitive to conservative mutations. Several ionizing groups are involved in catalysis and provide a platform for further mutagenesis analyses of scPPX. The N35H substitution is a first step to converting PPX into PPase. Whereas such a conversion may have no practical significance, it provides an insight into the mechanisms of both enzymes and the structural grounds of their different specificities. The N35H mutant mimics PPase with regard to metal binding, and like PPase, favors transition metal ions as cofactors. Further substitutions are required to confer PPase activity to scPPX.

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