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Function analysis of conserved amino acid residues in a Mn^{2+}-dependent protein phosphatase, Pph3, from *Myxococcus xanthus*

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The *Myxococcus xanthus* protein phosphatase Pph3 belongs to the Mg^{2+}- or Mn^{2+}-dependent protein phosphatase (PPM) family. Bacterial PPMs contain three divalent metal ions and a flap subdomain. Putative metal- or phosphate-ion binding site-specific mutations drastically reduced enzymatic activity. Pph3 contains a cyclic nucleotide monophosphate (cNMP)-binding domain in the C-terminal region, and it requires 2-mercaptoethanol for phosphatase activity; however, the C-terminal deletion mutant showed high activity in the absence of 2-mercaptoethanol. The phosphatase activity of the wild-type enzyme was higher in the presence of cAMP than in the absence of cAMP, whereas a triple mutant of the cNMP-binding domain showed slightly lower activities than those of wild-type, without addition of cAMP. In addition, mutational disruption of a disulphide bond in the wild-type enzyme increased the phosphatase activity in the absence of 2-mercaptoethanol, but not in the C-terminal deletion mutant. These results suggested that the presence of the C-terminal region may lead to the formation of the disulphide bond in the catalytic domain, and that disulphide bond cleavage of Pph3 by 2-mercaptoethanol may occur more easily with cAMP bound than with no cAMP bound.

Keywords: cAMP/enzymatic kinetics/Mg^{2+}- or Mn^{2+}-dependent protein phosphatase/*Myxococcus xanthus*/site-directed mutagenesis.

Abbreviations: cNMP, cyclic nucleotide monophosphate; *p*NPP, *p*-nitrophenyl phosphate; PPM, Mg^{2+}- or Mn^{2+}-dependent protein phosphatase; PPP, phosphoprotein phosphatase.

Protein phosphatases remove phosphate groups from various proteins that are the key components of a number of signalling pathways in eukaryotes and prokaryotes. Protein phosphatases that dephosphorylate Ser and Thr residues are classified into the phosphoprotein phosphatase (PPP) and the protein phosphatase Mg^{2+}- or Mn^{2+}-dependent (PPM) families (*1*). The PPP and PPM families are unrelated in overall amino acid sequence, but the PPM family displays some similarities with PPP enzymes in their overall fold and dinuclear metal centre (*2*). Analysis of a large number of prokaryotic genomes has shown that Ser/Thr protein phosphatases are also widely distributed in eubacterial and archaeal genomes.

Myxococcus xanthus is a Gram-negative soil bacterium, which demonstrates complex social behaviour. *Myxococcus xanthus* harbours about 100 Ser/Thr protein kinases and over 30 Ser/Thr protein phosphatases (*3*). To date, three *M. xanthus* protein phosphatases, Pph1, Pph2 and Pph3, have been characterized (*4*–*6*). *Myxococcus xanthus* Pph3 (MXAN_4398) has a strict requirement for Mn^{2+} for activity, and is insensitive to PPP inhibitors, such as okadaic acid and cyclosporin A, indicating that Pph3 is a PPM family protein phosphatase (*6*).

Crystal structure analyses of bacterial PPM members have been performed for tPphA from *Thermosynechococcus elongatus* (*7*), MtPstP from *Mycobacterium tuberculosis* (*8*, *9*), MspP from *Mycobacterium smegmatis* (*9*, *10*) and SaSTP from *Streptococcus agalactiae* (*11*). The core structures of these bacterial PPMs are nearly identical to that of the phosphatase core domain of human PP2C (*12*). However, the catalytic centre of these bacterial PPMs exhibit a third metal ion (M3), in addition to the dinuclear metal centre (M1-M2) at the catalytic site that is universally conserved in all PPM members. In addition, a loop (flap subdomain) above the active site is conserved among these bacterial PPMs. The flap subdomain is thought to regulate substrate binding and catalysis (*7*).

Myxococcus xanthus Pph3 has an additional C-terminal cyclic nucleotide monophosphate (cNMP)-binding domain. Unlike the PPP family, phosphatases that belong to the PPM family are monomeric enzymes, and no dedicated regulatory subunits of PPMs have been identified. However, a wide variety of additional domains that confer unique functions have been observed in various bacteria (*13*). In this study, the binding affinities of Pph3 for substrates and metal ions were determined using various point and deletion mutants, enabling us to consider the role of conserved amino acid residues in the catalytic reaction. We also investigated the function of the C-terminal region containing the cNMP-binding domain in Pph3.

Materials and Methods

Site-directed and deletion mutagenesis

The *pph3* gene (MXAN _4398) cloned into the expression vector pCold-TF (Takara Bio) was used as a template for PCR. Site-directed and deletion mutations were generated by the PrimeSTAR mutagenesis basal kit (Takara Bio), according to the manufacturer's instructions using the primer pairs described in Supplementary Table S1. The resulting PCR products were transformed into *Escherichia coli* BL21 (DE3). After confirmation of the desired mutations by DNA sequencing, the mutant enzymes were expressed and purified by the methods described later in the text.

Expression and purification of recombinant enzymes

Transformed *E. coli* cells were grown in Luria broth medium containing ampicillin (100 μg/ml) at 37°C. When the A_{600} of the culture reached 0.8, the *E. coli* cells were incubated at 15°C and were harvested after 24 h. Fusion proteins with an N-terminal hexahistidine tag were produced in the soluble fraction in *E. coli*, and purified by affinity chromatography on a Talon Cell Thru column (Clontech), and trigger factor was cleaved with human rhinovirus 3C protease at 4°C for 14 h.

Assay of protein phosphatase activity

The protein phosphatase activity was determined by measuring the release of inorganic phosphate from Thr phosphopeptide, RRA(pT)VA, as a substrate. A standard assay mixture contained 0.15 μg of enzyme, 15 mM $MnCl_2$, 7 mM mercaptoethanol and 0.1 mM Thr phosphopeptide in 100 mM Tris–HCl (pH 8.0) buffer. The enzyme reaction was carried out at 37°C for 1 h. After addition of Biomol Green reagent (Enzo Life Sciences), the absorbance of each well was read at 630 nm in a 96-well plate reader. When the phosphatase activity of mutant enzyme was low or absent, up to 15 μg of enzyme was used.

To determine the kinetic parameters K_m and K_{cat} for the substrate, phosphatase activities were measured at Thr phosphopeptide concentrations ranging from 10 to 600 μM, and the Mn^{2+} concentration was fixed at 15 mM. For the kinetic parameter K_m for Mn^{2+}, the Thr phosphopeptide concentration was fixed at 100 μM, and the Mn^{2+} concentration was varied from 0.5 to 200 mM. Protein concentrations were measured by the Bradford method (*14*). K_m and V_{max} values were determined using the Hanes–Woolf linearization method.

Measurement of cNMP binding to Pph3

To remove cNMP from purified enzymes, purified enzyme solutions were dialysed four times for 24 h against 500 ml of 50 mM Tris–HCl (pH 8.0) buffer. Next, 0.1 mM cAMP or 0.1 mM cGMP was added to the dialysed enzymes for 3 h on ice, and any unbound cAMP or cGMP was removed by using a DEAE-5PW column (Tosoh) equilibrated with 50 mM Tris–HCl (pH 8.0) buffer. Free cAMP or cGMP was separated from the enzymes by applying a 0–1.0 M NaCl linear gradient for 30 min at a flow rate of 0.5 ml/min. To measure the amount of bound cNMP, the enzymes were then mixed with an equal volume of 10% trichloroacetic acid, and the trichloroacetic acid-treated extracts were washed four times with 3 volumes of water-saturated ether (*15*). Samples were passed through an ODS-80TS column (Tosoh) under the following conditions: mobile phase, solvent A (5 mM tetrabutylammonium bromide in 20 mM potassium phosphate buffer, pH 3.5), solvent B (solvent A in 60% acetonitrile); gradient elution, 0 min, 95% (v/v) solvent A; 15 min, 60% solvent A; 18 min, 0% solvent A; flow rate, 1 ml/min and detection, 260 nm (*16*). In this condition, cAMP release from cAMP-binding protein was eluted from the column after 15.5 min.

Results and Discussion

Comparison of amino acid sequence and structure of Pph3 with other bacterial PPMs

All PPM enzymes have 9–11 conserved domains, with 8 highly conserved amino acids. Pph3 possesses a catalytic domain (approximately 250 residues) that includes 11 conserved domains and all critical amino acids identified in PPMs (Supplementary Fig. S1A). Comparison of the amino acid sequence of Pph3 with four previously solved structures of bacterial PPMs revealed that Pph3 shares 32–33% amino acid identity with tPphA, MtPstP, MspP and SaSTP. Between conserved domains 5a and 6, a putative flap subdomain at amino acids 150–180, which has sequence identity ranging between 30% and 39% when compared with the bacterial PPMs, is present in Pph3 (Supplementary Fig. S1B). Pph3 also contains an effector domain of the catabolite gene activator protein family at amino acid residues 278–387. The C-terminal region has high sequence identity with other protein phosphatases/cyclic nucleotide-binding domain proteins (Supplementary Fig. S1C). In addition to the primary structure, the secondary structure of catalytic region of Pph3 and the bacterial PPMs were also compared. The secondary structural elements of Pph3 and the bacterial PPMs closely correspond to each other (Supplementary Fig. S2).

Functions of proposed metal-binding residues

Human PP2C contains a binuclear ion centre at the catalytic site that activates a catalytic water molecule for nucleophilic attack of the phosphate group (*12*). Two catalytic metal ions (M1 and M2) within the binuclear metal centre of human PP2Cα are directly coordinated to the carboxylate groups of D60, D239 and D282, and E37 and D38 interact indirectly with M1 through hydrogen bonds, with metal-bound water molecules. In Pph3, all five of these amino acids are conserved with a glutamate residue at position 21 and four aspartate residues at positions 22, 37, 205 and 243.

In addition to the two metal ions, the structures of bacterial PPMs revealed that these enzymes have an additional metal ion (M3) at the catalytic site. In bacterial PPMs, M3 is coordinated by D118 and D192 in SaSTP (*11*) (corresponding to Pph3 D131 and D205); by D118, S160 and D191 in MtPstP (*8*) (D131, N173

and D205 in Pph3); by D119 and D193 in tPphA (*7*) (D131 and D205 in Pph3) and by H153 and D185 in MspP (*10*) (N173 and D205 in Pph3). S160 in MtPstP and H153 in MspP are located in the flap subdomain. To confirm the role of the conserved metal-ligand residues, D22, D37, D131 and D205 in Pph3 were replaced with Asn, and N173 in Pph3 was replaced with Ala. A mutant in which D243 was replaced by Asn could not be constructed using the PrimeSTAR mutagenesis basal kit.

The D22N and D205N mutants of Pph3 showed complete loss of activity against 0.3 mM RRA(pT)VA under increasing concentrations of enzyme and Mn^{2+} of 100-fold and 2- to 13-fold, respectively, as compared with standard assay conditions (Table I). In addition, the Pph3 D37N mutant showed vastly reduced activity, but did not show complete loss of activity. The D60N and D239N mutants of human PP2Cα (corresponding to the D37N and D205N mutants of Pph3) showed drastically reduced activity, and had 40- and 28-fold lower affinities for Mn^{2+}, respectively, whereas the D38N mutant (corresponding to D22N of Pph3) did not show reduced activity (*17*). Although D38A mutant (corresponding to D38N of PP2Cα) of mouse PP2Cβ showed drastically reduced activity (*18*). The Pph3 D37N mutant also showed an almost 8-fold increase in the K_m value for Mn^{2+}, but the K_m value for substrate displayed no change (Table II). On the other hand, mutation of D18, D34 and D193 (corresponding to D22, D37 and D205 in Pph3) to Ala in tPphA led to a complete loss of activity (*19*). These results indicate that these Asp residues in Pph3 also have an important role in the coordination of metal ions.

It is thought that mutation of D119 in tPphA specifically affects the coordination of M3, and M3 in tPphA is required for catalysis by providing a water molecule as a proton donor during catalysis (*19*). Therefore, the absence of M3 in the tPphA D119A mutant greatly reduces substrate turnover. In addition, Su *et al*. suggested that M3 is a general requirement for PPM-catalysed reactions, because mutation of the homologous Asp residue (D146A) in human PP2Cα led to a complete loss of its activity (*19*). Pph3 D131N (corresponding to D119 in tPphA) mutant retained about 20% specific activity, suggesting that the carbonyl group of Asn131 residue in the mutant may weakly coordinate M3. This mutation raised the K_m values for substrate and Mn^{2+} by 2.6- and 12-fold, respectively. These kinetic properties are similar to those of the D119N mutant of tPphA, suggesting that D131 in Pph3 may also be required for the coordination of M3 and may affect substrate turnover, rather than substrate binding. Recent research has further demonstrated that M1-M2 of tPphA is required for the binding of phosphorylated substrate, and M3 is involved in the subsequent dephosphorylation reaction (*20*).

The flap subdomain in bacterial PPMs is thought to be important for substrate binding and turnover. It is reported that Ser160 of MtPstP (*8*) and His153 of MspP (*10*) contact M3, but His161 of tPphA (*7*) and Asn160 of SaSTP (*11*) are too far away from M3. The S160A mutant of MtPstP and the H161A mutant of tPphA exhibited a slightly increased K_m and K_{cat} values for substrate, but these residues did not seem to affect the catalytic efficiency of the enzymes. In various bacterial PP2C homologues, Ser, His, Asn or Arg is located at the position of Asn173 in Pph3. The N173A mutant of Pph3 showed slight increases (1.8- and 1.5-fold) in the K_m values for substrate and Mn^{2+}, respectively, but it had greatly reduced specific activity, as well as K_{cat} and $K_{cat}\,K_m^{-1}$ values for substrate (~22 and 12%, respectively, of the wild-type enzyme) (Table II). These results indicate that N173 in the flap subdomain may be involved in substrate turnover. On the other hand, deletion of the flap subdomain in Pph3 led to complete loss of activity (Table I), suggesting that the flap subdomain in Pph3 may be an essential region for phosphatase activity or the deletion mutation may lead to changes in the structure and complete loss of its activity.

Functions of other conserved residues

In the active site of human PP2Cα, Arg33 plays a role in binding the substrate phosphate group and in stabilizing the transition state by means of the guanidinium side chain of Arg33, which forms bifurcated hydrogen bonds with two phosphate oxygen atoms (*17*). Ala replacement of Arg33 in human PP2Cα produced an

Table I. Specific activities of wild-type Pph3 and its variant enzymes.

Enzyme	Specific activity (nmol $min^{-1}mg^{-1}$)	Relative activity (%)
Wild-type Pph3	84.2 ± 3.0	100
R17A	4.70 ± 1 .31	5.6
D22N	ND	ND
D37N	0.80 ± 0.04	0.95
G40D	1.60 ± 0.10	1.9
H42Q	66.7 ± 21.2	79
D131N	18.3 ± 0.3	22
N173A	11.5 ± 0.5	14
D205N	ND	ND
E308A/G309V/G312A	78.7 ± 3.4	93
Flap deletion	ND	ND
cNMP deletion	184 ± 1	219

Phosphatase activity was assayed at 37°C for 1 h using 0.3 mM RRA(pT)VA. Data represent mean values ± SD from three measurements. ND: enzymatic activity non-detectable.

Table II. Kinetic parameters of wild-type Pph3 and mutants with single amino acid change in predicted metal-binding sites.

Enzyme	K_m (μM) (substrate)	K_m (mM) (Mn^{2+})	K_{cat} (min^{-1}) (substarte)	$K_{cat}\,K_m^{-1}$ ($mM^{-1}min^{-1}$) (substrate)
Wild-type Pph3	175 ± 15	3.70 ± 0.89	8.47 ± 1.29	48.4 ± 2.4
D22N	ND	ND	ND	ND
D37N	168 ± 18	29.1 ± 1.6	0.035 ± 0.004	0.21 ± 0.002
D131N	456 ± 134	44.5 ± 0.77	1.80 ± 0.09	3.95 ± 0.63
N173A	317 ± 32	5.57 ± 0.47	1.86 ± 0.27	5.87 ± 0.29
D205N	ND	ND	ND	ND

enzyme with a 7.7-fold higher K_m and a 2.5-fold lower K_{cat} for substrate as compared with wild-type enzyme. The Arg in domain 1 is also conserved in bacterial PPMs. As compared with wild-type enzyme, a Pph3 mutant with Arg17 replaced by Ala displayed a 2.6-fold higher K_m for substrate, and 10- and 25-fold lower K_{cat} and K_{cat} K_m^{-1} values, respectively, for substrate than those for wild-type enzyme (Table III). Su and Forchhammer recently analysed the role of the conserved Arg in tPphA using Arg13 variants and suggested that this Arg13 assists in substrate binding (*21*).

His62 of human PP2Cα has been proposed to act as a general acid during the cleavage of the P-O bond (*12*). Bacterial PPMs do not possess a His residue at the homologous position, whereas the His residues are present three residues downstream from the homologous position of human PP2C, except for MspP (Supplementary Fig. S1A). The H42Q mutant of Pph3 had a reduced K_{cat} as compared with the wild-type enzyme (44% decrease), but this decrease in K_{cat} was apparently lower than that of the human H62A mutant, suggesting that H42 in Pph3 does not function as an important residue in catalysis. The study of Pullen *et al*. (8) indicated that in MtPstP, the three-residue insertion at this position moves H43 7.2 Å away from the bridging water nucleophile as compared with the position of H62 in human PP2Cα, and a water molecule in the catalytic site may play the role of a general acid instead of the His residue. On the other hand, Su and Forchhammer suggested tPphA H39 residue has an important role for the substrate specificity (*20*). tPphA H39A variant did not impair the reactivity towards *p*-nitrophenyl phosphate (*p*NPP), but displayed reduced activity towards phosphopeptides. In contrast, Pph3 H42Q mutant also did not significantly reduce activity against *p*NPP, but showed enhanced activity towards Tyr and Ser phosphopeptides and a broad range of substrate specificity as compared with wild-type enzyme (Table IV). This result suggested that Pph3 H42 may also be involved in substrate recognition.

In *Arabidopsis thaliana,* the Gly246Asp mutant protein of HAB1 showed strongly reduced phosphatase activity (*22*). Gly246 in HAB1 (corresponding to Gly40 in Pph3) is localized in close proximity to the catalytic site. Asp replacement of Gly40 in Pph3 also led to greatly reduced phosphatase activity. No significant change was observed in the K_m value for substrate; however, this mutant enzyme showed ~4-fold increase in affinity for Mn^{2+}. Because Asp would be located near the catalytic site in the G40D mutant, this mutation may affect the location of metal ions or water molecules.

Effect of cAMP on Pph3 activity

Pph3 contains a conserved cNMP-binding domain in its C-terminal portion. A deletion mutant lacking the C-terminal region (amino acids 279–424) and a triple mutant (E308A/G309V/G312A) of the cAMP-binding motif were constructed in order to analyse regulation of its activity by cAMP. To determine whether cNMP binds to Pph3, the amount of cNMP that combined with Pph3 was analysed by HPLC using an ODS-80Ts column. After incubation of dialysed Pph3 with 0.1 mM cAMP or 0.1 mM cGMP for 3 h, unbound cAMP or cGMP was removed from Pph3 protein with a DEAE-5PW column. Pph3 contained 0.67 ± 0.02 mol of cAMP/mol of Pph3 after incubation with cAMP, but cGMP was undetectable after incubation with cGMP (data not shown). No cAMP or cGMP was detected in either the C-terminal region deletion or triple mutant enzyme. On the other hand, Pph3 incubated without cAMP also contained 0.23 ± 0.07 mol of cAMP/mol of Pph3, indicating that cAMP produced by *E. coli* may bind to Pph3 during expression of the recombinant Pph3 in *E. coli*, and cAMP may not be completely removed from Pph3 during dialysis.

Pph3 requires 2-mercaptoethanol for its activity. Pph3 has four Cys residues at amino acid positions 94, 116, 142 and 203. When the wild-type enzyme activity was assayed with or without cAMP in the

Table III. Kinetic parameters of wild-type Pph3 and mutants with conserved amino acid changes, except for putative metal-binding residues.

Enzyme	K_m (μM) (substrate)	K_m (mM) (Mn^{2+})	K_{cat} (min^{-1}) (substarte)	K_{cat} K_m^{-1} ($mM^{-1}min^{-1}$) (substrate)
Wild-type Pph3	175 ± 15	3.70 ± 0.89	8.47 ± 1.29	48.4 ± 2.4
R17A	448 ± 5	6.77 ± 1.22	0.84 ± 0.23	1.88 ± 0.48
G40D	198 ± 20	0.93 ± 0.15	0.13 ± 0.01	0.66 ± 0.06
H42Q	164 ± 16	3.33 ± 0.14	4.80 ± 1.53	29.3 ± 3.8
E308A/G309V/G312A	148 ± 5	3.79 ± 2.70	5.48 ± 0.23	37.0 ± 0.6
Flap deletion	ND	ND	ND	ND
cNMP deletion	171 ± 13	5.10 ± 0.36	28.7 ± 11.2	277 ± 65

Table IV. Substrate specificities of wild-type and H42Q enzymes.

Enzyme	pT-peptide		pY-peptide		pS-peptide		*p*NPP	
	Specific activity (nmol $min^{-1}mg^{-1}$)	Relative activity (%)	Specific activity (nmol $min^{-1}mg^{-1}$)	Relative activity (%)	Specific activity (nmol $min^{-1}mg^{-1}$)	Relative activirty (%)	Specific activity (nmol $min^{-1}mg^{-1}$)	Relative activity (%)
Wild	87.8 ± 1.1	100	13.1 ± 1.6	100	18.8 ± 5.9	100	1158.4 ± 20.1	100
H42Q	61.4 ± 5.2	69.9	42.0 ± 5.5	321.5	29.7 ± 8.0	158.2	872.2 ± 6.3	75.3

The sequences of the three peptides, pT-peptide, pY-peptide and pS-peptide, are RRA(pT)VA, RRLIEDAE(pY)AARG and RKR(pS)RAE, respectively.

presence of up to 100 mM 2-mercaptoethanol, the phosphatase activity increased with the increasing concentration of 2-mercaptoethanol (Fig. 1A). In contrast, in the absence of 2-mercaptoethanol the C-terminal deletion mutant showed higher activity as compared with the wild-type and triple mutant enzymes, suggesting that the C-terminal region may function as an inhibitor for Pph3 or the recombinant C-terminal deletion mutant may contain protein with unformed or incomplete disulphide bonds. To confirm the function of C-terminal region in Pph3, we constructed disulphide bond-disrupted mutants. From crystal structures of bacterial PPMs, we estimated that the disulphide bonds are formed between C94 and C116 or/and C142 and C203. Replacement of C116 in the wild-type enzyme with Ala increased the activity in the absence of 2-mercaptoethanol; however, mutation of C116 to Ala in the C-terminal deletion mutant had no effect on the activity in the absence of 2-mercaptoethanol (Table V). Although C203A mutation in the wild-type enzyme and C-terminal deletion mutant enzymes had no effect on phosphatase activity in the absence of 2-mercaptoethanol, suggesting that Pph3 has one disulphide bond between Cys94 and Cys116 or Cys116 and Cys142. In addition, there was no significant difference on phosphatase activity per nanomole of enzyme in the presence of 100 mM 2-mercaptoethanol between wild-type enzyme (5.1 ± 0.2 nmol min^{-1} nmol of $enzyme^{-1}$) and C-terminal deletion mutant enzyme (4.8 ± 0.3 nmol min^{-1} nmol of $enzyme^{-1}$). These results indicated that the C-terminal region of Pph3 has no direct inhibitory effect on Php3 activity, but affects the formation of the disulphide bond in the catalytic domain.

Table V. Phosphatase activities of disulphide bond-disrupted mutants.

Enzyme	0 mM 2-mercaptoethanol Specific activity (nmol min^{-1} mg of $protein^{-1}$)	100 mM 2-mercaptoethanol Specific activity (nmol min^{-1} mg of $protein^{-1}$)
Wild	3.6 ± 0.3	109.4 ± 3.9
Wild C116A	65.5 ± 7.5	106.6 ± 3.2
Wild C203A	6.0 ± 1.7	103.8 ± 0.4
C-terminal deletion	81.8 ± 9.9	151.0 ± 9.1
C-terminal deletion C116A	83.6 ± 15.4	150.5 ± 15.1
C-terminal deletion C203A	77.6 ± 18.3	138.3 ± 0.5

Phosphatase activity was assayed at 37°C for 1 h using 0.1 mM RRA(pT)VA in the absence of 2-mercaptoethanol or in the presence of 100 mM 2-mercaptoethanol. Data represent mean values ± SD from three measurements.

The phosphatase activity of the wild-type enzyme was higher in the presence of cAMP than in the absence of cAMP; however, the ratio of the increase in activity caused by the addition of cAMP decreased with the increasing 2-mercaptoethanol concentration (Fig. 1A). The specific activities of Pph3 with cAMP in the presence of 0, 10 and 100 mM 2-mercaptoethanol were ~2.2-, 1.3- and 1.1-fold higher, respectively, than those without cAMP. A triple mutant showed slightly lower specific activities than the wild-type enzyme without the addition of cAMP. In this experiment, there were no differences between the phosphatase activities of C-terminal deletion mutant or triple mutant with and without added cAMP (data not shown). Also, when wild-type and triple mutant enzymes were incubated in the presence of 40 mM 2-mercaptoethanol either with 0.1 mM cAMP or without cAMP for 0–5 h, the phosphatase activity of the wild-type and mutant enzymes increased with increasing time of incubation, and wild-type enzyme incubated with cAMP showed the highest activity when compared with wild-type enzyme without cAMP or triple mutant enzyme with and without

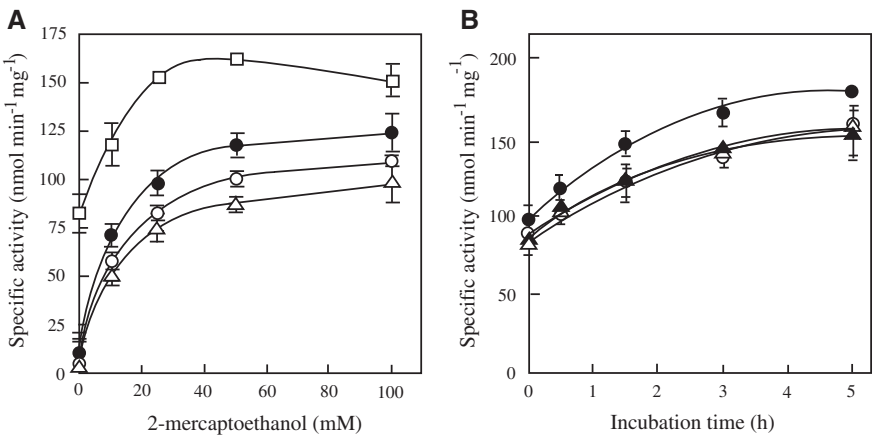

Fig. 1 Effect of cAMP on phosphatase activity. (A) Assay of phosphatase activity with or without cAMP in the presence of various concentrations of 2-mercaptoethanol. Phosphatase activity of the dialysed wild-type enzyme (circles) was assayed in the presence of 0–100 mM 2-mercaptoethanol either with 0.1 mM cAMP (closed symbols) or without cAMP (open symbols). Phosphatase activities of the C-terminal deletion mutant enzyme (squares) and triple mutant enzyme (triangles) were also measured without the addition of cAMP. The data are the means ± SD of four individual measurements. (B) Time course of phosphatase activity with or without cAMP in the presence of 50 mM 2-mercaptoethanol. The dialysed wild-type enzyme (circles) and triple mutant enzyme (triangles) were incubated in the presence of 40 mM 2-mercaptoethanol either with 0.1 mM cAMP (closed symbols) or without cAMP (open symbols) for 0–5 h at 15°C. After each incubation, phosphatase activity was measured at 37°C for 30 min in a reaction mixture containing 25 µl of 100 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (pH 8.0), 15 mM $MnCl_2$, 50 mM 2-mercaptoethanol and 0.1 mM Thr phosphopeptide. The data are the means ± SD of three individual measurements.

cAMP (Fig. 1B). The K_m values for substrate and Mn^{2+} of the triple mutant were similar to those of wild-type Pph3 (Table III). These results suggest that the disulphide bond cleavage of Pph3 by 2-mercaptoethanol may occur more easily with cAMP bound than with no cAMP bound. In *M. xanthus*, the cellular concentrations of cAMP and cGMP increase rapidly to maxima at 15–24 h of development, and then decrease (*23*, *24*). We previously reported that the *pph3* gene was expressed at different stages of development, with expression maximal during the aggregation stage (24 h) (*6*). Although the regulation of Pph3 activity may be weak, the phosphatase activity of Pph3 *in vivo* may also be regulated by cAMP.

Supplementary Data

Supplementary data are available at *JB online*.

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Conflict of interest
None declared.

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